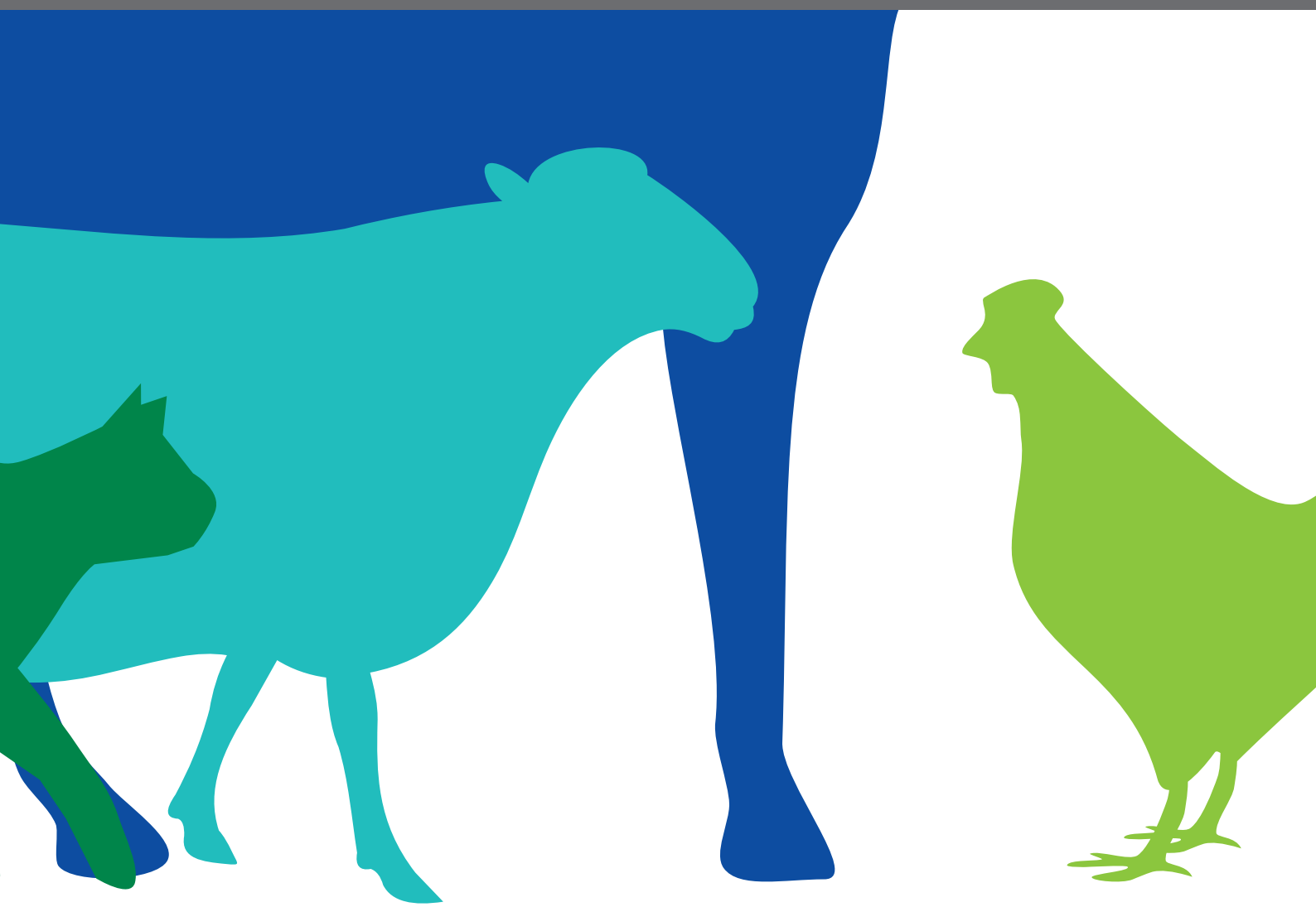
BOVINE TUBERCULOSIS – INTERNATIONAL PERSPECTIVES ON EPIDEMIOLOGY AND MANAGEMENT

EDITED BY: Andrew W. Byrne, Adrian R. Allen, Daniel J. O'Brien and Michele A. Miller

PUBLISHED IN: Frontiers in Veterinary Science



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ISSN 1664-8714
ISBN 978-2-88963-052-3
DOI 10.3389/978-2-88963-052-3

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BOVINE TUBERCULOSIS – INTERNATIONAL PERSPECTIVES ON EPIDEMIOLOGY AND MANAGEMENT

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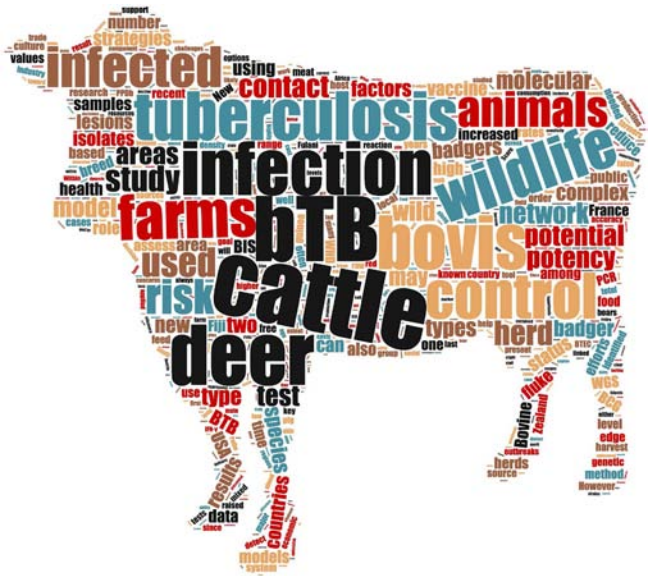

Image: "Tuberculous Word C(l)ow(d)", Andrew Byrne.

Bovine tuberculosis (bTB) is a significant zoonotic pathogen with a global distribution, and a considerable economic impact. It has a notoriously complex epidemiology, varying by affected region and often involving multiple-host species. Here we present an international collection of papers that address both national and international factors impacting on the control of bovine tuberculosis. We hope this Research Topic will provide a forum which may generate a greater understanding of the disease in a wider context, and inform future eradication efforts through the design of more effective interventions.

Citation: Byrne, A. W., Allen, A. R., O'Brien, D. J., Miller, M. A., eds. (2019). Bovine Tuberculosis – International Perspectives on Epidemiology and Management. Lausanne: Frontiers Media. doi: 10.3389/978-2-88963-052-3

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frontiers in Veterinary Science

EDITORIAL
published: 25 June 2019
doi: 10.3389/fvets.2019.00202

Editorial: Bovine Tuberculosis—International Perspectives on Epidemiology and Management

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Keywords: bovine tuberculosis, epidemiology, eradication, international, comparison

OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *15 April 2019*
Accepted: *06 June 2019*
Published: *25 June 2019*

Citation:
Byrne AW, Allen AR, O'Brien DJ and Miller MA (2019) Editorial: Bovine Tuberculosis—International Perspectives on Epidemiology and Management. Front. Vet. Sci. 6:202. doi: 10.3389/fvets.2019.00202

Editorial on the Research Topic

Bovine Tuberculosis—International Perspectives on Epidemiology and Management

INTRODUCTION

Bovine tuberculosis (bTB) remains a prominent zoonotic pathogen on the world stage, with significant impacts on animal and human health, and economic well-being. Eradication is hampered by a complex epidemiology, which in many countries involves wildlife hosts. Indeed, despite advances in understanding gleaned from national programs of bTB eradication, much of our understanding of transmission mechanisms, diagnostics, control, and multi-host infection systems remains opaque.

In this collection of *Frontiers in Veterinary Science*, as editors, we felt these limitations could best be addressed by adopting an international perspective. Localism understandably focuses on the fine details of problems at hand, but can perhaps overlook issues that only become apparent when compared to the experiences of others.

Below we summarize the papers published in this truly international collection, and highlight some themes. We trust readers will find these articles as stimulating to read as they were to edit.

Theme: Modeling as a Tool for Understanding bTB

Modeling approaches were used to gain insights and make inferences on a number of different problems in bTB management.

Ladreyt et al. in their paper "*In silico* Comparison of Test-and-Cull Protocols for Bovine Tuberculosis Control in France," developed a stochastic simulation model to explore the potential impacts of differing test-and-cull options relative to whole herd depopulations in terms of epidemiological effectiveness, cost and acceptability to stakeholders. The authors suggest the model will be of utility for decision support for comparing alternative control protocols.

In "Exploring the Fate of Cattle Herds With Inconclusive Reactors to the Tuberculin Skin Test," Brunton et al. used statistical survival models to track the future risk of herds which retained inconclusive reactor (IR) animals. The authors reported significant increased future risk in herds with IR animals detected, relative to negative herds, and suggested that careful decision-making around the management of IR reactors needs to be employed to help mitigate such risk.

Statistical models were used by Frankena et al. in their paper "A New Model to Calibrate a Reference Standard for Bovine Tuberculin Purified Protein Derivative in the Target Species" to determine the potency of an in-house developed, reference standard of *M. bovis* purified protein derivative (PPDb). Secondarily, the model determined the precision and accuracy of the test relative to a standard (guinea pig) potency test. Such work will be important for ensuring uniformity of standards of PPD for bTB test diagnostics, as the Bovine International Standard (BIS) supply is limited.

Simulation and mathematical modeling has been a fundamental tool for bovine tuberculosis control, especially where wildlife reservoir species are involved. In "Modeling as a Decision Support Tool for Bovine TB Control Programs in Wildlife," Smith and Delahay concentrate on the badger-TB episystem to highlight methodological approaches used to model disease dynamics and control interventions. The paper also highlights how future data collection could be integrated into modeling endeavors, and how such models could be optimally utilized.

Theme: Host Genetics

The use of genetic selection to improve animal health has emerged from recent advances in genomics and their application to epidemiological data. Resistance to bTB is a heritable trait in cattle, and provides an additional tool by which the disease can be controlled.

In their Perspective article, "Can We Breed Cattle for Lower Bovine TB Infectivity?," Tsairidou et al. pose a counterpoint to existing efforts to use genomic selection in cattle to improve resistance to bTB. Specifically, they raise the possibility that infectivity may be a separate genetic trait in cattle—indicating that some animals could be predisposed to being bTB "super-spreaders." They also show by simulation modeling, how including selection for an infectivity phenotype alongside resistance could deliver reduced bTB prevalence for cattle industries.

In their Original Research article, "Impact of Genetic Selection for Increased Cattle Resistance to Bovine Tuberculosis on Disease Transmission Dynamics," Raphaka et al. describe their development and evaluation of a simulation model which details the outcomes of varying intensities of genetic selection for bTB resistance in UK dairy cattle. Their findings show that adding genetic selection to bTB control strategies can aid in the reduction of bTB prevalence and severity of breakdowns. The authors suggest the use of genetic selection tools, alongside more traditional test and slaughter methods, could enhance eradication schemes.

Theme: Pathogen Genetics

Genomic methods are revolutionizing traditional molecular epidemiological approaches to disease source attribution, principally due to their much superior resolution. Application to bTB infectious systems promises to improve our knowledge of transmission dynamics.

Orloski et al. provide a current description of the diversity of *M. bovis* isolates in cattle and farmed cervids in "Whole Genome Sequencing of *Mycobacterium bovis* Isolated from Livestock in the United States, 1989-2018." The authors conclude that the use of whole genome sequencing (WGS) can reduce time and costs associated with epidemiological investigations, provides a powerful tool for advancing our understanding of transmission, and may improve eradication programs.

Similarly, in "Whole Genome Sequencing for Determining the Source of Mycobacterium bovis Infections in Livestock Herds and Wildlife in New Zealand," Price-Carter et al. demonstrated the increased resolution of WGS for identifying sources of infection in outbreaks, especially in areas complicated by wildlife reservoirs. WGS was also able to provide information on the evolution of *M. bovis* within New Zealand animal populations, becoming a key component in their eradication strategy.

Theme: Wildlife Reservoirs

In multi-host epidemics, control or eradication of bTB in domestic hosts is often unachievable if disease control in wildlife reservoir populations is not simultaneously implemented.

As highlighted by both Buddle et al. and Gormley and Corner there are few options available to control TB in wild populations that are both effective and socially acceptable. Buddle et al. in "Efficacy and Safety of BCG Vaccine for Control of Tuberculosis in Domestic Livestock and Wildlife," highlight Bacillus Calmette Guerin (BCG) vaccination of wildlife as one such approach. The authors highlight the evidence to suggest BCG can induce protection in European badgers, white-tailed deer, wild boar, and brushtail possums. Gormley and Corner explored the palatability of wildlife interventions to different stakeholders and value systems in their review "Wild Animal Tuberculosis: Stakeholder Value Systems and Management of Disease." The authors suggest that factors influencing consensus on management approach can depend on the species in question, the economic cost-benefit and ethical considerations. The review identified that interventions that are acceptable in one region, may not be agreed upon in another region, even amongst broadly similar stakeholder groups.

Vaccination with BCG has been shown to reduce disease in humans caused by *M. tuberculosis*, and Palmer and Thacker have also recently shown its potential for wildlife in "Use of the human vaccine, *Mycobacterium bovis* Bacillus Calmette Guerin in deer." Decreased disease severity in vaccinated deer would likely be accompanied by decreased disease transmission. Progress on the development of oral baits for vaccines will facilitate the effort to implement this potentially valuable tool for addressing *M. bovis* infection in deer, and subsequent spread to livestock.

Bouchez-Zacria et al. in "The Distribution of Bovine Tuberculosis in Cattle Farms Is Linked to Cattle Trade and Badger-Mediated Contact Networks in South-Western France,

2007–2015," used a network modeling approach to investigate cattle farms' bTB risk in France, utilizing both cattle trade data in concert with badger contact networks based on inferred badger home ranges, and *M. bovis* molecular typing. This work highlighted how both spatial relationships and trade relationships between farms, along with linkages associated with badger territorial behavior can be attributed to bTB risk, highlighting the complexity of multi-host epidemics.

Human-caused aggregations of animals are a challenging tuberculosis management problem. In "Baiting and Feeding Revisited: Modeling Factors Influencing Transmission of Tuberculosis Among Deer and to Cattle," Cosgrove et al. extend previous modeling work to investigate how spatial and temporal persistence, density and attractiveness of feeding sites for wild deer affect both prevalence in deer and subsequent interspecies transmission. They show why feeding of deer is a relevant issue not only for hunters and wildlife managers, but for cattle producers and agriculture agencies as well.

The essential role of on-farm biosecurity in managing bovine tuberculosis is one of the focal points of VerCauteren et al's. "Persistent Spillback of Bovine Tuberculosis From White-Tailed Deer to Cattle in Michigan, USA: Status, Strategies, and Needs." The authors provide a much-needed summary of biosecurity research undertaken in Michigan to date, and lessons learned. In addition, they make a case for management tools as yet un-, or at least under-utilized: vaccination of wild deer, precision culling of deer on farms, and, notably, strategic habitat manipulations to spatially redistribute deer.

Also focusing on white tailed deer in the USA, Cross et al. "Bovine Tuberculosis Management in Northwest Minnesota and Implications of the Risk Information Seeking and Processing (RISP) Model for Wildlife Disease Management" focuses on how deer hunters sought and acquired information on potential human health risks stemming from *M. bovis* exposure via hunting. Understanding how stakeholders obtain knowledge and form perceptions is crucial to implementing disease management in which they are necessary participants.

A pair of studies in this Research Topic addresses reservoirs of *Mycobacteria* in European wildlife. In "Mycobacterium caprae Infection of Red Deer in Western Austria–Optimized Use of Pathology Data to Infer Infection Dynamics," Nigsch et al. provide for the first time in English a detailed description of the *M. caprae* outbreak spilling over to sympatric pastured cattle. They advocate for the use of a summary measure of detailed pathology findings—the Patho Score—as a quick and easy means of drawing broader inferences about the epidemiology of the disease in red deer, with implications for control in cattle.

In France, Réveillaud et al. recount the design and implementation of a nationwide system for *M. bovis* testing. "Infection of Wildlife by *Mycobacterium bovis* in France Assessment Through a National Surveillance System, Sylvatub" details the structure and integration of a surveillance system that acquires samples from diverse governmental and non-governmental partners, via both active and passive means. Thus, far, in addition to cattle, infection has been detected mainly in badgers and wild boar, although not to the extent noted elsewhere in Europe.

Many countries where *M. bovis* cycles between cattle and free-ranging wildlife declare the necessity of eliminating the disease, but such goals are typically easier proclaimed than achieved. Exactly how can eradication be methodically pursued in the face of real-world logistical, cost and policy constraints? In "Roll-Back Eradication of Bovine Tuberculosis (TB) From Wildlife in New Zealand: Concepts, Evolving Approaches, and Progress," Nugent et al. characterize a Bayesian "Proof of Freedom" framework currently being implemented on an area-specific basis, as well as how, incorporating decision theory, the approach is being scaled up to the national level.

Theme: The Importance of Diagnostics

As well as the diagnostic regent variation (see Frankena et al.), host physiological and immunological status can affect the performance of diagnostic tests. Kelly et al. in "Association of Fasciola gigantica Co-infection with Bovine Tuberculosis Infection and Diagnosis in a Naturally Infected Cattle Population in Africa" present evidence to suggest an association between a liver fluke species' infection and bTB risk in different cattle breeds in Cameroon. Parasite co-infection is believed to affect the balance between Th1 and Th2 arms of the immunological response to infection, potentially impacting either diagnosis or disease progression. Kelly et al. report a complex interaction between the presence of bTB lesions and the performance of the interferon-gamma ante-mortem test in mixed breed animals, but not Fulani cattle.

In "Validation of a Real-Time PCR for the Detection of Mycobacterium tuberculosis Complex Members in Bovine Tissue Samples," Lorente-Leal et al. discuss their development and validation of a real time PCR assay for the detection of members of the *Mycobacterium tuberculosis* complex (MTBC) organisms in bovine tissues. Their assay exhibits good sensitivity and specificity relative to culture, and whilst the latter remains the gold standard for disease confirmation, the speed of the PCR assay could rapidly increase turnaround times, which is potentially of substantial epidemiological importance.

Hadi et al. describe in "Development of a Multidimensional Proteomic Approach to Detect Circulating Immune Complexes in Cattle Experimentally Infected with *Mycobacterium bovis*," the preliminary findings of a novel proteomic method for the diagnosis of bTB infection in cattle. They identify immune complex proteins from the MTBC in experimentally infected cattle suggesting potential for this type of diagnostic approach. They also identify ways in which the method can be improved before validation on a larger dataset.

Effective management of bTB requires development of accurate diagnostic tests in the wide spectrum of susceptible hosts. However, the conventional tuberculin skin test has low sensitivity in camelid species. In "Development and Evaluation of a Serological Assay for the Diagnosis of Tuberculosis in Alpacas and Llamas," Infantes-Lorenzo et al. describe the development of an ELISA based on the antigen P22 that had high specificity and sensitivity. This provides improved methods of detecting *M. bovis* and *M. microti* in New World camelids.

Theme: "The Human Component"

The "human component" of bTB epidemiology and control was highlighted in a number of papers relating to societal values and ethics of bTB control, as well as human zoonotic risk.

In "Risk Perceptions and Protective Behaviors Toward Bovine Tuberculosis Among Abattoir and Butcher Workers in Ethiopia," Fekadu et al. describe the findings of their modeling study elucidating the risk factors associated with consumption of raw meat, and contraction of bTB by abattoir workers in Ethiopia. The data showed that despite workers' knowledge of the dangers involved in eating raw meat, and awareness of bTB risk, there was little uptake of preventative behavior, particularly in male workers and older demographics. The authors recommend tailoring public health interventions toward not only increasing awareness of zoonotic risk, but also influencing behavioral change.

In "TB Control in Humans and Animals in South Africa: A Perspective on Problems and Successes," Meiring et al. draw from the lessons of human TB control in South Africa and provide a comparison of these steps to bTB. Reduction in human TB incidence in this high burden country has been achieved through antimicrobial treatment and increasing awareness, both of which are lacking for bTB. Lack of effective movement controls, mandatory testing program, veterinary resources including state diagnostic laboratories, point-of-care tests for cattle, and presence of bTB in wildlife populations contributes to persistence of disease. A multi-sector approach, as seen for human TB, is needed to address bTB.

Although bTB is a global disease, it can be neglected in smaller nations. Borja et al. describe findings of bTB in Fiji in "A Retrospective Study on Bovine Tuberculosis in Cattle on Fiji: Study Findings and Stakeholder Responses." Despite a bTB control program, goals were not being achieved due to lack of training of staff conducting TB testing, absence of standard data collection and unregulated movement of cattle. Revision of the program resulted in increased detection but also farmers' concerns. This study highlights the technical and social challenges to effective disease control.

Perceptions of cattle farmers are critical to implementing tuberculosis management strategies that ultimately rely upon their proactive cooperation and compliance. In "Negotiated Management Strategies for Bovine Tuberculosis: Enhancing Risk Mitigation in Michigan and the UK," Little compares experiences with cattle producers in the two countries facing the need for heightened biosecurity on their farms. Rather than relying solely upon voluntary compliance, she demonstrates the value of negotiated outcomes in obtaining producer support for more stringent regulations necessitated by the presence of *M. bovis* in wildlife.

Finally, in their Hypothesis and Theory article, "Bovine Tuberculosis in Britain and Ireland – A Perfect Storm? The Confluence of Potential Ecological and Epidemiological

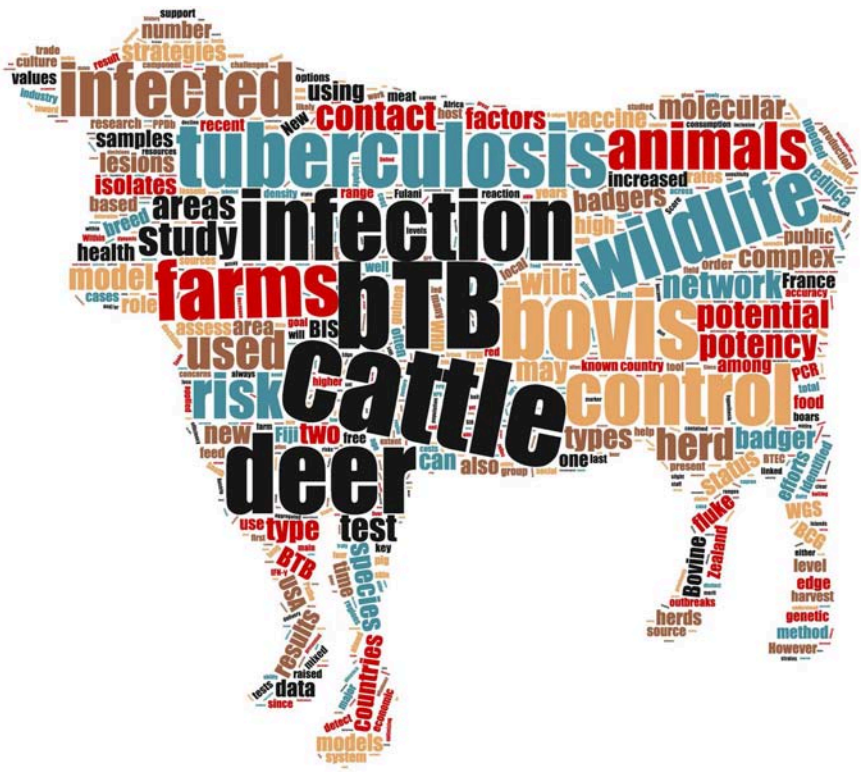

FIGURE 1 | Frequency weighted "word cloud" using abstracts from the Research Topic "Bovine tuberculosis—international perspectives on epidemiology and management."

Impediments to Controlling a Chronic Infectious Disease," Allen et al. focus on the ongoing problems of eradicating bTB in Britain and Ireland—asking, why are these territories so different from their continental European neighbors? Is there a cocktail of unique epidemiological and ecological factors that are hindering efforts? If so, what could they be, and how can they be addressed? Suggested factors included the presence of a wildlife reservoir, differing diagnostic approaches, variation in pathogen genetics, co-infection, the structure and density of animal movement trade networks, and the potential for environmental persistence and a benign climate.

CONCLUSION

This Research Topic was contributed to by 150 authors, representing over 15 countries. As highlighted, some common themes emerged from these contributions (**Figure 1**). The value, utility, and contribution to understanding bTB made from different modeling approaches was prominent. Wildlife was a significant component of the epidemiology of bTB in a number of different countries (Ireland, the UK, France, USA, South Africa, and New Zealand), with disease risk management technically and sociologically challenging. Genetic tools were found to be a core enabler to derive new insights into bTB epidemiology at both the host and pathogen levels. The importance of vaccines and diagnostics, their performance characteristics, and their standardization remains highly relevant. Finally, the "human component" was a significant theme, in terms of societal values, ethics and perceived (and realized) cost-benefits of interventions, as well as the practical risk of *M. bovis* infection as a zoonotic pathogen. These themes reflect the reality that bTB control across countries is a multi-factorial problem requiring significant specialist input from various disciplines, in conjunction with "buy-in" from stakeholders across broader society, in order to move efforts from control to eradication.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 23 October 2018
doi: 10.3389/fvets.2018.00265

In silico Comparison of Test-and-Cull Protocols for Bovine Tuberculosis Control in France

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *07 June 2018*
Accepted: *02 October 2018*
Published: *23 October 2018*

Citation:
Ladreyt H, Saccareau M, Courcoul A and Durand B (2018) In silico Comparison of Test-and-Cull Protocols for Bovine Tuberculosis Control in France. Front. Vet. Sci. 5:265. doi: 10.3389/fvets.2018.00265

Whole depopulation of cattle herds (WHD) confirmed infected by bovine tuberculosis (bTB) has led since the 1950s to a drop of herd incidence in France below 0.1% in 2000, justifying the current officially bTB free (OTF) status of the country. However, this protocol is expensive, ethically questionable, and difficult for breeders to accept because the number of confirmed animals in an infected herd is often very low. A test-and-cull protocol combining at least three screening sessions of the entire herd followed by the slaughter of all the non-negative animals has been used for some years. The aim of this work was to evaluate *in silico* the epidemiological effectiveness, the public costs and the acceptability to farmers of this test-and-cull protocol as well as of several ones. A stochastic compartmental model of within-herd bTB spread was used. Six test-and-cull protocols were compared: two versions of the official protocol and four alternatives with varying delays between screenings, and varying tests used. Protocols were simulated for an average French beef herd, and compared to WHD. Three key indicators were computed: the failure probability of the protocol (a failure being defined as an herd recovering its OTF status recovery while still infected, indicator of epidemiological effectiveness), its overall public cost and the percentage of farmers who would have dropped it to switch to WHD (indicator of acceptability to farmers). Failure probability ranged from 1.4 to 12.4% and was null (by definition) for WHD. The median cost varied between 2.7 and 78 K€ for the test-and-cull protocols, vs. 120 K€ for WHD. The percentage of dropout ranged from 7.8 to 22%. The optimal tradeoff between epidemiological effectiveness, public costs, and acceptability to farmers was obtained for protocols with an increased delay (6 months instead of 2 in the currently used protocol) between the last two screening sessions, with either 3 or 2 screening sessions. This study may help improving the official test-and-cull protocol applied in France under European Union regulation, by suggesting alternative protocols, very effective, cheaper, and more acceptable than WHD.

Keywords: bovine tuberculosis, dynamic modeling, cattle herd, disease control, test-and-cull protocol

INTRODUCTION

The European Commission recognizes most of the European countries officially bTB free (OTF) but the infection remains endemic in cattle herds in several parts of Europe such as Spain, Ireland, some regions of United Kingdom, and some regions of Italy (1–5). France has been OTF since 2001 (Decision 2001/26/EC), but this status, which is essential for trade, is threatened by the upsurge of the disease in cattle farms since 2004 (6).The surveillance and control of bTB in Europe focus on animal screening (in slaughterhouses and farms) and the elimination of infection in detected infected herds. The surveillance and control protocols vary according to the local epidemiological situation. In France, in areas with recent outbreaks or where wildlife is involved in bTB transmission, surveillance consists in yearly screening tests, and disease control protocols tend to be drastic. In areas where *Mycobacterium bovis* has not been detected for a long time, surveillance can be reduced to animal screening every 4 years, or can only be based on meat inspection in slaughterhouses, although screening tests are still performed each year in specific herds considered at-risk (e.g., herds producing raw milk or those identified by contact-tracing from recent outbreaks). Disease elimination protocols implemented in outbreaks have also evolved in France since the very first mandatory measures defined in the 1950s. Protocols became progressively more drastic until 1999, when whole depopulation became mandatory in herds where the infection by *M. bovis* had been confirmed (Ministerial Decree of 4 May 1999). These measures allowed an almost complete eradication of bTB in France, reducing the prevalence of 25% of herds infected in 1955 to less than 0.1% in 2001, thus justifying obtaining the officially bTB free (OTF) status (6, 7).

In this context, whole depopulation remains the recommended method of disease elimination in infected herds. In practice, however, it becomes less and less adapted to the epidemiological situation in France, where bTB prevalence remains very low (around 100 herds reported as infected per year) despite a slight increase since 2004 (6). This measure first entails significant public costs, partly due to compensations paid to farmers for all the slaughtered animals, of which only a small number are infected: the average cost for an outbreak (compensations and disinfection) was 107 k€ in 2014 (6). More globally, in 2012, of the 193 million euros dedicated to surveillance and eradication plans by the EU, more than a third was attributed to tuberculosis (8), and more than 17 million euros were spent for bTB control in 2014 in France (6). Studies are then needed to reduce bTB control costs as it is done in other countries (9). A second drawback of whole herd depopulation is that this disease elimination protocol is difficult to accept for obvious ethical reasons for farmers and welfare reasons for animals. In addition, since the within-herd prevalence of the infection is low, very few of the slaughtered animals are confirmed to be infected. Although this does not imply that negative animals are uninfected, the breeders have the impression to have unnecessarily culled their animals. This low acceptability of the protocol can lead to a real lack of effectiveness of the control strategy, which is a source of concern for the animal health authorities (10). Finally, besides cost and acceptability problems, the effectiveness of whole herd depopulation can sometimes be questioned with the recurrence of bTB in some farms after restocking (11). These factors motivated the evolution of French regulations toward a gradual reintroduction of a test-and-cull protocol. This selective slaughter of only animals reacting to a combination of tests was thus authorized under certain conditions throughout France in 2014.

Only one test-and-cull protocol is currently authorized, which has not been evaluated yet. The question arises whether it may be improved in terms of epidemiological effectiveness, public costs and acceptability to the farmer. The current test-and-cull protocol provides for three screening tests with 2 months between each. All three controls have to be consecutively negative to allow the herd regaining its OTF status. The two first screening tests associate single intradermal tuberculin skin test (SIT), gamma-interferon assay (IFN-γ), and a serology test, whereas the third one uses the single intradermal comparative cervical skin test (SICCT). In practice, in areas where the infection by atypical mycobacteria is known to be frequent in cattle, SIT may be replaced by SICCT to increase the specificity of screenings.

The aim of this work was to compare, through modeling and simulation, several test-and-cull scenarios in a bTB-infected farm, to determine the most epidemiologically effective scenario, while evaluating its public costs and its acceptability to the farmer.

MATERIALS AND METHODS

Model

The model was a stochastic compartmental model operating in monthly time steps. Only females involved in reproduction were represented, as other animals were assumed to play only a minor role in the epidemiological system, either because of their short lifespan (calves, beef cattle), or because they are very few (bulls). Each heifer or cow was represented by its age (in years) and its health state, with S (susceptible) for non-infected animals, E (latent) for infected animals that do not excrete the bacteria yet and do not have lesions, and I (infectious) for infected animals having lesions and excreting the bacteria. The dynamics of bTB

TABLE 1 | Parameters of the demographic process included in the model of within-herd bTB dynamics.

Parameter	Value*	Source
Size of the herd	141 animals	(13–15)
Maximal age of cows	15 years	(12)
Stabling period	November to March	(12)
Yearly culling rate	35%	(16)
Age of culled animals	≥ 4 years	(12)
Culling period	January to March	(12)

**Values characterizing of an average French beef cattle herd.*

in a cattle herd, from the *M. bovis* introduction to the elimination of infection was represented by three processes:

- The demographic process: The age structure resulted from the cull of animals, the culling rate being assumed to vary according to the month and to the age class (null for heifers, >0 for cows). The size of the herd was assumed constant and the herd closed: slaughtered animals (because of routine slaughter or due to disease control) were replaced by young animals born in the same herd.
- The infectious process: animals were assumed to be grouped into batches according to their age class, distinct batches being kept in separate buildings or on distant pastures. The transmission of *M. bovis* was thus assumed to only occur between animals of the same batch. However, because of the aging of animals, the composition of batches changed every year, and animal transfers between batches allowed *M. bovis* to spread inside the herd. *M. bovis* transmission intensity was assumed to vary according to whether the animals are housed inside a stable (high intensity of within-batch transmission) or allowed to graze (low intensity of within-batch transmission), the transmission parameter was thus assumed to vary accordingly.
- The detection and control process: it combined ante mortem tests (SIT, SICCT, IFN-γ, and serology), post mortem and confirmation tests (routine or detailed carcass inspection, PCR, bacterial culture, and histology), as well as the culling of all (whole herd depopulation) or specific (test-and-cull) animals. At the individual level, it was assumed that ante-mortem tests may allow detecting animals in the E and I states (according to the sensitivities of these tests), whereas only animals in the I state could be detected by carcass inspection (again according to the sensitivity of routine or detailed inspection). The detection and control process was represented by a succession of steps, one or several tests being implemented at each step on one or several animals. Moves from one step to another depended on the results of these tests, and on those of routine carcass inspection. This representation allowed the model to simulate detection and control programs of arbitrary degree of complexity.

The model parameters were estimated from field data using Approximate Bayesian Computation (ABC) methods. The duration of the latent state was thus estimated 3.5 months (95% credible interval: 2–8 months), the transmission parameter was estimated 0.43 month^{-1} (95% CI: 0.16–0.84) inside buildings, and 0.08 month^{-1} (95% CI: 0.01–0.32) on pastures. Based on these estimates, the model was then validated using an independent dataset.

The model was implemented using the R software. A detailed description of its structure, parameterization and validation can be found in Bekara et al. (12).

Parameters

Demographic and Infection Process

As most of herds detected infected in France are beef herds, only this type of herd (i.e., breeding and suckling herds) was addressed in our study, and the values of the parameters of the demographic process (**Table 1**) were chosen to represent a typical French beef herd, in 2017. Three animal batches were considered, 1-, 2-, and 3-year old heifers, and cows with their calves.

At the beginning of each simulation, the values of the two transmission parameters (inside buildings and on pastures) and of the duration of the latent period were randomly drawn from the joint posterior distribution produced by the ABC estimation procedure (12). Moreover, the infection was assumed to be brought in the herd by the introduction of a single infected animal (I health state), at a randomly chosen month of the first simulated year.

Modeled Surveillance and Control Measures

Two sets of possible surveillance measures (feasible under present French field conditions) were represented in the model. Herds were assumed subjected to routine screening, based on skin tests of animals over 1 year of age. If at least one reactor was detected, the herd was placed under movement restriction until

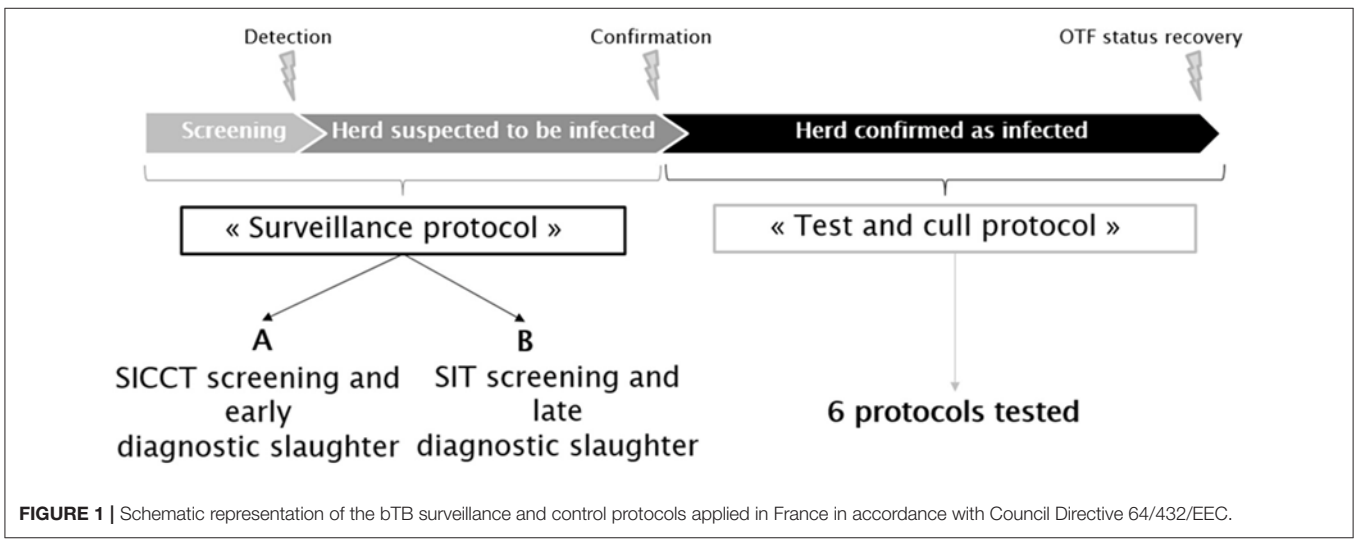

FIGURE 1 | Schematic representation of the bTB surveillance and control protocols applied in France in accordance with Council Directive 64/432/EEC.

confirmation (or not) of bTB infection. This phase (from the routine skin testing until the confirmation of infection) will be hereafter called the "surveillance protocol" (**Figure 1**). Two such surveillance protocols were distinguished. The first one, termed below "A," used a SICCT annual screening of the herds, followed by the slaughter of non-negative animals to infirm or confirm the suspicion. The second one, termed below "B," used a SIT annual screening of the herd. Reactors are retested in the following days with IFN-γ; the non-negative animals being then slaughtered for confirmatory purposes. When bTB infection is confirmed, the herd is kept under movement restriction during the implementation of the test-and-cull protocol, which ends when the herd is reported as free from bTB infection (the OTF status of the herd is recovered).

The current official test-and-cull protocol is based on three series of tests called "controls" (**Figure 2**) carried out on the entire herd (animals over 1 year of age) every 2 months. The first two controls combine a SIT ("SIT Official" protocol) or a SICCT ("SICCT Official" protocol, in the context of a surveillance of type A), an IFN-γ test, and a serological test. The third control consists of a SICCT. Moving from control X to control X+1 requires all the tested animals to be negative to all tests; otherwise the reactors are culled and the protocol moves back to the 1st control, 2 months later. The OTF status of the herd is recovered when the three controls are negative consecutively.

Based on these two reference protocols, several parameters were modified to specify alternative protocols: the inter-control time periods, the number of controls required and the type of tests used. Four new protocols were thus studied (**Figure 2**): a protocol where the time period between control 2 and control 3 was increased from 2 to 6 months (called "Increased delay" protocol), a protocol where a control was removed ("Two controls" protocol), a protocol where a control with SICCT, IFN-γ and serology was added ("Four protocols" protocol), and a protocol using only SICCT ("SICCT only" protocol).

TABLE 2 | Sensitivities and specificities of tests used for bTB surveillance and control in the model of within-herd bTB dynamics.

Test	Sensitivity (median [CI 95%])	Specificity (median [CI 95%])	References
SIT	0.81 [0.53; 0.94]	0.91 [0.63; 1.00]	(17)
SICCT	0.75 [0.61; 0.86]	1 [0.99; 1.00]	(17)
IFN bovine and avian PPD	0.70 [0.55; 0.92]	0.94 [0.88; 0.97]	(17)
IFN ESAT6	0.79 [0.64; 0.89]	0.99 [0.98; 1.00]	(17)
Serology	0.60 [0.31; 0.86]	0.93 [0.84; 0.97]	(17)
PCR	0.86 [0.65; 0.96]	1 [1.00; 1.00]	(17)
PCR NRL	1*	1	NRL
Histology	0.66 [0.41; 0.84]	1 [0.95; 1.00]	(17)
Culture	0.74 [0.46; 0.94]	1 [0.73; 1.00]	(17)
Routine necropsy (meat inspection)	0.71 [0.37; 0.92]	1 [0.99; 1.00]	(17)
Detailed necropsy (suspected animals)	0.96 [0.82; 1.00]	1 [0.99; 1.00]	(17)

**In the context of confirmation of positive PCR results obtained by local veterinary laboratories.*

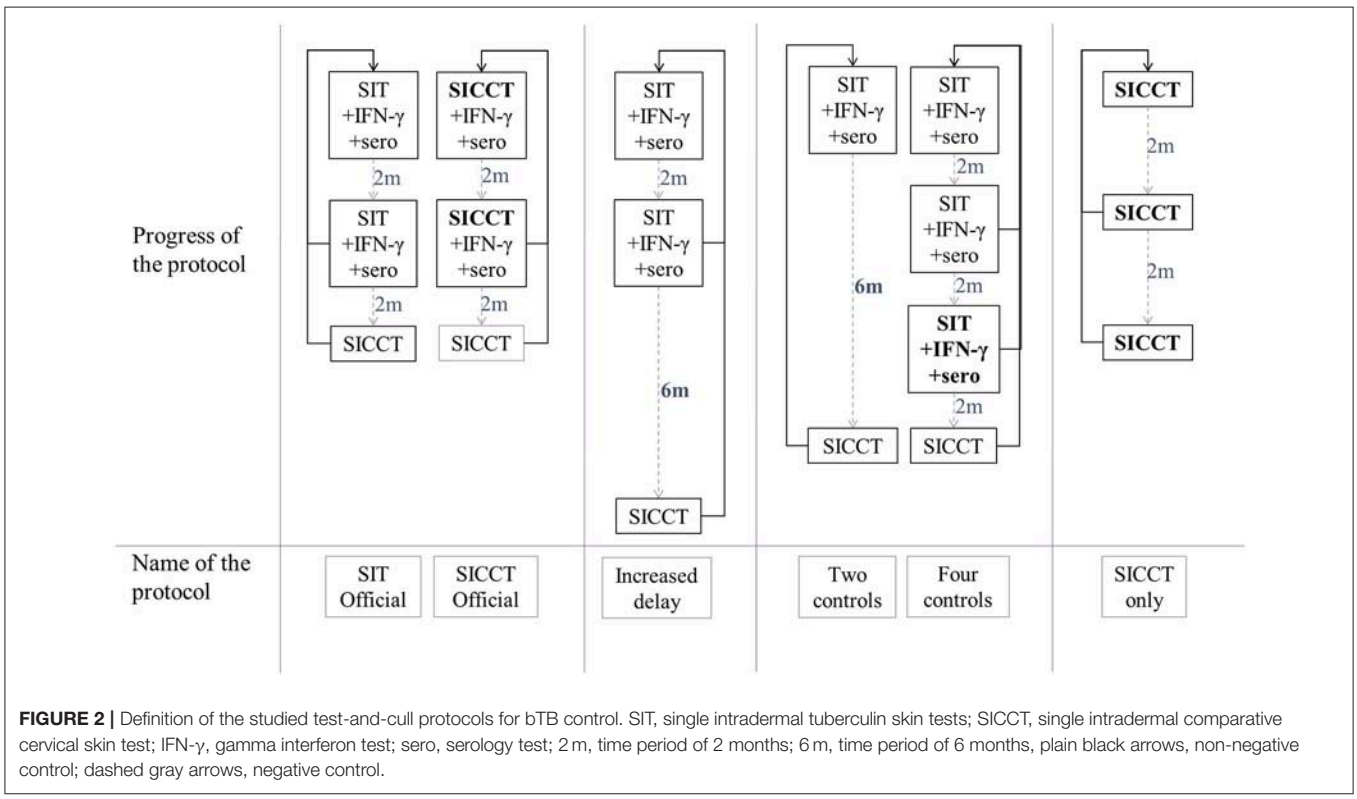

FIGURE 2 | Definition of the studied test-and-cull protocols for bTB control. SIT, single intradermal tuberculin skin tests; SICCT, single intradermal comparative cervical skin test; IFN-γ, gamma interferon test; sero, serology test; 2 m, time period of 2 months; 6 m, time period of 6 months, plain black arrows, non-negative control; dashed gray arrows, negative control.

These scenarios were compared to each other, and to whole depopulation protocol.

Tests sensitivities and specificities were fixed according to the literature and to the expertise of the French national reference laboratory (NRL) for bTB (**Table 2**).

Indicators

Epidemiological Effectiveness

The failure probability of the protocol was the probability that a herd would wrongly regain its OTF status while still infected (some infected animals, either latent E or infectious I, are still present but remain undetected). This corresponds to a failure of the system of detection of infected animals (e.g., lack of test sensitivity, animals not tested), and thus of the epidemiological effectiveness of the protocol. The number of infectious animals-months was considered a proxy for the risk of transmission of the infection to the neighboring farms and to the breeder or the farm staff. The number of infectious animals-months was computed as the sum across all infected animals of their total infectivity time (e.g., duration in months spent in I state).

Public Costs

The proportion of susceptible (S) animals among those culled during the test-and-cull protocol was an indicator of both cost and epidemiological effectiveness, as these false-positive animals are unnecessarily culled and compensated. It was computed as the number of susceptible animals culled during the test-and-cull protocol over the number of culled animals during the test-and-cull protocol (i.e., the number of animals to be compensated).

The overall public costs combined compensations paid to the farmer (for slaughtered animals, calculated based on expert opinions, data provided by French veterinary services (French Ministry of agriculture) of departments Dordogne and Cote d'Or and presented in **Table 3**) and the laboratory and veterinary costs (prices of analyses, veterinary visits and acts, presented in **Table 4**). The total farmer compensation costs were calculated for each simulation by summing the average compensation costs of each slaughtered animal, according to its age group. The total laboratory and veterinary costs were calculated by multiplying the number of tests and veterinary visits by their respective unitary costs.

TABLE 3 | Average compensation paid to beef cattle farmers per slaughtered animal according to the age group, calculated from compensation reports of seven French herds having been subjected to a test-and-cull protocol between 2014 and 2017, for bTB control.

Age group	Average compensation cost (€)
0–1 year	507
1–2 years	712
2–3 years	1232
3–6 years	906
6–10 years	758
10–15 years	759

(Source: French Ministry of Agriculture).

Acceptability

Seven acceptability indicators were defined. First, the total number of culled animals (during surveillance protocol and test-and-cull protocol) was calculated (**Table 7A**, indicator 1 on **Figure 3**). Besides, the number of culled animal confirmed infected with *M. bovis* by culture (**Table 7A**) was computed as a proxy for acceptability: indeed, the animals culled but not confirmed infected by culture (even if they are infected) are most often seen by breeders as animals "slaughtered for nothing," which is one of their biggest sources of frustration. The total time needed for disease elimination and OTF status recovery (**Table 7B**, indicator 2 on **Figure 3**) represented the total time needed for the herd to actually get rid of the infection and correctly regain its OTF status. It could include periods when the herd had temporarily recovered its OTF status but was still infected (see "requalified herd" period while still I animals on **Figure 3**). The delay between a confirmation and an OTF status recovery represented the duration of strict movement restriction of the herd (**Table 7B**, indicator 3 on **Figure 3**), which is source of non-acceptability. Modeling allowed computing the duration of the wrongly movement restriction of the herd (**Table 7B**, indicator 4 on **Figure 3**) by measuring the delay between the slaughter of the last infected animal (E or I) (i.e., the "real elimination of the infection" moment on **Figure 3**) and the OTF status recovery (**Figure 3**). The number of veterinary visits (**Table 7A**) corresponded to the number of controls needed to eliminate the infection and recover the OTF status. This number of visits could be greater than the number of controls provided for by the protocol, when one or more controls had been unfavorable, leading to restart the protocol.

The global acceptability was finally evaluated by a synthetic indicator corresponding to the percentage of simulations for which the farmer would have dropped out the test-and-cull protocol before its end (percentage of drop out). The acceptability of the measures by the breeders is a difficult notion to evaluate without sociological survey. It was assumed, according to personal communications from local representatives of veterinary services, that farmers would drop out the protocol if the number of animals confirmed infected exceeded 3, or if the duration of the protocol exceeded 26 months.

TABLE 4 | Unit costs of veterinary acts and laboratory analyses used for bTB control in France.

Act/Analysis	Average price (€)	Source
Veterinary visit	27.7	(18)
Blood sample	2.77	(18)
SIT	2.77	(18)
SICCT	6.93	(18)
IFN	50	LDA 24, personal communication, 2017
Serology	12	LDA 24, personal communication, 2017; LDA 21, personal communication, 2017
PCR	50	LDA 24, personal communication, 2017
Culture	50	LDA 24, personal communication, 2017
Histology	50	Pricing grid LAPVSO, Vet diagnostics

For each scenario, 1,000 simulations were conducted, leading to proportions (for binary indicators) or to distributions of indicator values, reported below based on medians, 2.5 and 97.5% percentiles. Finally, three key indicators were represented graphically: the failure probability, the overall public costs, and the percentage of dropout. The six studied test-and-cull protocols were then separately ranked according to each of these three key indicators, and the global rank of each protocol was computed as the average value of these three ranks.

RESULTS

Epidemiological Effectiveness

Among the test-and-cull protocols (**Figure 2**), the "Increased delay" had the lowest failure probability: respectively 1.4 and 1.5% after an A (i.e., SICCT annual screening and early diagnostic slaughter) and a B (i.e., SIT annual screening and retesting with IFN-γ before diagnostic slaughter) surveillance protocols (**Table 5**). On the contrary, the official protocols presented the highest failure probability: 8.3% after an A surveillance protocol ("SICCT Official" protocol) and 12.4% after a B surveillance protocol ("SIT Official" protocol). In general, the failure probability was slightly higher after a B surveillance protocol. After an A surveillance protocol, the number of infectious animals-months remained low regardless of the test-and-cull protocol (median of 1 to 2 animal-months). This number was higher after a B surveillance protocol (median of 3 to 4 animal-months).

Public Costs

Although the difference was moderate, the B surveillance protocol always induced higher public costs than the A one (**Table 6B**). Whole herd depopulation was clearly the most expensive protocol with a median overall cost of 120 K€ per infected herd. The "Four controls" protocol was the most expensive of the test-and-cull protocols, with median values between 73.2 and 78 K€. However, the others were not much cheaper except the "Two controls" which costed more than 20 K€ less, and the "SICCT only" which was the cheapest protocol, costing between 2.7 and 4.8 K€. In this latter case, very few animals were culled during the protocol (between 0 and 1 in median **Table 6A**) leading to lower compensations. In addition, this protocol did not use IFN-γ at all, which is an expensive screening test.

The proportion of S animals among the animals culled during the protocol was high and reached 100% in median after an

TABLE 5 | Epidemiological effectiveness indicators of test-and-cull protocols to control bTB in an average French beef cattle herd, compared to whole herd depopulation.

Protocol	Failure probability (%)		Number of infectious animals-months[a]	
Surveillance	**A**	**B**	**A**	**B**
Test-and-cull				
SIT Official	7.3	12.4	2 [0–92]	4 [0–140]
SICCT Official	8.3	/[b]	2 [0–80]	/
Increased delay	1.4	1.5	1 [0–50]	4 [0–92]
Four controls	5.0	5.0	2 [0–52]	3 [0–77]
Two controls	4.4	6.5	1 [0–54]	3 [0–111]
SICCT only	7.5	5.4	2 [0–65]	3 [0–89]
Whole depopulation	0 by def.	0 by def.	0 by def.	0 by def.

[a] *Median, brackets: 2.5 and 97.5% percentiles.*
[b] *"SICCT Official" can only be implemented in the context of a surveillance of type A, which uses SICCT for annual screening.*

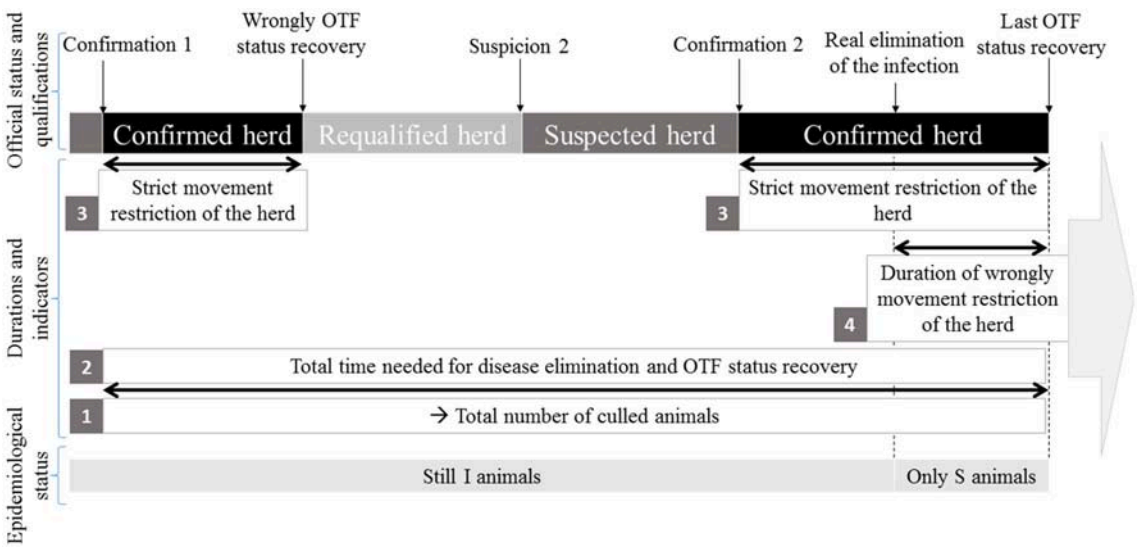

FIGURE 3 | Representation of four indicators [total number of culled animals (1), total time needed for disease elimination and OTF status recovery (2), strict movement restriction of the herd (3), duration of wrongly movement restriction of the herd (4)] in parallel with the evolution of the official and epidemiological status of the herd.

TABLE 6 | Public costs indicators of test-and-cull protocols to control bTB in an average French beef cattle herd, compared to whole herd depopulation.

Protocol	Number of culled animals during protocol[a]		Proportion of S among animals culled during protocol (%)[a]	
Surveillance	**A**	**B**	**A**	**B**
A				
Test-and-cull				
SIT Official	39 [25–107]	49 [28–131]	100 [83–100]	95.9 [66–100]
SICCT Official	20 [10–66]	/	100 [69–100]	/
Increased delay	40 [26–101]	49 [27–135]	100 [83–100]	95.9 [69–100]
Four controls	50 [37–127]	54 [34–131]	100 [88–100]	98.1 [82–100]
Two controls	22 [11–74]	29 [12–90]	100 [79–100]	96.4 [70–100]
SICCT only	0 [0–12]	1 [0–25]	0,0 [0–0]	0 [0–0]
Whole depopulation	141	141	100 [94–100]	99.3 [84–100]

Protocol	Lab/vet costs (K€)[a]		Compensation costs (K€)[a]		Overall cost (K€)[a]	
Surveillance	**A**	**B**	**A**	**B**	**A**	**B**
B						
Test-and-cull						
SIT Official	20.1 [18–55]	26.9 [18–69]	35.6 [23–102]	44.8 [25–130]	56 [41–158]	71.7 [43–201]
SICCT Official	20.3 [18–63]	/	18.4 [9–62]	/	40 [27–131]	/
Increased delay	20.2 [18–57]	27.1 [18–69]	36.0 [24–95]	44.2 [25–112]	56.9 [42–151]	71.3 [31–185]
Four controls	27.1 [23–68]	29.5 [22–69]	46.0 [34–115]	48.5 [31–120]	73.2 [58–182]	78 [54–187]
Two controls	11.2 [9–41]	17.7 [9–47]	20.3 [10–68]	26.4 [10–82]	31.6 [20–106]	44.8 [20–126]
SICCT only	2.7 [2–10]	3.6 [2–13]	0.0 [0–12]	0.9 [0–22]	2.7 [2–21]	4.8 [2–35]
Whole depopulation	0 [0–1]	0.2 [0–1.8]	120 [115–125]	120 [115–125]	120 [115–125]	120 [116–126]

[a] *Median, brackets: 2.5 and 97.5% percentiles.*

A surveillance, for all the protocols except the "SICCT only," meaning that all infected animals had been culled during the surveillance phase (**Table 6A**). Oppositely, for the "SICCT only," this percentage was null, due to the 100% specificity of SICCT. In the other protocols however, almost all culled animals were false positives. After a B surveillance, the percentage was smaller but remained very high (between 95.9 and 99.3% of culled animals in median were S).

Acceptability

After both A and B surveillances, the "Four control" protocol was the test-and-cull protocol leading to the highest number of culled animals (between 55 and 66 in median) (**Table 7A**). The "SICCT only" protocol only induced the culling of 6 and 14 animals in median. Whatever the protocol, an A surveillance induced fewer culling (between 13 and 8 animals). After an A surveillance, medians of the number of culture-confirmed animals were null regardless the test-and-cull protocol (**Table 7A**). Indeed, almost all animals culled were S (**Table 6A**). The median reached one confirmed animal after a B surveillance, regardless the test-and-cull protocol.

The median number of veterinary visits (or control sessions) needed to eliminate the infection and recover the OTF status showed that in most cases, after an A surveillance, the minimal number of controls was performed (i.e., the number of controls provided for by the protocol), although in some cases, the number of required visits was important (97.5% percentile between 6 and 10 visits). After a B surveillance however, an additional control, in median, was necessary to eliminate the infection and recover the OTF status (**Table 7A**). After an A surveillance, the total time needed for disease elimination and OTF status recovery varied in median between 6 months for the "SIT Official" protocol and 10 months for the "Increased delay" one (**Table 7B**). After a B surveillance, this duration was always about two months longer in median, varying from 8 months for the "SIT Official" and "SICCT only" protocols to 12 months for the "Increased delay" protocol. In whole depopulation, the regulatory depopulation duration is 30 days. However, it is necessary to add the time needed for the cleaning-disinfection operations, the repopulation, and the realization of the tests on the new animals to recover the OTF status. This duration is therefore hardly comparable to that of test-and-cull protocols.

Regardless the surveillance protocol, the median duration of strict movement restriction was similar to the total time needed for disease elimination and OTF status recovery (**Table 7B**). This shows that in most cases, there were no periods of herd

TABLE 7 | Acceptability indicators of test-and-cull protocols to control bTB in an average French beef cattle herd, compared to whole herd depopulation.

Protocol	Total number of culled animals[a]		Number of culled animal confirmed infected with *M. bovis* by culture[a]		Number of veterinary visits[a]	
Surveillance	**A**	**B**	**A**	**B**	**A**	**B**
A						
Test-and-cull						
SIT Official	39 [25–119]	49 [28–138]	0 [0–6]	1 [0–20]	3 [3–9]	4 [3–10]
SICCT Official	20 [10–74]	/	0 [0–7]	/	3 [3–9]	/
Increased delay	40 [26–111]	49 [27–135]	0 [0–6]	1 [0–22]	3 [3–8]	4 [3–10]
Four controls	55 [42–138]	66 [47–159]	0 [0–6]	1 [0–12]	4 [4–10]	5 [4–11]
Two controls	27 [16–89]	40 [22–123]	0 [0–6]	1 [0–15]	2 [2–6]	3 [2–7]
SICCT only	6 [3–29]	14 [4–49]	0 [0–6]	1 [0–17]	3 [3–10]	4 [3–11]
Whole depopulation	141 (by definition)	141 (by definition)	0 [0–4]	1 [0–9]	/	/

Protocol	Total time needed for disease elimination and OTF status recovery (months)[a]		Duration of strict movement restriction (months) [a]		Duration of wrongly movement restriction (months)[a]		Percentage of drop out (%)	
Surveillance	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
B								
Test-and-cull								
SIT Official	6 [6–32]	8 [6–34]	6 [6–11]	8 [6–17]	6 [4–7]	6 [4–7]	10.7	22
SICCT Official	7 [6–32]	/	7 [6–11]	/	6 [5–7]	/	11.3	/
Increased delay	10 [10–26]	12 [10–28]	10 [10–25]	12 [10–27]	10 [8–11]	10 [8–11]	8.5	20.1
Four controls	8 [8–33]	10 [8–34]	8 [8–20]	10 [8–21]	8 [6–9]	8 [6–9]	9.6	16
Two controls	8 [8–33]	10 [8–34]	8 [8–20]	10 [8–21]	8 [6–9]	8 [6–9]	7.8	21.1
SICCT only	7 [6–33]	8 [6–34]	6 [6–18]	8 [6–21]	6 [6–6]	6 [6–7]	12.8	22

[a] *Median, brackets: 2.5 and 97.5% percentiles.*

infection with false OTF status. However, the 97.5% percentiles showed that these two durations could differ up to 21 months. Thus, these situations were rare, but when they occurred, they lasted a long time (between 6 and 21 months), except for the "Increased delay" protocol where the difference was only 1 month. Duration of the wrongly movement restriction was also most of the time equal to the total time needed for disease elimination and OTF status recovery after an A surveillance (**Table 7B**). This means the infection was eliminated from the herd when the last infected animals were culled, during the surveillance period. The necropsy confirming the infection, the herd was declared infected and the test-and-cull protocol started although there was no more infected animal. Only the "SICCT Official" and "SICCT only" scenarios had differences of 1 month. However, after a B surveillance, the median durations of wrongly movement restriction were shorter than the total time needed for disease elimination and OTF status recovery. Infection was eliminated from the farm 2 months in median after the confirmation and the implementation of the test-and-cull protocol.

After an A surveillance, the "Two controls" protocol had the lowest percentage of drop out: 7.8% (**Table 7B**). After a B surveillance however, the "Four control" protocol was the one with the lowest but still quite high percentage of drop out of 16%. The "Two controls" implied 21.1% of drop out. "SICCT only" had the higher percentage of drop out in both A and B cases, but implied 12.8% of drop out after an A surveillance while it reached 22% of drop out after a B surveillance, like the "SIT Official" protocol.

Indeed, considering the percentage of drop out as the main indicator for acceptability, we can note that acceptability was better after an A surveillance, regardless the test-and-cull protocols.

Global Analysis and Ranking of the Protocols

The failure probability of the protocols function on their overall public cost is plotted in **Figure 4**. The percentage of drop out is indicated as a label for each protocol. In this representation, the optimal protocols should be located in the lowest left part of the graph, (attention needs also to be paid to the acceptability). **Figure 4** confirms that B surveillance (blue markers) induced increased public costs (blue markers being always on the right of red markers) and decreased acceptability (superscripts of blue markers being always higher than superscripts of red ones) and effectiveness (blue markers being higher than red ones except for the "SICCT only" protocol). These considerations should lead to prefer the A surveillance protocol. Each scenario was

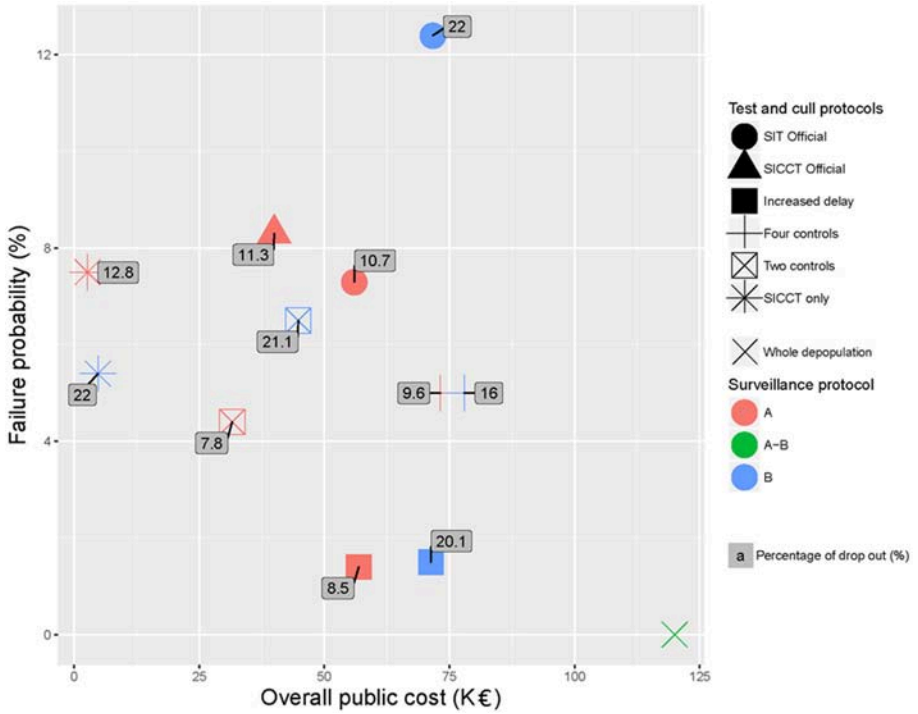

FIGURE 4 | Failure probability of the test-and-cull protocols for bTB control after both A and B surveillances schemes, according to their overall public cost and their percentage of drop out (labels) compared to whole depopulation.

then ranked for each indicator separately (**Table 8**). **Figure 4** and **Table 8** show that in both A and B cases, the "SICCT only" protocol was very economical. However, after an A surveillance its failure probability was the second highest (ranked 6th out of 7 protocols, see **Table 8**) and its percentage of drop out was the highest (ranked 7th out of 7). After a B surveillance, although its failure probability was enhanced (ranked 4th out of 6), its percentage of drop out was the second highest (ranked 4th out of 6, placed equal with "SIT Official"). Therefore, in both cases, the "SICCT only" protocol does not appear to be appropriate for bTB control. The "SIT Official" protocol was among the less effective and most expensive ones, especially after a B surveillance. Its percentage of drop out was the highest after a B surveillance (placed equal with the "SICCT only") and the 4th highest after an A surveillance. Similarly, the "SICCT Official" protocol was poorly ranked for each of the three indicators.

After an A surveillance (red markers), the "Two controls" and "Increased delay" protocols appeared to be the two best protocols. Both had a reasonable acceptability (<9% of drop out, 1st and 2nd rank), a reasonable failure probability (<5% of failure, 2nd and 3rd rank) and induced reasonable public costs (<60 K€TF and 2nd and 5th rank). The "Two controls" protocol was cheaper but had a higher failure probability whereas the "Increased delay" one was almost twice more expensive but with a much lower failure probability.

The average of the three ranks (failure probability, public costs and drop out percentage) allowed obtaining a global ranking of these protocols, while attributing the same importance to each of the three criteria (**Table 8**). The "Two controls" protocol was the best tradeoff (average rank of 2) after an A surveillance. After a B surveillance, the best tradeoff was obtained by the "Increased delay" protocol with an average rank of 2.3, closely followed by the "Four controls" and "SICCT only" protocols (average rank of 3) (**Table 8**).

DISCUSSION

In this study, we evaluated the official test-and-cull protocols implemented in France, in accordance with Council Directive 64/432/EEC as well as four alternative protocols. Their epidemiological effectiveness, the public costs they induce and their acceptability to farmers were compared.

For that purpose, a previously published and validated model was used (12), model that allowed representing the "true" health state of the animals and the detection and control events, in order to quantify events such as incorrectly assigning a bTB free status to the farm (19, 20). This model was chosen because it allowed parameterizing easily alternative surveillance and control protocols, and because its parameters were estimated from field data collected in France, and validated independently: Bekara et al. (12) performed both an internal validation using a leave-one-out cross-validation procedure, and an external validation to demonstrate the ability of the model to reproduce observational bTB data collected in France between 1980 and 2010, that were not used for parameter estimation. The model was parameterized based on French

TABLE 8 | Ranking of test-and-cull protocols to control bTB in an average French beef cattle herd, compared to whole herd depopulation, according to three key indicators, and average rank considering the three indicators being at the same level of importance.

Protocol	Rank for failure probability*		Rank for overall public cost*		Rank for percentage of drop out*		Mean rank**	
Surveillance	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Test-and-cull								
SIT Official	5	6	4	4	4	4	4.3	4.7
SICCT Official	7	/	3	/	5	/	5	/
Increased delay	2	2	5	3	2	2	4	2.3
Four controls	4	3	6	5	3	1	4.3	3
Two controls	3	5	2	2	1	3	2	3.3
SICCT only	6	4	1	1	6	4	4.3	3
Whole depopulation	1	1	7	6	7	6	5	4.3
Total number of protocols	7	6	7	6	7	6	7	6

**1is the protocol with the lowest value, 6 or 7 is the one with the highest one.*
***The smaller the rank, the better the scenario.*

data with an average beef herd size (141 animals) that may be greater than in most of European countries. According to the Directorate General of the European Commission responsible for statistical information at Community level (Eurostat), the last calculated average size of beef herds would be 70 animals (21). The model could easily be adapted to different breeding contexts.

Results suggest that the official protocol could probably be improved, as alternative protocols appeared more effective, acceptable while inducing lower public costs.

The proportion of non-infected animals among the slaughtered animals appeared high, except with the "SICCT only" protocol. Following an A surveillance (based on SICCT annual screening and slaughter of positive animals to confirm suspicions), *M. bovis* was often very quickly eliminated from the infected farms (i.e., either at the time of the confirmation of infection, or in the first months following this confirmation). Moreover, the low specificity of the SIT and IFN-γ tests (17) led to the cull of many susceptible animals. This phenomenon was mitigated when using surveillance protocol B (based on SIT annual screening, IFN-γ on positive animals and slaughter of non-negative animals to confirm suspicions), but the simulated proportion of non-infected animals among the culled animals remained high, with a minimum value of 95.9% for protocols other than the "SICCT only." Indeed, even though the simulated scenario rankings were relatively similar following an A or a B surveillance, the surveillance protocol had a strong impact on the epidemiological effectiveness, the public costs and the acceptability to farmers. After an A surveillance, and therefore a drastic management of the suspicion, most herds did not contain infected animals at the start of the test-and-cull protocol, unlike after a B surveillance. Epidemiological effectiveness, costs and acceptability of the test-and-cull protocols were then enhanced. For example and according to acceptability, herds managed under surveillance protocol A had a shorter duration of strict movement restriction (about 2 months in median) than those managed under surveillance protocol B. The farmer can therefore be prepared to the fact that the protocol will last longer if the surveillance protocol of his herd was B.

We investigated the balance between the costs that can be invested in test-and-cull protocols and the consequences of choosing a specific scheme. It all depends on the goal: if it is to eradicate the infection, the most effective and acceptable protocols will have to be chosen, regardless of their cost. Indeed, without good acceptability, the actors will not follow the measures and the strategy will lose of power. In this case, "Increased delay" protocol is to be implemented. If, however, one is willing to accept a small percentage of outbreaks wrongly regaining their bTB free-status, then less effective but less expensive scenarios may be chosen, such as the "Two controls" protocol in the context of reducing public expenditure. In both cases, the good effectiveness of the "Increases delay" and "Two controls" protocols highlights the importance of long inter-control delays for the epidemiological effectiveness of the scenarios. This appears to be valid even when very few animals are actually infected.

Field observations bring support to the results we obtained. According to a study on the typology of French farms that were subjected to the test-and-cull official protocol between 2014 and 2017 (22), infection was laboratory-confirmed in <4 animals in 95% of the farms. In our simulations, we obtained a very low number of laboratory-confirmed infected animals (between 0 and 1 in median) for the official scenarios, which is thus consistent with field observations. In the same way, the number of control sessions observed in the field (on average 3.3 visits) was close to the figure we obtained (median of 3 to 4 visits for the official protocols).

In the model, births were assumed to compensate for animal culls, herd size thus remaining constant. This simplifying assumption may not accurately represent reality. First, some breeders decide to reduce the size of their herd when starting the test-and-cull protocol, for reasons of biosecurity and easier management of the batches (personal communication 2017: F.

Chevalier, French national referent for bTB). Then, if many animals are slaughtered (rightly or wrongly), it can surpass the amount of births and prevent renewal. Thus, the number of young cattle in the model could be over-estimated. However, these young cattle probably play a minor role in infectious dynamics and are, in practice, not tested because they are too young. The impact of this simplification on our results is therefore assumed to be low.

The overall indicator of acceptability (percentage of drop out) was calculated taking into account the number of confirmed animals and the duration of the test-and-cull protocol. The number of animal reacting to tests may also have been a relevant parameter, but it was too difficult to determine an adequate threshold. In the field, when the number of reactors is high, veterinary services can advise or even force the breeder to shift from the test-and-cull protocol to whole herd depopulation. However, no official or empirical value exists to support that. This is why we decided not to take into account this parameter for the definition of acceptability.

The six studied test-and-cull protocols have been compared and ranked according to 3 key indicators, and according to an overall rank that gave the same level of importance to each. However, in real life, the choice of a control strategy does not always obey a pure epidemiological, economic or social rationality (23). Using a multicriteria decision analysis method (MCDA) would allow investigating more precisely the overall ranking of the protocols according to the expectations of the decision-makers (24).

Even though we focused on test-and-cull protocols applied or feasible in France, some of the protocols we analyzed are also relevant at the European level. The bTB surveillance and control protocols used in EU countries are indeed not country-specific, as these protocols and the tests they include stem from the European legislation (especially the Council Directive 64/432/ECC). As an example, test and cull is very common in the United Kingdom, where bTB can reach in some areas the highest prevalence of the EU (except in Scotland which is OTF), or in Spain (1, 4). For example, England and Northern Ireland eradication program incorporate the surveillance protocol we called "A" (13, 25, 26). A SICCT is performed on the whole herd and reactors are immediately removed for slaughter. If postmortem evidence of *M. bovis* infection cannot be demonstrated in any of the slaughtered reactors, OTF herd status that was suspended may be restored after one single skin test of all the animals with negative results, minimum 60 days later. However, if the infection is confirmed or if more than two animals had reacted to the SICCT, the herd loses its OTF status and enters in a "test and cull protocol" close to one of those we tested: the "SICCT only." Indeed, two consecutively negative SICCT on the whole herd are required to restore the OTF herd status while we modeled a three control protocol. Although other protocols used in EU countries could easily be implemented and compared using the model we used (which was designed for that purpose), it can be expected that the main results would remain valid, such as the reduced failure probability when inter-control delays are lengthened, or the positive effect of type A surveillance on epidemiological effectiveness, on cost and acceptability.

In conclusion, this study aimed at contributing to the identification of points on which decision-makers should act to improve the detection and control of bTB. It appears that there is room for an improvement of the present official test-and-cull protocol implemented in France in line with Council Directive 64/432/EEC, in particular by allowing an increase in the time interval between controls. Decision-makers may use this study to communicate with field actors and justify the needed modification of the French regulations concerning the bTB control. As tests and protocols are close between European countries it could be interesting to extend this study to other regions facing bTB taking into account their specificities in terms of epidemiological situation and cattle breeding.

AUTHOR CONTRIBUTIONS

HL, MS, AC, and BD conceived and designed the study. HL, MS, and BD encoded the model and indicators. HL, MS, AC, and BD performed the analysis. HL wrote the manuscript. HL, MS, AC, and BD revised the manuscript. All the authors approved the submitted version of the manuscript.

ACKNOWLEDGMENTS

The authors thank the French Ministry of Food and Agriculture, Directorate General for Food (DGAl) and the School of French Veterinary Services (ENSV) which funded Helena Ladreyt's internship grant. The authors also warmly thank Fabrice Chevalier (DGAl), Maria-Laura Boschiroli (NRL), Taeb Slassi and Christophe Constant (French Veterinary Services) for their expertise on bTBmanagement in France and the communication of the data, and Valentine Poirier and Adrien Bouveret (interns) for communicating the field data. This work was supported by the Laboratoire d'Excellence Integrative Biology of Emerging Infectious Diseases program (grant ANR-10-LABX-62-IBEID).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 28 September 2018
doi: 10.3389/fvets.2018.00228

Exploring the Fate of Cattle Herds With Inconclusive Reactors to the Tuberculin Skin Test

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Bovine tuberculosis (TB) is an important animal health issue in many parts of the world. In England and Wales, the primary test to detect infected animals is the single intradermal comparative cervical tuberculin test, which compares immunological responses to bovine and avian tuberculins. Inconclusive test reactors (IRs) are animals that demonstrate a positive reaction to the bovine tuberculin only marginally greater than the avian reaction, so are not classified as reactors and immediately removed. In the absence of reactors in the herd, IRs are isolated, placed under movement restrictions and re-tested after 60 days. Other animals in these herds at the time of the IR result are not usually subject to movement restrictions. This could affect efforts to control TB if undetected infected cattle move out of those herds before the next TB test. To improve our understanding of the importance of IRs, this study aimed to assess whether median survival time and the hazard of a subsequent TB incident differs in herds with only IRs detected compared with negative-testing herds. Survival analysis and extended Cox regression were used, with herds entering the study on the date of the first whole herd test in 2012. An additional analysis was performed using an alternative entry date to try to remove the impact of IR retesting and is presented in the **Supplementary Material**. Survival analysis showed that the median survival time among IR only herds was half that observed for clear herds (2.1 years and 4.2 years respectively; $p < 0.001$). Extended Cox regression analysis showed that IR-only herds had 2.7 times the hazard of a subsequent incident compared with negative-testing herds in year one (hazard ratio: 2.69; 95% CI: 2.54, 2.84; $p < 0.001$), and that this difference in the hazard reduced by 63% per year. After 2.7 years the difference had disappeared. The supplementary analysis supported these findings showing that IR only herds still had a greater hazard of a subsequent incident after the IR re-test, but that the effect was reduced. This emphasizes the importance of careful decision making around the management of IR animals and indicates that re-testing alone may not be sufficient to reduce the risk posed by IR only herds in England and Wales.

Keywords: bovine, tuberculosis, SICCT, inconclusive, tuberculin

OPEN ACCESS

Edited by:
Andrew William Byrne, Agri Food and Biosciences Institute, United Kingdom

Reviewed by:
Tracy Ann Clegg, University College Dublin, Ireland
Paul R. Bessell, University of Edinburgh, United Kingdom

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *22 June 2018*
Accepted: *04 September 2018*
Published: *28 September 2018*

Citation:
Brunton LA, Prosser A, Pfeiffer DU and Downs SH (2018) Exploring the Fate of Cattle Herds With Inconclusive Reactors to the Tuberculin Skin Test. Front. Vet. Sci. 5:228. doi: 10.3389/fvets.2018.00228

INTRODUCTION

Bovine tuberculosis (TB) caused by *Mycobacterium bovis* occurs throughout the world, being particularly prevalent in Africa and South America. In Europe, countries that had not achieved Officially Bovine Tuberculosis Free Status (OTF) status in 2016 included Bulgaria, Croatia, Cyprus, Greece, Ireland, Italy, Portugal, Romania, Spain, and the United Kingdom (1). Bovine TB is one of the most important animal health issues in England and Wales, with prevalence of the disease in some parts of England being the highest in the European Union (2). Control of the disease is based on detection and slaughter of infected cattle using immunological testing of cattle herds, restriction of movement from infected herds and carcase inspection of animals at slaughter. Additional testing may be performed in herds perceived to be at risk, e.g., contiguous to an infected herd, or in animals prior to movement. More rigorous testing is applied to herds in which disease is suspected or confirmed.

In England, Defra's strategy for achieving OTF status for England published in 2014 saw the regionalisation of control measures to take account of the spatial heterogeneity of incidence risk (3). The overall incidence rate for England as a whole was 10.2 per 100 herd years at risk in 2016 (4), but this varied considerably across the High Risk (HRA), Edge, and Low Risk (LRA) areas of England [12.8, 3.4, and 0.3 herd years at risk respectively (5)]. In the HRA and Edge area, herds are tested on an annual basis, with herds in some parts of the Edge area being tested every 6 months, whereas in the LRA, herds are tested every 4 years. Tailored control measures are applied to each area in order to meet the objectives of the eradication strategy, which are to achieve OTF status, and more specifically to reduce incidence in the HRA, stop and reverse the spread of disease in the Edge area, and maintain or further reduce incidence in the LRA.

Wales has tested all herds annually since 2008, and in 2016, the TB incidence rate in Wales was 7.0 per 100 herd years at risk (6). Wales has also moved toward a regional approach to TB eradication, by establishing Low, Intermediate, and High TB Areas defined by disease incidence risk. A number of changes to TB control were introduced in October 2017 as part of the Welsh Government's eradication programme (7). In Scotland, which is officially free of tuberculosis, herd-level risk-based surveillance is used for a more targeted approach to routine tests. Herds defined as low-risk are excluded from routine testing.

The primary test used to detect infected animals is the single intradermal comparative cervical tuberculin (SICCT) test, which is based upon injection of bovine and avian tuberculins alongside one another in the skin of the neck. Cattle infected with *M. bovis* tend to show a greater response to bovine tuberculin than avian tuberculin, distinguishing infection with *M. bovis* from infection with other mycobacteria (8). However, while the test is estimated to have high specificity (nearly 100%) (9), the sensitivity of the test at the animal level when using standard interpretation has been estimated to be around 80% but could be as low as 50% (8, 10).

Inconclusive reactors (IRs) to the skin test are defined in England and Wales as animals that demonstrate a reaction to the bovine tuberculin that is less than 4 mm larger than an avian reaction under standard interpretation of the test, or less than 2 mm larger than an avian reaction under severe interpretation. In 2015, there were 2,785 herds in England in which only IRs were detected and which went on to have a re-test, and 21% of these herds had positive reactors (i.e., an incident) at the re-test (5). In Wales, there were 970 IR-only herds of which 21% had an incident at the re-test (6). Animals in these herds at the time of the IR result may be infected, yet the herds will not usually be subject to movement restrictions unless there is a recent history of TB in the herd. In England, 1,420 IRs were slaughtered in 2016 and 13.4% were found to have visible lesions (4). In Wales, 862 IRs were slaughtered in 2016 and 2.9% had visible lesions (6). This could have implications for efforts to control TB if undetected infected cattle move out of those herds over the 60-days period prior to the re-test. This has been demonstrated in Ireland where Clegg et al. (11) reported that between 11.8 and 21.4% of IRs slaughtered before being re-tested were infected with *M. bovis* at post mortem, compared with between 0.13 and 0.22% of animals with a negative SICCT test.

A change in policy for the management of IRs was introduced in England in November 2017. The policy now requires that all IRs in the HRA and Edge Area with a negative result on re-testing must remain restricted for life to the holding in which they were identified. This also applies to IRs in infected herds in the LRA. In comparison, the Welsh eradication programme aims to remove IRs detected in chronically infected herds, under specific circumstances, alongside any reactors. These proactive approaches to managing the risks of IRs are appropriate in light of current knowledge, yet the factors associated with the fate of IR herds are still not well understood. Analysis of 2016 surveillance data has shown that in the HRA and Edge areas of England, herds with a history of TB had a significantly greater risk of having a confirmed incident at the IR retest (4). However, the association between a herd having an IR-only test result and the time to a subsequent incident has not been explored in England and Wales. To improve our understanding of the risk that IRs represent, this study aims to assess whether there are differences in the time to a subsequent incident in herds with only IRs detected compared with herds that test negative at a whole herd test.

MATERIALS AND METHODS

Study Population and Data Extraction

A retrospective cohort study followed cattle herds in England and Wales between 1st January 2012 and 31st December 2016. Data describing TB testing and incidence for the study period were obtained from the Animal and Plant Health Agency's Sam database. The study population included all unrestricted herds (TB-free) in the high-risk and edge areas of England and Wales that had a whole-herd type test (WHT) in 2012. This included a small number of routine herd tests (5% of all WHT included) which in some cases might not include all animals in the herd. Herd demographic data, information relating to the first WHT in 2012 and the first subsequent incident (test where reactors were disclosed or infected animals detected at slaughter) were obtained. The number of incidents in the 10 years prior to the 2012 WHT, and the annual rolling county-level incidence at

the end of 2012 were also obtained. The dataset was prepared using Microsoft SQL Server 2012 and extracted for cleaning and analysis using Stata 14 (Stata Corporation, College Station, TX, USA).

Herds entered the study on the date of their first WHT in 2012. Herds with a positive test result at the first 2012 WHT, or an incident linked to this test, were excluded. The remaining herds were grouped into two cohorts: those with a clear test result at the 2012 WHT ("clear herds") and those that had only IRs detected ("IR only herds"). The outcome was defined as a subsequent incident (i.e., reactors detected at a subsequent test or infected animals detected at slaughter) during the follow-up period. Herds were censored either on the date of the test that disclosed an incident or at the end of the study period, whichever was earlier. Herds lost to follow-up due to the closure of the farm contributed time at risk until the date they were archived in Sam. Time was measured in days, but scaled up to years for the analysis.

The hypothesis being tested was that the hazard of a subsequent incident is different between herds in which IRs have been detected and herds which test negative.

Statistical Analyses

Descriptive analyses were performed to examine the number of herds in each cohort (clear herds or IR only herds), and the number of incidents during the follow-up period. The median survival time in years for each cohort was estimated using the Kaplan-Meier method (12). Differences in survival time between the two cohorts were analyzed using the log-rank statistic.

Cox regression was used to examine the association between first WHT status in 2012 and the hazard of a subsequent incident. Other explanatory variables examined for an association with the hazard of a subsequent incident were herd type, herd size, the season in which the 2012 WHT took place, the number of incidents in the previous 10 years, geographical risk area and annual rolling county-level incidence at the end of 2012. These other explanatory variables were then individually added to a model with first WHT status in 2012 to assess whether they resulted in a change in the hazard ratio for the primary exposure. Herd size, the number of incidents in the previous 10 years and county-level incidence were analyzed as both continuous and categorical variables, and those that resulted in the greatest change in the hazard ratio for first WHT status in 2012 were used in the analysis. Efron's method for dealing with ties was used since there were a large number of tied events in the dataset due to the large number of herds and the resolution of the temporal unit (days). All variables associated with the hazard of a subsequent incident with a $p < 0.20$ in univariable analyses were considered for inclusion in a multivariable model.

The multivariable analysis was performed in a stepwise manner with the variable first WHT status in 2012 ("clear" or "IR only") forced into the model as the primary exposure variable. The outcome variable was occurrence of a subsequent incident. Confounders were then sequentially added to the model in a forward stepwise manner, starting with the variable that resulted in the greatest change in the hazard ratio for first WHT status in the univariable analysis. An interaction between herd type and location was considered. The likelihood ratio test and Akaike's Information Criterion (AIC) were used to compare models (13). Model fit was assessed using Harrell's C concordance statistic and by plotting the Cox Snell residuals and deviance residuals, as recommended by Dohoo et al. (14).

To test the assumption of proportional hazards, a log-minus-log survival plot was generated for first WHT status adjusted for variables included in the final model. The correlation between the Schoenfeld residuals of each variable and transformed time was assessed using the Chi-squared test. A $p < 0.05$ was taken as evidence against the null hypothesis that the hazards were proportional. In addition, graphs of the scaled Schoenfeld residuals over time were plotted for each variable to look for nonlinear relationships between the residuals and time or influential outliers. Interactions between each of the variables and log time were assessed by extending the model to include time varying coefficients using the tvc command in Stata. Model fit could not be assessed using the Cox-snell and deviance residuals after the inclusion of the time-varying coefficients, so models were assessed using the likelihood ratio test and AIC.

An additional analysis was performed using the date of the first subsequent clear herd test after the first WHT as the entry date, thereby excluding herds that were disclosed as infected at the IR retest. The purpose of this was to try to remove the impact of the IR retesting and ensure that all herds were starting out on comparable testing regimes. The results of this analysis are presented in the **Supplementary Material**.

RESULTS

Descriptive Analysis

There were 30,600 unrestricted herds that had a WHT in 2012, and overall, the median percentage of animals tested per herd at the first WHT in 2012 was 98%. Of the 30,600 herds, 27,289 (89%) tested negative (clear), and 3,311 (11%) only had IRs (IR only) at the first WHT in 2012. Overall, 30% of herds went on to have a subsequent incident within the follow-up period. A greater percentage of IR only herds went on to have a subsequent incident compared with clear herds (63 and 27% respectively) (Z-test to compare two proportions: $p < 0.001$) (**Table 1**).

The percentage of herds that suffered a subsequent incident was greater among herds with three or more incidents in the 10 years prior to the 2012 WHT, dairy herds, and increased with herd size (**Table 1**). In addition, herds appeared to be more likely to have a subsequent incident if they were located in the high-risk area of England and in a county where incidence was greater than the median incidence across all counties at the end of 2012 (**Table 1**). The percentage of herds that had a subsequent incident did not vary with the season in which the 2012 WHT took place. Among IR only herds, 53% of subsequent incidents were disclosed by an IR retest, whereas among clear herds, 19% of subsequent incidents were disclosed by an IR retest (*Z*-test to compare two proportions: $p < 0.001$). The median number of skin test reactors was lower among incidents disclosed by an IR retest than among incidents disclosed by other tests (0 vs. 1 respectively; Wilcoxon rank-sum test: $p < 0.001$). However, the median numbers of IRs and reactors to the gamma interferon test

was zero among incidents disclosed by an IR retest and among incidents disclosed by other tests.

Seven herds were excluded from the analysis as they had an archive date (date herd closed down) that fell before the date of the first WHT in 2012 and they were not tested again within the follow-up period. This left 30,593 herds under observation. There were 9,326 herds with a subsequent incident, which occurred at a median follow-up time of 1.8 years (range: 0.02–4.9), while 21,267 herds were censored at a median follow-up time of 4.5 years (range: 0.03–5.5). There were 3,705 herds lost to follow-up because the business closed down. More clear herds were lost to follow-up (13.1%) than IR only herds (3.8%).

The median survival time among IR only herds was over half that observed for clear herds. Median survival time was also reduced among herds with more than 200 animals, dairy herds, and herds with 3 or more incidents in the previous 10 years (**Table 2**).

TABLE 1 | Number and percentage of herds that had a subsequent incident, stratified by each explanatory variable.

Variable	*N*	Missing	Herds with a subsequent incident		
			n	%	95% CI[a]
FIRST WHT STATUS IN 2012					
Clear	27,289	0	7,231	26.5	26.0–27.0
IRs Only	3,311		2,095	63.3	61.6–64.9
SEASON IN WHICH 2012 WHT TOOK PLACE					
Spring	9,935	0	2,976	30.0	29.1–30.9
Summer	3,996		1,198	30.0	28.6–31.4
Autumn	7,474		2,253	30.1	29.1–31.2
Winter	9,195		2,899	31.5	30.6–32.5
NUMBER OF INCIDENTS IN THE PREVIOUS 10 YEARS					
0–2	27,639	0	7,376	26.7	26.2–27.2
3 or more	2,961		1,950	65.9	64.1–67.5
GEOGRAPHICAL RISK AREA					
England high-risk	17,145	0	6,595	38.5	37.7–39.2
England Edge	3,311		636	19.2	17.9–20.5
Wales	10,144		2,095	20.7	19.9–21.5
ANNUAL ROLLING COUNTY LEVEL INCIDENCE AT THE END OF 2012					
0–14.6 per 100 herd years at risk	17,431	0	3,983	22.9	22.2–23.5
>14.6 per 100 herd years at risk	13,169		5,343	40.6	39.7–41.4
HERD TYPE					
Beef	23,713	0	6,087	25.7	25.1–26.2
Dairy	6,447		3,189	49.5	48.3–50.7
Other	440		50	11.4	8.7–14.7
HERD SIZE					
0–10	4,941	1,563	453	9.2	8.4–10.0
11–50	8,697		1,755	20.2	19.4–21.0
51–100	5,488		1,802	32.8	31.6–34.1
101–200	5,164		2,336	45.2	43.9–46.6
201–300	2,196		1,218	55.5	53.4–57.5
>300	2,551		1,700	66.6	64.8–68.4

[a] *Confidence interval.*

There was a difference in the survival functions of the clear and IR only cohorts (**Figure 1**) and this observation was supported by the results of the log-rank test (**Table 3**). Significant differences in survival were also observed between herds grouped according to their TB history, geographical area, county level incidence, production type, and size (**Figures 2B–F**). The survival of herds did not appear to vary according to the season in which their 2012 WHT took place (**Figure 2A**), although the log-rank test indicated there was some evidence of a difference ($p = 0.04$).

Assessment of the Hazard of Subsequent Incidents Among Clear and IR Only Herds

A Cox regression was performed to assess the hazard of a subsequent incident within the two cohorts. There were strong associations between each of the explanatory variables and the hazard of subsequent incidents in the univariable analysis (**Table 4**). Factors found to be associated with increased relative hazard of a subsequent incident were having an IR only test result at the 2012 WHT, having the first 2012 WHT in autumn or winter compared with spring, a recent history of TB, increased county-level incidence, being a dairy herd (compared to a beef herd), and increasing herd size. Herds in the edge area of England, and those in Wales, had a reduced incidence rate when compared to the high-risk area of England. Herds classed as production type

TABLE 2 | Median, minimum, and maximum survival time in the clear and IR only cohorts, and by each explanatory variable.

Variable	Level	Survival time (years)		
		Median	Min	Max
First WHT status in 2012	Clear	4.21	0.02	5.46
	IR	2.07	0.02	5.09
Season in which 2012 WHT took place	Spring	4.63	0.02	4.84
	Summer	4.36	0.05	5.46
	Autumn	4.11	0.02	5.28
	Winter	4.08	0.02	5.08
Number of incidents in the previous 10 years	0–2	4.22	0.02	5.46
	3 or more	2.36	0.02	5.42
Geographical risk area	England high-risk	4.07	0.02	5.28
	England Edge	4.26	0.05	5.46
	Wales	4.31	0.12	5.19
Annual rolling county level incidence at end of 2012	0–14.6 per 100 herd years at risk	4.27	0.04	5.46
	>14.6 per 100 herd years at risk	4.31	0.12	5.19
Herd type	Beef	4.23	0.02	5.46
	Dairy	3.76	0.02	5.22
	Other	4.25	0.18	4.99
Herd size	0–10	4.34	0.03	5.25
	11–50	4.36	0.05	5.42
	51–100	4.25	0.06	5.46
	101–200	4.11	0.02	5.22
	201–300	3.40	0.02	5.15
	>300	2.57	0.02	5.24

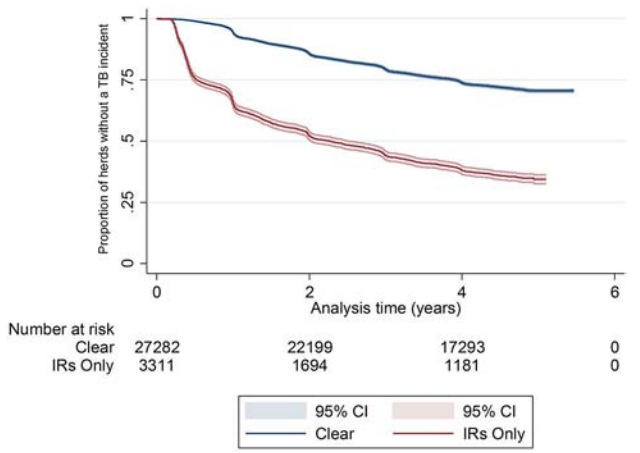

FIGURE 1 | Kaplan-Meier survival estimates for herds according to first WHT status in 2012.

TABLE 3 | Results of the log-rank tests for equality of survivor functions.

Variable	Chi-squared	*P*-value
First WHT status in 2012	3,008.9	<0.001
Season in which 2012 WHT took place	8.51	0.037
Number of incidents in the previous 10 years	2,635.7	<0.001
Geographical risk area	1,238.86	<0.001
Herd type	1,535.93	<0.001
Herd size	4,388.12	<0.001
Annual rolling county level incidence at end of 2012	1,207.05	<0.001

"other" also had a reduced incidence rate compared with beef herds (**Table 4**).

The initial multivariable Cox regression model included first WHT status in 2012, herd size, the number of incidents in the 10 years before the first WHT in 2012, herd type, county-level TB incidence and geographical risk area. The plot of the Cox-Snell residuals (**Figure 3**) indicated that the model was a poor fit, and the plot of the deviance residuals over time (**Figure 4**) revealed a number of observations that were not well fit by the model, particularly those herds with the shortest survival time. However, the Harrell's C statistic was 0.75 indicating that the model correctly predicted the sequence of two observed failures 75% of the time. Assessment of the proportionality of the hazards using the log-minus-log plot (**Figure 5**) indicated that the ratio of hazards varied over time. The Chi-squared test of the correlation between the Schoenfeld residuals of each variable and transformed time generated a $p < 0.05$ for all variables except local incidence, indicating that the proportional hazards assumption had been violated. The log-minus-log plot illustrated a change in the ratio of hazards around 60 days, which correlated with the timing of IR retests. This indicated that an analysis of the time to a subsequent incident may not be appropriate given the differences in follow-up testing between the cohorts, and that time varying coefficients should be included to model interactions between the explanatory variables and time.

The final extended Cox regression model contained first WHT status, herd size, recent history of TB, herd type, local incidence and geographical risk area, and included interactions between time and first WHT status, herd size, TB history, risk area and herd type. The relative hazard of having a subsequent incident was 2.7 times greater among herds that were IR only at the 2012 WHT compared with herds that had a clear test result (after adjusting for herd size, testing following the 2012 WHT, recent history of TB, herd type, local incidence and geographical risk area) (**Table 5**). The interaction with time indicated that the increased relative hazard of having a subsequent incident among IR only herds decreased by 63% each year. This means that according to the model, the relative hazard of 2.7 in year one is reduced to 1.34 in year two, and drops to 0.89 by year three. This change in relative hazard over time is presented in **Figure 6**. This shows that the effect disappears (i.e., the relative hazard = 1) by around 970 days, or 2.7 years.

DISCUSSION

Understanding the level of infection that could be present among IRs is important for directing control measures. In Ireland, Clegg et al. (11) found that IRs that passed the IR retest and then moved herds within 6 months were 12 times more likely to have a positive result at the next test, or have lesions detected at slaughter, compared to all animals in Ireland. Our analysis has shown that the time interval before a new TB incident in IR only herds was around half that of herds with a negative whole herd test; and that the hazard of a subsequent incident was 2.7 times greater for IR only herds compared with clear herds after accounting for the influence of traditionally accepted drivers of TB. This difference in hazard decreased over time by 63% per year.

The number of incidents in the 10 years prior to the study was consistently associated with an increase in the hazard of a subsequent incident. This is in agreement with other studies where TB history has been identified as a risk factor for future incidents (15–17). Herd size has frequently been associated with increased disease risk (1, 15, 18, 19), but this association can be difficult to interpret. An effect of increasing herd size may simply reflect changes in other risk factors related to farm management, or it may have implications on the sensitivity and specificity of the test at herd level (20).

Dairy herds located within areas subject to badger culling in England were shown to have a greater risk of TB than beef herds in the same areas (21). It has also been shown in separate analyses for England and Wales that the effect of herd type is reduced after adjusting for herd size and location (4, 6). In this study, there was no difference in the rate of subsequent incidents among dairy compared with beef herds, after adjusting for herd size, location and other factors that were not included in the country-level analyses described above (4, 6). However, the time-varying coefficient for herd type was significant for dairy. This

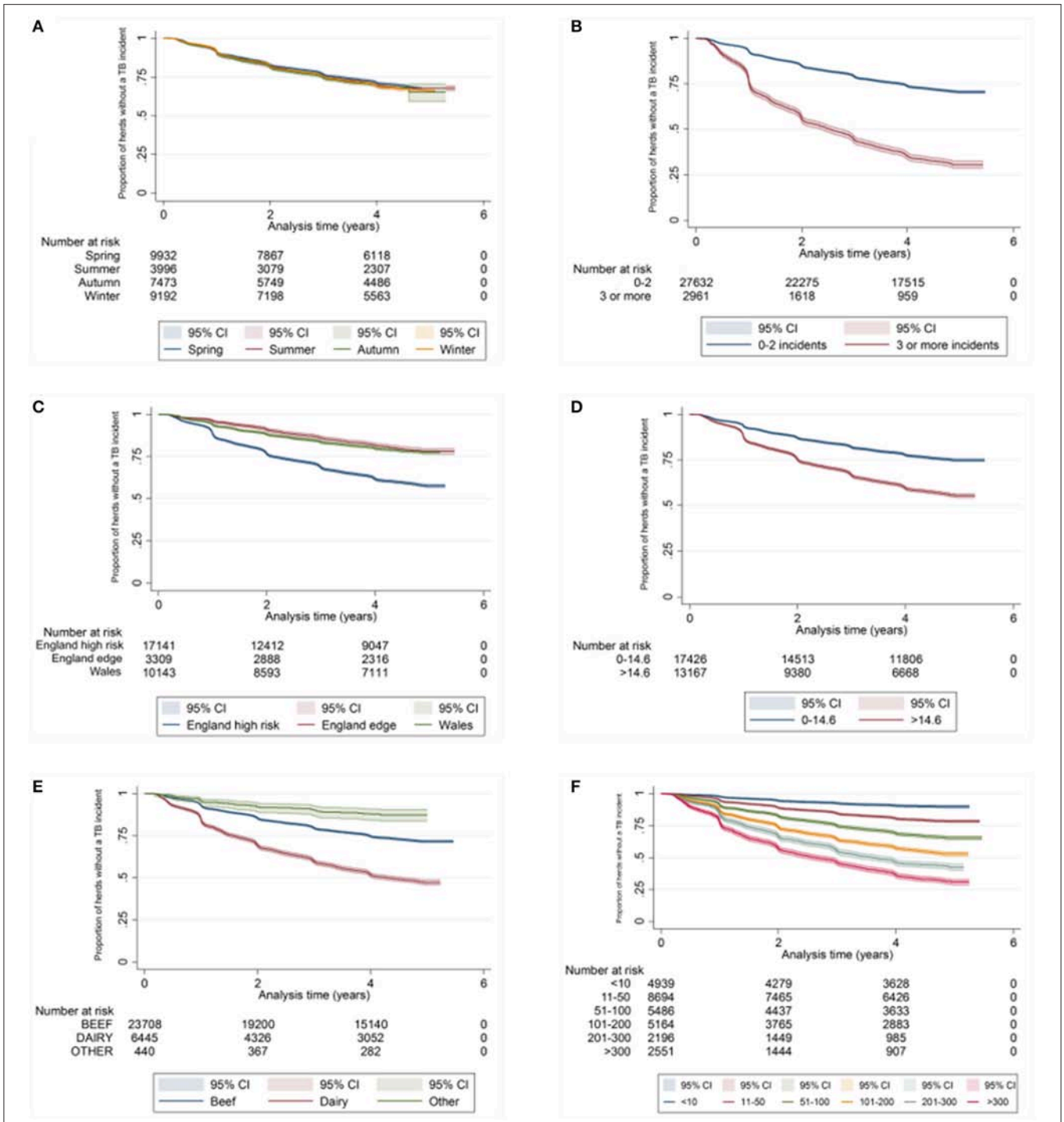

FIGURE 2 | Kaplan-Meier survival estimates for herds according to season in which 2012 WHT took place **(A)**, number of incidents in the previous 10 years **(B)**, geographical risk area **(C)**, annual rolling county level TB incidence at end of 2012 **(D)** herd type **(E),** and herd size **(F)**.

suggests that the hazard of a subsequent incident among dairy herds increases by 14% each year. This may be related to the longer life expectancy of dairy cattle compared to beef cattle, meaning that dairy cattle are at risk of exposure to TB for longer than beef cattle (21, 22). Both O'Hagan et al. (23) and Downs et al. (24) have shown that dairy SICCT reactors are less likely to have visible lesions than beef reactors, which could indicate that infected dairy cattle are detected through SICCT surveillance earlier than beef cattle. Therefore, one might expect IRs from beef herds to pose a higher future risk than IRs from dairy herds.

TABLE 4 | Results of the univariable Cox regression analysis of factors associated with the rate of subsequent incidents.

Variable	Level	HR[a]	95% CI[b]		*P*-value
First WHT status in 2012	Clear	*1.00*			
	IRs only	3.58	3.41	3.76	<0.001
Season in which first WHT took place	Spring	*1.00*			
	Summer	1.06	0.99	1.13	0.105
	Autumn	1.08	1.02	1.14	0.007
	Winter	1.06	1.01	1.11	0.031
Number of incidents in the previous 10 years	<3	*1.00*			
	3 or more	1.50	1.49	1.52	<0.001
Geographical risk area	England high risk	*1.00*			
	England Edge	0.43	0.40	0.47	<0.001
	Wales	0.47	0.44	0.49	<0.001
Annual rolling county level incidence at end of 2012	0–14.6 per 100 herd years at risk	*1.00*			
	>14.6 per 100 herd years at risk	1.07	1.07	1.07	<0.001
Herd type	Beef	*1.00*			
	Dairy	2.26	2.16	2.36	<0.001
	Other	0.44	0.33	0.58	<0.001
Herd size	1–10	*1.00*			
	11–50	2.21	1.99	2.45	<0.001
	51–100	3.82	3.44	4.23	<0.001
	101–200	5.74	5.19	6.35	<0.001
	201–300	7.71	6.92	8.59	<0.001
	>300	10.49	9.45	11.63	<0.001

[a] *Hazard ratio.*
[b] *Confidence interval.*
Ratios in italics represent the reference groups.

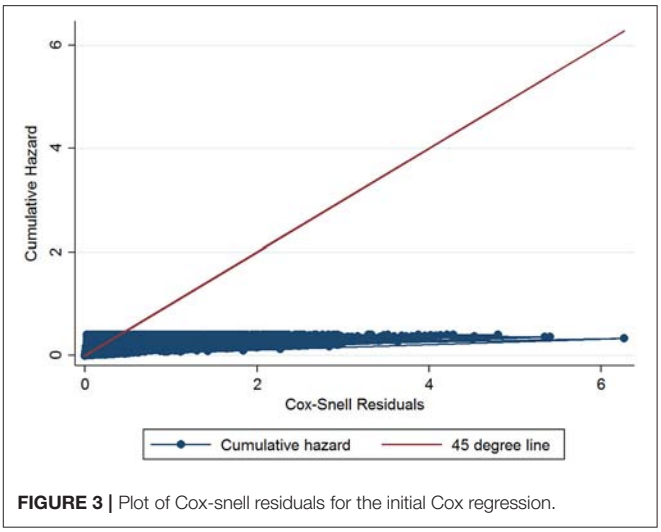

FIGURE 3 | Plot of Cox-snell residuals for the initial Cox regression.

Increased county-level incidence was associated with an increased hazard of a subsequent incident, and herds in the edge area of England and in Wales had a reduced hazard compared with herds in the high risk area of England. Olea-Popelka et al. (15) and Green et al. (25) both showed that increased local prevalence of TB is associated with an increased risk of infection. Johnston et al. (26) found regional variation in risk factors for TB incidents, and Brunton et al. (27) reported spatial heterogeneity in the factors associated with the spread of endemic TB. The significant time-varying coefficient for Wales is interesting, and indicates that the hazard for herds in Wales reduces over time. This was not seen for herds in England, so could be related to differing policies on IRs in the two countries.

The TB testing regime in England and Wales is determined by factors such as location, animal movements and disease history. As such, it varies considerably between herds across both cohorts. However, there are also structural differences in the data due to the TB control policy. IRs have a subsequent test following disclosure of IRs, which does not take place in herds where all the cattle tested negative to the whole herd test. This increases the probability of IR-only herds having a subsequent incident compared with herds that tested clear, since increased testing increases the chances of detecting disease. This is further complicated by the fact that animals that have a second IR test result at the follow up test will automatically be classified as reactors. This means that there is a bias toward detecting cases within the IR only cohort. Unfortunately, the structure of the data did not allow the analysis of individual test data for each herd to explore the impact of this further. Instead, the time-varying coefficients were included to model how the relative hazard of a subsequent incident amongst IR only herds compared with clear herds varied over time. A reduction in the hazard ratio over time was observed, which indicates that the hazard for IR only herds becomes comparable to that of clear herds after around two and a half years. If the effect of re-testing was the only reason that IR only herds had a greater hazard of a subsequent incident, then we would expect the hazard ratio to reach 1.0 after the 60 days retest. The fact that it takes over 2 years to reach 1.0 suggests that the hazard of a subsequent TB incident is still higher among IR only herds than herds that tested negative to a whole herd test once the effect of re-testing has been removed.

An additional analysis was performed to try to remove the impact of the IR re-testing by ensuring that all herds were starting out on comparable testing regimes, and the results of this analysis are presented in the **Supplementary Material**. The results of this additional analysis indicate that there is still a significantly greater hazard of a subsequent incident amongst IR only herds compared with clear herds, but that this is reduced once the effect of re-testing is removed. This aligns with the finding that the hazard ratio is still greater than 1.0 after the 60 days re-test has passed. However, the additional analysis needs to be interpreted cautiously as the sample size for the IR cohort was reduced by almost half (46%) due to missing or inaccurate values within the subsequent clear test variable used as the new entry date. The clear herd cohort was less affected by missing values (15%). This introduces a considerable bias to the additional analysis and makes it difficult to draw firm conclusions from this about the fate of IR only herds compared to clear herds after they get through the IR testing regime.

There is potential for the misclassification of IRs due to the imperfect test for TB. The influence of disease prevalence on the predictive value of the test also introduces the potential for

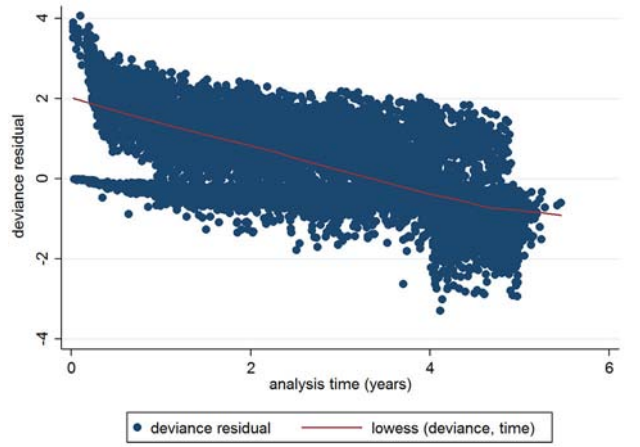

FIGURE 4 | Plot of deviance residuals for the initial Cox regression.

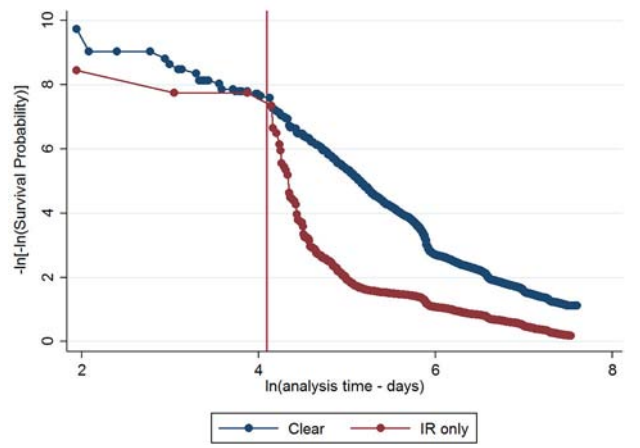

FIGURE 5 | Log-minus-log survival plot for first WHT status adjusted for herd size, the number of incidents in the 10 years before the first WHT in 2012, herd type, county level TB incidence and geographical risk area. A reference line has been added to indicate the change in the HR at 60 days.

misclassification across risk areas. For example, the low positive predictive value of the test when prevalence is low means that IRs in the low-risk areas may be false positives, while the low negative predictive value of the test when prevalence is high means that IRs in high-risk areas may be false negatives. Even if perfect classification were possible, the nature of IRs is that their infection status is uncertain. They may be uninfected animals that have been exposed to other mycobacteria, or they may be infected animals that do not respond adequately to the test due to factors such as immunosuppression or co-infection (8). This uncertainty makes managing the potential risk that IRs pose challenging, and highlights the need for evidence to understand this risk.

The finding that the hazard of a subsequent incident reduces over time among IR only herds indicates that the policy in England and Wales for dealing with IRs is having an effect. However, these herds still appear to be at greater risk of having an incident after the IR re-testing regime. This could reflect

TABLE 5 | Multivariable extended Cox regression model of factors associated with a subsequent incident amongst clear and IR only herds, including time varying coefficients.

Variable	Level	HR[a]	95% CI[b]		*P*-value
MAIN COVARIATES					
First WHT status in 2012	Clear	*1.00*			
	IRs only	2.69	2.54	2.84	<0.001
Herd size	1–10	*1.00*			
	11–50	1.92	1.70	2.17	<0.001
	51–100	3.00	2.66	3.39	<0.001
	101–200	3.93	3.49	4.43	<0.001
	201–300	4.65	4.09	5.30	<0.001
	>300	6.18	5.45	7.02	<0.001
Number of incidents in the previous 10 years		1.19	1.17	1.21	<0.001
Herd type	Beef	*1.00*			
	Dairy	0.98	0.93	1.04	0.547
	Other	0.61	0.45	0.82	0.001
Annual rolling county level incidence at end of 2012	0–14.6 per 100 herd years at risk	*1.00*			
	>14.6 per 100 herd years at risk	1.05	1.05	1.06	<0.001
Geographical risk area	England high risk	*1.00*			
	England Edge	0.90	0.80	1.02	0.088
	Wales	0.80	0.75	0.86	<0.001
TIME-VARYING COEFFICIENTS					
First WHT status in 2012	Clear	*1.00*			
	IRs only	0.37	0.34	0.39	<0.001
Herd size	1–10	*1.00*			
	11–50	1.20	1.05	1.38	0.008
	51–100	1.26	1.10	1.44	0.001
	101–200	1.32	1.16	1.51	<0.001
	201–300	1.46	1.26	1.69	<0.001
	>300	1.40	1.21	1.61	<0.001
Number of incidents in the previous 10 years		1.02	1.01	1.04	0.008
Geographical risk area	England high risk	*1.00*			
	England Edge	1.04	0.93	1.17	0.464
	Wales	0.88	0.83	0.94	<0.001
Herd type	Beef	*1.00*			
	Dairy	1.14	1.07	1.21	<0.001
	Other	0.62	0.44	0.88	0.007

[a] *Hazard ratio.*
[b] *Confidence interval.*
Ratios in italics represent the reference groups.

that the testing is not removing all potentially infected animals from the herd, or there may be other factors which put these herds at a greater risk of having a TB incident that we have yet to understand. This is important information for both policy makers in England and Wales, and those in other countries looking to learn from the English and Welsh experience in tackling bovine TB. The evidence from this analysis suggests

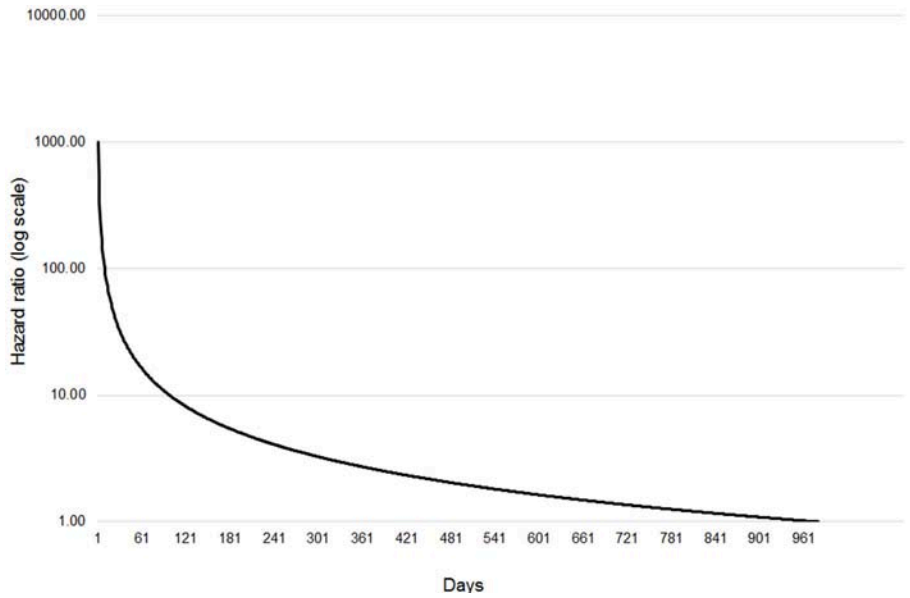

FIGURE 6 | Change in relative hazard over time amongst IR only herds compared with clear herds, adjusted for herd size, the number of incidents in the 10 years before the first WHT in 2012, herd type, county level TB incidence and geographical risk area, and interactions between time and first WHT status, herd size, the number of incidents in the 10 years before the first WHT in 2012, herd type, and geographical risk area.

that the new policy decision in England, restricting IRs with a negative re-test to the herd in which they were detected for life, should help reduce any residual risk associated with an IR for disease spread. This approach has been implemented in Ireland since 2012 (28) following the analysis of the fate of IRs by Clegg et al. (29).

The present study has shown that the hazard of a subsequent TB incident is greater among IR only herds than herds that tested negative to a whole herd test, and that the hazard ratio decreases over time, but remains greater than 1.0 after the IR re-testing regime. This emphasizes the importance of careful decision making around the management of IR animals and indicates that re-testing alone may not be sufficient to reduce the risk posed by IR only herds. Further characterisation of IRs is needed to determine whether the differences observed here are related to management or biological factors. This may be best achieved through an animal-level analysis so that the risk of retaining individual IR animals in a herd in England and Wales can be understood. Our findings correlate with the Irish findings, indicating that the risks of IRs are unlikely to be country and context specific. This provides further evidence of the risk that IRs pose for the spread of TB, which can support the development of policies in other countries relating to the management of IRs.

AUTHOR CONTRIBUTIONS

LB designed the study, performed the analysis, and drafted the manuscript in part fulfillment of the requirements for the degree of Master of Science in Veterinary Epidemiology at the Royal Veterinary College, University of London. AP generated the dataset and edited the manuscript. DP and SD provided advice on study design and analysis, made additions to the text, and edited the manuscript.

ACKNOWLEDGMENTS

We wish to thank Paul Upton (APHA) for his expertise and assistance in collating the data, and to Professor Glyn Hewinson (APHA) for reviewing the manuscript. We thank Defra for funding data provision and contributions from SD and AP through projects EA3131 and SB4500.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00228/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 03 October 2018
doi: 10.3389/fvets.2018.00232

A New Model to Calibrate a Reference Standard for Bovine Tuberculin Purified Protein Derivative in the Target Species

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OPEN ACCESS

Edited by:
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Tyler C. Thacker, Agricultural Research Service (USDA), United States

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *08 June 2018*
Accepted: *07 September 2018*
Published: *03 October 2018*

Citation:
Frankena K, Jacobs L, van Dijk T, Good M, Duignan A and de Jong MCM (2018) A New Model to Calibrate a Reference Standard for Bovine Tuberculin Purified Protein Derivative in the Target Species. Front. Vet. Sci. 5:232. doi: 10.3389/fvets.2018.00232

Since 1986, use of a Bovine International Standard (BIS) for bovine tuberculin has been required to ensure national and international uniformity regarding the potency designation of bovine tuberculin Purified Protein Derivative (PPDb) preparations produced by multiple manufacturers. The BIS is the unique golden standard in the guinea pig potency assay, representing 100% potency, where potencies of production batches are calculated as relative potencies in comparison with the potency of the BIS which was set at 32,500 international Unit (IU) per mg. The stock supply and lifetime of the BIS is limited.The aim of this study was to develop a model to determine the potency of a newly produced in-house Reference Standard (RS) for PPDb with great accuracy in the target species (cattle) and to prove its precision and accuracy in the guinea pig potency test. First simulations were done to estimate the required number of cattle needed. Then, 30 naturally bTB infected cattle were subjected to a tuberculin skin test using multiple injections of both the RS and the BIS. Both were applied randomly in the same volume and concentration (1 dose). The potency of the RS against the BIS was directly derived from the least square means (LSMEANS) and was estimated as 1.067 (95% CI: 1.025–1.109), equal to a potency of 34,700 ± 1,400 IU/mg. In six guinea pig potency assays the RS was used to assign potencies to production batches of PPDb. Here, precision and accuracy of the RS was determined according to the parallel-line assay. Relative potencies were estimated by exponentiation of the common slope. The corresponding 95% confidence intervals were obtained according to Fieller's theorem. In sensitized guinea pigs, the relative potency of the RS against the BIS was 1.115 (95% CI: 0.871–1.432), corresponding to an absolute potency of 36,238 IU/mg (95% CI: 28,308–46,540).In conclusion: the method used to determine the potency of the RS against the BIS in naturally bTB infected cattle, resulted in a highly accurate potency estimate of the RS. The RS can be used in the guinea pig test to assign potencies to PPDb production batches with high precision and accuracy.

Keywords: *mycobacterium bovis*, tuberculin, bovine international standard, new reference standard, potency estimation, guinea pigs, cattle

INTRODUCTION

Bovine tuberculosis (bTB) is a zoonotic livestock infection most frequently caused by the bacterium *Mycobacterium bovis* which is often found to be endemic in cattle but which can infect several species of mammals and also marsupials. *Mycobacterium bovis*, belongs to a group of related Mycobacterium species, known as the *Mycobacterium tuberculosis* complex (MTBC), members of which cause tuberculosis in humans and multiple animal species including bovines (1, 2). TB in bovines is a chronic disease characterized by granulomatous lesions in multiple parts of the body and clinical signs such as coughing, reduced milk production etc. Infections can remain subclinical for many years, even if multiple organs are affected (3, 4).

Throughout the last century, extensive control programs resulted in eradication of bTB in many countries, including most EU Member States, Australia, Canada, Switzerland and many states in the USA. Important control measures in cattle include regular and systematic testing of cattle herds, compulsory slaughter of test-positive animals, movement restrictions out of infected herds and post-mortem slaughterhouse surveillance (5–7). However, bTB is still present in various countries, especially in those where a wildlife reservoir of *M. bovis* is present (8). To control and eradicate bTB, multiple tests have been developed to detect infected cattle. The widely used tuberculin skin tests are based on the development of a delayed type hypersensitivity reaction in cattle infected by a MTBC after an intradermal injection with bovine (*M. bovis*) tuberculin Purified Protein Derivative (PPDb) (9, 10). Details about the skin test are in Annex B Directive 64/432/EEC (10) and in OIE guidelines (9).

Since the development of the tuberculin skin tests in cattle, various companies worldwide commenced production of PPDb. To ensure national and international uniformity regarding the potency designation of the PPDb preparations it was essential to define a bovine tuberculin standard. In 1986, after multiple assays in both cattle and guinea pigs, the Bovine International Standard (BIS) was officially established by the World Health Organization (WHO) as the standard for PPDb. The BIS is freeze-dried and stored in glass ampoules, each ampoule containing 1.8 mg of PPDb. Based on the results of an international collaborative study, organized by the WHO, the activity of the contents of each ampoule was defined as 58,500 IU of PPDb (11, page 20), hence the ampoule contained 32,500 IU/mg PPDb Since 1987, the BIS is internationally distributed, on behalf of the WHO, by the International Laboratory for Biological Standards, Hertfordshire, England (11, 12).

Because the stock of the BIS is limited—and reported as seemingly being at the end of its lifetime due to formation of aggregates in some ampoules (13)—, the International Laboratory for Biological Standards encourages manufacturers of PPDb to produce their own Reference Standard (RS) to be used in the guinea pig and cattle potency test. The aim of this study was to determine the potency of a new RS for PPDb with great accuracy in the target species (cattle) and to prove its precision and accuracy in the guinea pig potency test, the prescribed release test for PPD. The new RS was comparable to the BIS in composition and potency in cattle and guinea pigs. A trial in natural bTB infected cattle was designed and performed to determine the potency of the RS in cattle with great precision and accuracy. In the guinea pig test the RS is used to assign potency to individual production batches of PPDb. The accuracy and precision of the RS in the assignment of the potency to production batches of PPDb in the guinea pig potency test was shown in 6 trials. Additionally, the potency of the new RS was compared with the BIS in sensitized guinea pigs.

METHODS

The study consisted of 2 parts: I. Calibration of the RS against the BIS in naturally bTB sensitized cattle. II. Prove of accuracy and precision of the RS in the guinea pig potency test. Here the RS is used to assign potency to individual production batches of PPDb.

Production of the Reference Standard

It was decided by Prionics Lelystad B.V. to produce the RS according to the same formula as was used for the BIS with respect to the *M. bovis* strain, volume of the vials, concentration of protein and buffer. Therefore, 15 L of homogenous bulk of PPDb, derived from *M. bovis AN5,* in glucose-phosphate buffer (R31 medium, Hyclone, UK) was formulated with a final concentration of 1 mg/mL (±0.05 mg). Formulation and sterile filtration was performed using Standard Operating Procedures with the exception of adding phenol to the formulated final product. The phenol concentration, due to the phenol present in the starting material of the concentrated tuberculin PPD, is estimated to be 0.03%. No extra phenol was added to the buffer used to formulate the final product. Phenol can evaporate during the freeze-dry process and is hazardous for the environment. The formulated final product was filled into 6 ml vials each vial containing 1.8 mL (±0.02). Freeze drying was done in a Klee Freeze Dryer using program TUB MSL/WSL (freezing of the samples for 3 h at −35°C; drying under vacuum at −33°C at 8.0E-2 mbar for 112 h; drying at 25°C at 1.0 E-2 mbar for 24 h). After freeze drying the vials were closed, capped, labeled and stored in sealed plastic bags at 2–8°C. Before use the content of a vial with the RS is reconstituted in 1.8 ml of Water For Injection (WFI) containing 0.42% phenol to give an end solution in R31 buffer of 0.45% phenol and 1 mg/mL of PPD.

Calibration of the RS Against the BIS in Naturally Infected Cattle

Cattle

The trial was carried out on the Longtown Veterinary Research Farm of the Central Veterinary Research Laboratory of the Department of Agriculture, Food and the Marine (DAFM) in Ireland. Thirty naturally infected steers, all between 14 and 24 months old, were selected from herds in which *M. bovis* infection was confirmed (see **Supplementary Material** for sample size calculation). Cattle which had given a positive skin response, in the single intradermal comparative tuberculin test (SICCT), i.e., showing an increase at the bovine site equal to 4 mm or more than any increase at the avian site, were selected. The time interval between the SICCT on the farms of origin and the study was at least 60 days. All animals also failed (tested positive

in) the gamma interferon (y-IFN) Bovigam® test (Thermofisher Scientific, Lelystad) 2 weeks prior to the study, indicating they were responsive to tuberculin.

Trial Design and Testing Procedure in Cattle

For a RS to be considered as a valid standard, its potency must be estimated with high precision and accuracy. Therefore, the 95% confidence interval was set as the estimated potency ±10% of that potency.

In cattle, four injection sites at each side of the mid-neck are routinely used to perform a potency assay. Therefore, RS and BIS were applied twice on the left side and twice on the right side of the neck. Within the sides of the neck, the injections were randomly allocated to the four injection sites according to one of the 6 unique combinations. For each side, one of the 6 combinations was randomly selected using PROC SURVEYSELECT of SAS version 9.4 (14).

Four injection sites were marked and clipped, using a battery powered hair clippers, on both sides of the mid-neck of the 30 bovines, followed by measurement of the initial skinfold thickness with a caliper before injection. Subsequently cattle were injected (according to the randomization scheme) with a volume of 0.1 mL of BIS or RS at a concentration of 1.0 mg/ml. Skinfold thickness was measured again at 72 h post-injection and the increase in skinfold thickness developed between 0 and 72h was calculated. Injections and measurements were all performed by the same person (AD).

The cattle study was approved by the Health Products Regulatory Authority (HPRA), Dublin, Ireland (project authorization number: AE19113/P008).

Statistical Analysis

The differences in skinfold thickness were statistically analyzed at a significance level of 5% with a linear mixed model using the increase in diameter of skin at the injection sites (in mm) as outcome variable [PROC MIXED of SAS (14)]. Estimation method used was restricted maximum likelihood (REML) and variance component was specified as covariance structure. The initial model included tuberculin batch (RS, BIS), side (left, right) and site (1–4) of injection and animal was included as random effect. Relative potency can be derived directly from the least square means (LSMEANS) for both batches and its 95% CI from the 95% CI of the difference in LSMEANS. Next, the potency of the RS was calculated by multiplying the relative potency of the RS with the known potency of the BIS i.e. 32,500 IU/mg.

Proof of Accuracy and Precision of the RS in the Guinea Pig Potency Test

Guinea Pigs and Sensitization

Dunkin Hartley guinea pigs were obtained from ISO 9001 certified breeders of SPF guinea pigs. The guinea pigs were infected with 0.0008 mg of wet mass of living virulent *M. bovis* of strain AN5 by intramuscular injection of 0.5 ml into the left hind leg of each animal. Infection was 5 weeks prior to the skin test. At the moment of infection, the weight of the guinea pig was between 400 and 600 grams.

Trial Design and Assay Procedure

Tuberculin skin tests were performed in six trials (labeled T1 to T6) with each 9 guinea pigs. Per guinea pig four injection sites on both sides of the flanks were available. According to the incomplete balanced Latin square design three PPDb batches can be assayed per trial (production batches and/or standards). Batches (production batches and standards) are assayed in three dilutions which were randomly allocated to the injection sites according to the incomplete balanced Latin square design (15). This design can be analyzed as a parallel-line assay (16).

Five weeks after infection, flanks were shaved and treated with depilatory crème leaving enough space for 4 injection sites on each side. Subsequently each guinea pig received eight injections of 0.2 ml of bovine tuberculin PPD. Syringes were coded making persons involved in the GP trials blind for the precise content of any syringe. Diameters of delayed type hypersensitivity reactions, visible as reddish circles around the injection sites, were measured with calipers between 24 and 28 h later. For details of the procedure for skin testing in guinea pigs, see Annex B Directive 64/432/EEC (10), OIE guidelines (9) and the European Pharmacopeia Monograph 0536.

In the six trials, the potency of 4 production batches of PPDb was determined in the guinea pig potency assay using both the RS and the BIS as standard. The four production batches were respectively: A (batch 102402), B (batch 104008), C (batch 110404), and D (batch 112003). In each potency assay, BIS and RS were included and one of the tuberculin batches A–D. Batch A was used in three potency tests, batches B, C, and D each in one potency test. Furthermore, each PPDb was tested in three dilutions 1:200; 1:1,000 and 1:5,000 (concentrations for the RS and the BIS being 0.005, 0.001, and 0.0002 mg/ml. In general, these concentrations result in well measurable skin reactions and acceptable confidence limits (9). Also, these concentrations are 5-fold dilutions, being equidistant at the log scale.

Statistical Analysis

The goal of the analysis was to determine whether production batches get comparable potency estimates when assayed against either one of both standards (RS or BIS), additionally the relative potency of the RS to the BIS in guinea pigs was calculated. Guinea pig trials were statistically analyzed using generalized linear mixed models [PROC MIXED of SAS (14)] using diameter of skin reaction (in mm) as the outcome variable and guinea pig was included as random effect. Analysis was performed according to a parallel-line assay, as described by Finney (16), with pairs of tuberculins: Batches A, B, C, or D against either RS or BIS and RS against BIS. The independent variables were tuberculin batch (production batch or standard) (Batch), logarithm of the concentration (Logconc), square of Logconc ($Logconc^2$) and the interaction between Batch and Logconc (Batch*Logconc). $Logconc^2$ was included in the model to assess whether or not significant curvature was present. From the interaction Batch*Logconc it can be concluded whether or not both batches show parallel lines. Guinea pig (animal) was included as random effect. The

assumption was that the diameter outcomes were directly proportional to the logarithm of the tuberculin concentration. For assaying the RS against the BIS a pooled analysis of all six trials was performed. Therefore, the variable Trial was added to the model as fixed effect, as well as the interaction between Batch and Trial. Insignificant variables ($p > 0.05$) were removed from the model using backward model building, except for Batch and Logconc, and Trial in case of the pooled analysis.

Relative potencies were estimated by exponentiation of the common slope. The corresponding 95% confidence intervals were obtained, according to Fieller's theorem (16) which is especially suited for interval estimation of ratios (17) using PROC IML of SAS (14). Finally, the relative potencies of the four additional tuberculin batches against RS were converted into actual potencies by multiplication with the potency estimate of RS from the cattle trial (which appeared to be 34,700 IU/mg). The relative potency of the RS against the BIS was converted to actual potency (expressed in IU) by multiplication with 32,500 IU which is the potency of BIS.

According to the regulations of the European Commission (10), the OIE (9) and the monograph 01/2008 /0536 of the European Pharmacopeia[1], potency testing of PPDbs in guinea pigs is only valid when the confidence limits are between 50 and 200% of the estimated potency. Furthermore, the estimated potency is not less than 66% and not more than 150% of the stated potency (9).

Guinea pig trials were approved by the Animal Ethic Committee (DEC) of the Animal Science Group of Wageningen University & Research (registration number 1625085300).

[1]European Pharmacopeia 9.0. *Monograph 01/2008/0536.*

RESULT

Cattle Trial

Descriptive Analysis

In total 240 observations were available, half of which were observations on RS injection sites and the other half on BIS injection sites. The average increase in skinfold thickness (between 0 and 72 h) was 6.88 mm (SD 2.81) and 6.48 mm (SD 2.61) for RS and BIS respectively.

Potencies

Statistical analysis showed that the variables Batch ($p = 0.01$) and Site ($p < 0.001$) were significantly related to the increase in skinfold thickness. LSMEANS for RS and BIS were 6.90 and 6.46 mm, respectively. The relative potency of RS against BIS (with stated potency of 32,500 IU/mg) was therefore estimated as 6.89540/6.4630 = 1.0669 (95% CI: 1.016–1.118) and the absolute potency of RS is then 1.067*32,500 = 34,674 IU/mg (95% CI: 33,020–36,335) or roughly 34,700 ± 1,650. This CI is smaller than the anticipated ±3,000 used in the simulation which is due to smaller variations between bovines (2.5 mm where 3.0 mm assumed) as well as within bovines (1.3 mm where 2.0 mm assumed). In the final model, the intra-class correlation due to the random bovine effect was 0.77.

Guinea Pig Trials

Descriptive Analysis

From all injections administered ($n = 432$), 34 resulted in a zero-response (diameter 0.00 mm). All originating from the lowest dose in the potency test (0.0002 mg/ml concentration). From historical data it is known that the lowest dose in the potency test can generate a zero-response. These were treated

TABLE 1 | Skin response (mm) of guinea pigs after injection with three concentrations of Bovine International Standard (BIS), Reference Standard (RS) and for production batches (A–D) using an incomplete balanced Latin square design; for RS and BIS data of six trials were pooled, for tuberculin A data of three trials were pooled.

Tuberculin batch	Concentration (mg/ml)	*N*	Mean	Std Dev	Minimum	Maximum
RS	0.0002	34	12.00	2.08	8.11	17.84
	0.001	48	15.70	1.92	10.31	18.97
	0.005	48	18.99	1.88	14.83	23.62
BIS	0.0002	36	12.04	1.81	7.25	16.16
	0.001	48	15.88	1.73	11.59	21.49
	0.005	48	18.67	2.00	14.90	24.75
A	0.0002	23	12.40	2.14	6.94	15.36
	0.001	24	16.07	1.40	13.58	18.69
	0.005	24	18.40	1.95	14.46	21.74
B	0.0002	4	12.75	3.68	9.20	17.90
	0.001	8	15.04	2.70	11.77	19.56
	0.005	8	20.23	1.65	17.64	22.47
C	0.0002	7	10.95	1.66	8.83	13.35
	0.001	8	15.78	2.37	12.69	20.05
	0.005	8	19.51	1.99	16.38	21.50
D	0.0002	6	12.02	1.63	9.85	14.12
	0.001	8	15.72	2.60	11.41	19.96
	0.005	8	19.69	0.97	18.10	20.85

as missing values and excluded from the analyses because there was no response to the lowest concentration of PPD. Besides that, inclusion of the zero-responses made the distribution of the outcome variable (diameter) non-Gaussian, preventing the valid use of generalized linear mixed models. Additionally, measurements of 36 skin reactions were labeled "weak" meaning they were measured with less accuracy due to an unclear distinction between the reddish hypersensitivity reaction and the normal skin. However, these measurements were not excluded from the analysis, as no clear decision rules exist when to exclude such observations. **Table 1** shows the average skin responses of all tuberculins as well as the corresponding minima, maxima and standard deviations. **Figure 1** shows the average responses of RS and BIS over the 6 trials.

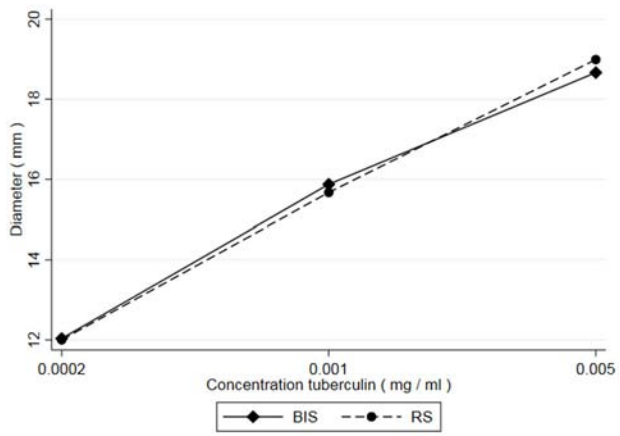

FIGURE 1 | Average skin responses (diameter) of guinea pigs ($n = 54$) after injection with 3 concentrations (equidistant on a log scale) of Bovine International Standard (BIS) and a Reference Standard (RS).

Parallel Line Assay

The initial model of the single trial analysis included the terms Batch, Logconc, Logconc^2, Site, and the interaction between Batch and Logconc. The effect of Site was not significant ($p > 0.05$) for any trial and was eliminated from the model. Logconc^2 was significant for the batch pair (A, BIS) in trial T2 and the interaction term Batch*Logconc was significant for (RS, BIS) and (A, RS) in T2 and for (RS, BIS) in T3. Therefore, these trials were deemed invalid and excluded from further analysis. The final models for T1 and T4-T6 included only Batch and Logconc as independent variables (**Table 2**).

Potencies

Table 2 displays the relative and absolute potencies and corresponding Fieller's 95% CI of RS compared to BIS, based on data of individual trials and of the four valid trials (T1, T4-T6) pooled. Potencies were not significantly different between RS and BIS because 1.0 is included in all confidence intervals of the relative potency of RS against BIS. The estimated potency of RS based on analysis of the pooled valid trials was 1.115*32,500 = 36,238 IU/mg (95% CI: 28,308–46,540).

Table 2 also shows the potency estimates of tuberculin batches A, B, C, and D against RS and BIS. These potency estimations were included to check whether the potency estimations of

TABLE 2 | Estimated relative and absolute potencies (IU/mg) of pairs of tuberculin batches with 95% CI's in six guinea pig trials.

Trial	Batch pair	N	Rel. pot. (95% CI)	Potency (95% CI)
T1	RS, BIS	48	1.230 (0.757–2.025)	39,975 (24,603–65,813)*
	A, BIS	48	1.233 (0.828–1.853)	40,073 (26,910–62,223)*
	A, RS	48	1.095 (0.739–1.627)	37,997 (25,643–56,457)**
T2	RS, BIS	48	Non-parallel	–
	A, BIS	48	Significant curvature	–
	A, RS	48	Non-parallel	–
T3	RS, BIS	45	Non-parallel	–
	A, BIS	45	0.782 (0.444–1.338)	25,415 (14,430–43,485)*
	A, RS	46	1.081 (0.760–1.542)	35,133 (24,700–50,115)**
T4	RS, BIS	41	1.132 (0.678–1.893)	39,280 (23,527–65,687)*
	C, BIS	43	1.103 (0.710–1.689)	35,848 (23,075–54,893)*
	C, RS	44	0.957 (0.639–1.417)	33,208 (22,173–49,170)**
T5	RS, BIS	39	0.954 (0.585–1.565)	31,005 (19,013–50,863)*
	D, BIS	42	1.181 (0.794–1.756)	38,383 (25,805–57,070)*
	D, RS	41	1.189 (0.766–1.827)	41,258 (26,580–63,397)**
T6	RS, BIS	41	1.131 (0.745–1.761)	36,785 (24,213–57,233)*
	B, BIS	42	0.975 (0.656–1.465)	31,688 (21,320–47,613)*
	B, RS	39	0.852 (0.559–1.285)	29,564 (19,397–44,590)**
T1, T4, T5, T6	RS, BIS	169	1.115 (0.871–1.432)	36,238 (28,308–46,540)*

Relative potencies multiplied by 32,500 (potency assigned to BIS).

**Relative potencies multiplied by 34,700 (potency assigned to RS when calibrated against bis in cattle).*

these additional tuberculin batches against RS were more or less similar to the potency estimations against BIS, which will be the case if the potency estimate of RS as found in cattle is valid. Potencies estimated using RS differ between +2,900 (trial 5, batch B) to −2,700 (trial 4, batch D) compared to BIS. The overall effect of trial was not significant ($p = 0.16$) and also no significant differences were present between individual trials (all p-values > 0.16).

DISCUSSION

Dobbelaer et al. (18) stated that potency estimations in guinea pigs can differ significantly from the potencies in the natural host. Therefore, a cattle trial was designed, performed and analyzed to assign a potency to a reference standard (RS) PPDb. The suitability of the new RS as a *M. bovis* reference standard to assign potency to individual production batches of PPDb was assessed in the guinea pig potency test, the prescribed release test for PPD[1]. Data from tuberculin skin tests in naturally bTB infected cattle and *M. bovis* infected guinea pigs were used to determine the potency of RS compared to the potency of BIS.

To obtain an unbiased potency estimation of the RS, any interference with the potency estimation by the inclusion of other tuberculin batches or tuberculin concentrations, which are not used in practice, should be avoided. Therefore, the cattle trial solely included RS and BIS, in only one dose of 0.1 ml of 1 mg/ml (which is the standard dose of injection in the field) (18).

In the cattle trial, the potency of RS was slightly higher than the potency of BIS. When rounded to the nearest hundred, the potency estimate of RS was 34,700 IU/mg, indicating a difference of 6.8% compared to the potency of BIS.

In guinea pig trials, commonly two test tuberculins are assayed against a standard tuberculin and the common slope of these three tuberculins is then used to estimate the relative potencies of the test tuberculins against the standard tuberculin. However, the most unbiased estimation of the potency of a tuberculin should be solely based on observations of one tuberculin against the standard and by that preventing any influence of the third batch. Therefore, we applied pairwise estimations of potencies, i.e., RS against BIS and of production batches against either RS or BIS. The potency of RS was estimated at 36,238 IU/mg, indicating a difference of 10.3% compared to the potency of BIS. The relative potency estimate of batch A against BIS in trial T3 (TUB 13/009B_Ba) is remarkably lower compared to T1 (0.782 vs. 1.233, p-value of trial 0.06) while the relative potency of batch A against RS was very similar (1.081 vs. 1.095) (**Table 2**). This could be due to an aberrant quality of BIS in the particular ampoule used in trial T3. It is well known that the quality of BIS is decreasing after 30 years of storage. The relative potencies of the four production batches were somewhat lower against RS than against BIS in 3 out of the 4 valid trials.

The cattle model as described in this paper is shown to be an excellent model for precise estimation of the potency of a new RS. Therefore, it is highly recommended to determine the potencies of a new bovine RS in the natural host, i.e., in naturally bTB infected cattle.

However, according to the European Pharmacopeia[1] the guinea pig potency test is the prescribed release test for production batches of bovine tuberculin PPD. Therefore, it was needed to show the suitability of the new RS needed in the guinea pig model as well. Our results are in accordance with the hypothesis of Dobbelaer et al. (18) that homologous tuberculins result in equal potencies in guinea pigs and in cattle. Indeed, the BIS, the RS and the four production batches used in this study are homologous tuberculins.

AUTHOR CONTRIBUTIONS

MdJ, KF, LJ, and TvD: conceptualization. KF, LJ, TvD, MG, and AD: data curation. KF and MdJ: formal analysis. MG, LJ, and MdJ: supervision. KF: Writing—original draft preparation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00232/full#supplementary-material

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Conflict of Interest Statement: LJ and TvD were employed by company Thermo Fisher, Prionics Lelystad B.V., Lelystad, The Netherlands.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with one of the authors KF.

frontiers in Veterinary Science

REVIEW
published: 06 November 2018
doi: 10.3389/fvets.2018.00276

Modeling as a Decision Support Tool for Bovine TB Control Programs in Wildlife

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Computer modeling has a long history of association with epidemiology, and has improved our understanding of the theory of disease dynamics and provided insights into wildlife disease management. A summary of badger bovine TB models and their role in decision making is presented, from a simple initial SEI model, to SEIR (inclusion of a recovered category) and SEI_1I_2 (inclusion of two stages of disease progression) variants, and subsequent spatially-explicit individual-based models used to assess historical badger management strategies. The integration of cattle into TB models allowed comparison of the predicted impacts of different badger management strategies on cattle herd breakdown rates, and provided an economic dimension to the outputs. Estimates of R_0 for bovine TB in cattle and badgers are little higher than unity implying that the disease should be relatively easy to control, which is at odds with practical experience. A cohort of recent models have suggested that combined strategies, involving management of both host species and including vaccination may be most effective. Future models of badger vaccination will need to accommodate the partial protection from infection and likely duration of immunity conferred by the currently available vaccine (BCG). Descriptions of how models could better represent the ecological and epidemiological complexities of the badger-cattle TB system are presented, along with a wider discussion of the utility of modeling for bovine TB management interventions. This includes consideration of the information required to maximize the utility of the next generation of models.

Keywords: badger, model, decision making, bovine tuberculosis, simulation

OPEN ACCESS

Edited by:
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Reviewed by:
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National Veterinary Institute, Sweden
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *02 July 2018*
Accepted: *17 October 2018*
Published: *06 November 2018*

Citation:
Smith GC and Delahay RJ (2018) Modeling as a Decision Support Tool for Bovine TB Control Programs in Wildlife. Front. Vet. Sci. 5:276. doi: 10.3389/fvets.2018.00276

INTRODUCTION

Mathematical models are both a simplification of reality and a reflection of our current understanding. As a working hypothesis of our supposed reality they can consequently only be shown to be wrong (1). A good model should only include necessary parameters, although the definition of "necessary" depends on the model's purpose. There are three main types of model: statistical, mathematical and simulation. Statistical models find relationships between parameters and will not be considered here. There is a continuum from mathematical to simulation models, but in general the former are used to investigate how a system works, while the latter, usually mechanistic, can be used to investigate management options.

Bovine tuberculosis (bTB, caused by *Mycobacterium bovis*) is a serious disease of cattle and control can be made more challenging by the involvement of wildlife reservoirs (2). In the UK and Republic of Ireland, European badgers (*Meles meles*) are implicated in the persistence and

spread of infection to cattle (3, 4). In both countries management of the risks of transmission to cattle has focused on culling badgers (5, 6). As badgers are native this imposes certain practical restrictions and attracts controversy. There has also been substantial Government investment in recent years in the development of a badger vaccine (7, 8) with small-scale deployment for research and operational purposes (9, 10).

M. bovis in badgers is a chronic progressive condition, which can lead to debilitating disease and death, although many infected badgers survive for years and prevalence can average about 10–20% or higher (11). Principal sites of infection are the lungs and associated lymph nodes. Badgers may exhibit a range of responses to infection ranging from latency (host infected but bacteria are effectively contained), to generalized disease (12) when they are likely to be most infectious, potentially shedding bacteria in sputum, feces, urine, or pus from wounds or abscesses (13). Once infectious, onward transmission of *M. bovis* occurs by aerosol transmission among animals in close contact, via bite wounding (14), and indirectly through environmental contamination (15, 16). Transmission to cattle is thought to be through contact with bacteria in the environment rather than via direct contact (17, 18).

Mathematical modeling has a long history with the badger-TB system. This has ranged from modeling the dynamics of infection in badger populations, to complex two host badger and cattle systems, and simulating the impact of management to inform disease control policy (see below). Modeling is often referred to as an iterative process. Models can be used to investigate the theoretical aspects of disease ecology and management, data are investigated to determine parameter values, and the models can determine where the data are deficient. If the model output is sensitive to parameter estimates that are uncertain or poorly measured, then this can be used to define new research questions and hence to guide the collection of empirical data to fill gaps and reduce uncertainties. These new data are then incorporated and the process repeated. This iteration rarely occurs in reality since people who generate empirical data and those who write models often work independently. Our research team (the UK National Wildlife Management Centre and its precursors), are therefore relatively unusual in this regard, being responsible for both the longest field study of badgers and bovine TB epidemiology (19), and the evolution of a series of models describing this system. Since reviews of badger/bTB models are already available [e.g., (20, 21)], we provide a historical narrative of the development of these models, the roles they have played in supporting decision-making, and our perspective on the future of modeling in this complex and challenging area of disease management.

HISTORICAL REVIEW

Early badger/bTB models investigated population dynamics in detail since this was the first opportunity to examine data from an ongoing study, resulting in a simple SEI (susceptible, exposed and infectious disease categories) model (22). This work summarized the known information on population dynamics (e.g., fertility and mortality rates). The resultant model suggested that disease induced mortality was 2.5 times natural mortality and thus exerted a high level of population suppression. The model was used to determine R_0, the expected number of secondary cases produced by one infected case in a completely susceptible population. This is a measure of the transmission potential of a disease and the estimated R_0 lay between 1.9 and 9.7, which reflected the level of parameter uncertainty. This model also explored pseudo-vertical transmission (i.e., mother to offspring transmission via close contact or ingestion of infected milk), the potential presence of asymptomatic carriers of infection, environmental reservoirs and inactive (short-term non-infectious) cases. With hindsight we can see that consideration of these phenomena illustrates the short-fall in empirical evidence on disease progression at the time (23).

The next model was an SEIR (SEI plus a recovered category) model and a parameter search used to refine population and epidemiological values (24). However, the inclusion of a "recovered" class was not itself tested, and has not been implemented in most other models. A further variant was the SEI_1I_2 model which permitted two levels of infectiousness (associated with early and advanced disease) and pseudo-vertical transmission (25). Investigation of six potential model structures suggested that those with two levels of infectiousness had some support.

The construction of an individual-based simulation model permitted the inclusion of territoriality and spatial components (26), which resulted in disease clusters and removed the clear relationship between disease prevalence and population suppression. The use of social groups also meant that the threshold density for disease persistence was now considered as the average minimum social group size that would permit disease maintenance. Although this model also suggested substantial disease-induced population suppression, the effect was reduced by the spatial clustering of disease (26). This was the first model to assess different historical badger management strategies (27): Gassing, Clean Ring, Interim and Live Test strategies (see **Table 1** for definitions). Model outputs suggested that the most efficient strategies were Gassing and the Clean Ring since they may remove foci of infection. The model also explored badger vaccination and concluded that it would take between 10 and 30 years to eradicate bTB with a perfect vaccine, depending on the efficacy of delivery. A later version investigated fertility control (through the use of a theoretical oral contraceptive) and concluded that in isolation this would not eradicate bTB in badgers but that disease control was possible when combined with high levels of culling (30). A simple generic model was used to simulate combined vaccination and fertility control and concluded that the reduced efficacy of vaccination, relative to culling, disappeared when allied with fertility control, and thus a combined approach could be effective (31).

A revision of Smith et al. (25) was the first model to predict limited population suppression (32), which was supported by the field data (33). This model also suggested that culling lactating females only had a limited impact on disease control, which supported the prevailing policy of releasing them.

A return to a simple model investigated the effects of social perturbation [the process of disruption of the social structure of

TABLE 1 | A summary of historical badger control strategies used in England.

Control Strategy	Approach	Estimated Efficacy of control[1]	Area[2]
Gassing	Gassing setts where badgers confirmed with bTB.	90%	Up to 10 km^2
Clean Ring	Cage trap and shoot social groups in an expanding ring where confirmed with bTB	80%	Mean 9 km^2
Interim	Cage trap and shoot badgers on and around confirmed cattle breakdowns	70%	Mean 12 km^2
Live Test	Trial strategy of cage trap and shoot in response to an antibody test.	80%	Mean 1 km^2

[1]*from Smith et al. (28),* [2]*from Krebs et al. (29).*

populations subjected to culling: Swinton et al. (34)], which could theoretically increase absolute numbers of infected animals. Both this and a subsequent study (31) also investigated the effect of fertility control, and suggested that lethal control was generally more effective. However, Smith and Cheeseman (31) found that permanent sterility combined with vaccination could be just as effective as lethal control, and would permit disease elimination without risking population extinction. Using updated parameters, a simple mathematical model of badgers and cattle concluded that R_0 was lower than previous estimates, at about 1.1 (35), and was supported by subsequent empirically-derived estimates of 1 to 1.2 (11). These findings suggest that control would require less than a 20% reduction in transmission rates to eliminate disease, although this appears to contrast with field experience.

Simulation models then added cattle, firstly as a simple homogenous set of herds connected to each badger social group (36). This model was used to assess the live test strategy (36), and other historical and prospective strategies (28) including vaccination (37). These studies concluded that the use of a live test required better test sensitivity and that more badgers per group needed testing and that Gassing and the Clean Ring were the most effective historical strategies. The model identified proactive widespread vaccination as the most effective vaccination strategy requiring vaccinating at least 40% of badgers every year to eliminate disease and that combined strategies gave the best initial reduction in cattle herd breakdown rates. Since the models were generating results that could inform policy, there was merit in ensuring the results were robust, so a second independent model was developed using the same input data. Reassuringly, this model gave very similar results (38).

Most of the data came from a field study of bTB epidemiology in badgers (39–42). When the latest models were subjected to sensitivity analysis, the outputs were found to be sensitive to the two infectious classes (particularly the more infectious category, and their mortality rates). This led to more detailed field research, which allowed disease categories and survival rates to be refined (43) and incorporated into subsequent models.

Between 1998 and 2005 a large scale field experiment took place in England, to determine the role of badger culling as a means of controlling bTB in cattle. Results of the Randomised Badger Culling Trial (RBCT) demonstrated that cattle herd breakdown rates were significantly reduced within proactively culled areas, but increased around the edges (4). Subsequent investigations identified significant spatial disruption of badger social group territories after culling (44), which tied in with previous observations of post-cull badger populations, including enhanced movement of surviving animals [reviewed by (45)]. The long-term field data from the Woodchester Park study demonstrated a clear link between badger movement rates and prevalence of bTB in an undisturbed population (46), suggesting that enhanced movements of badgers following culling might have adverse epidemiological outcomes. Thus, the model could now be updated by changing badger behavior (movement probabilities) to generate the pattern of herd breakdowns seen in the field. This approach of pattern-oriented modeling had recently been taking root in ecological models (47, 48). In a subsequent model, badger movement was simulated to match data from field studies (45), and the contact rate amongst badgers increased until the simulated rise in the herd breakdown rate matched that observed during the RBCT (49). The revised model also included a more realistic cattle layer incorporating individual farms and cattle movements, allowing investigation of pre-movement cattle testing, and including farm economics so that a partial cost-benefit analysis could be conducted. Even if most of the badger control costs were borne by the farmer the model concluded that, due to perturbation, the cost-benefit analysis was nearly always negative. Preventing badger immigration, or if perturbation did not occur, an economic benefit was more likely than not (49). If the Government bore the cost of badger culling then even without perturbation, most scenarios indicated an overall economic loss (50).

The Smith et al. (50) model was revised and updated with further field data, and used to investigate different bTB control strategies. In Wales the model was used to inform a decision on what badger management approach to take in an Intensive Action Area (IAA) identified by Government (51–53). The IAA was subjected to badger vaccination, and following 4 years of treatment the model was used to determine the effects of a lack of vaccine in the fifth year (54). This indicated that the fifth year of vaccination would add relatively little to the overall benefit, and no discernable benefit if vaccination was delayed by a year. This suggests that, following 4 years of treatment, herd immunity was raised to a level sufficient to justify a break in vaccination effort. In Northern Ireland, simulations investigated selective badger culling to inform proposals for a trap, live test and cull or vaccinate (TVR) approach (55), which is currently being trialed (56). In England the model was used to assess different culling and vaccination policies and concluded that in order to realize a benefit, badger culling would need to continue for at least 4 years and that low culling efficacy or an early cessation to culling could lead to an increase in the number of herd breakdowns (57).

Other models have investigated different selective or combined badger management strategies (58–60). Supporting previous results, these studies indicated that badger culling may reduce disease prevalence, but alone cannot eradicate bTB, and that combined vaccination strategies may be the most effective. None of the models have found that a single strategy is the most effective, generally agreeing that combined approaches are required, together with strong cattle measures. The deployment of such approaches in the field would provide data to test these predictions. The inability of models to easily eradicate bTB with single approach methods contrasts with the available estimates of R_0, which have suggested that control should be easier to achieve.

Although the principal driver for interest in bTB is to control the disease in cattle, there has been substantially less modeling focused on cattle. However, models of bTB in New Zealand were used to investigate cattle management. These indicated that improved cattle testing (61) and cattle management (62) alone were insufficient to eradicate bTB in the presence of the local wildlife vector (the brushtail possum *Trichosurus vulpecula*). A further model indicated the potential benefits of increased cattle testing, and reduced cattle movement in combination with wildlife vector control (63). These results, combined with output from other models (64–70) were used to inform the eradication strategy (https://ospri.co.nz/our-programmes/tbfree/about-the-tbfree-programme/about-bovine-tb/history-of-tb/).

Other wild mammal species can be infected with *M. bovis* and some may act as maintenance hosts, with potential onward transmission to cattle. In Spain, wild boar *Sus scrofa* and red deer *Cervus elaphus* appear most important as wild reservoirs of infection (71) and in North America white-tailed deer *Odocoileus virginianus* are involved in transmission to cattle (72). A model of bTB in white-tailed deer assessed various vaccination and targeted removal strategies and concluded that vaccination (alone or combined with targeted removal) needed to be undertaken annually to achieve a detectable reduction in prevalence (73), and currently an oral vaccination approach is under investigation (74). However, to date modeling has been applied to a far lesser extent to these situations compared to the badger-cattle bTB system.

The historical evolution of modeling described above clearly indicates where models have been used to inform decision making on bTB control in wildlife. In the badger bTB system, the interplay between field studies and modeling, and the use of models to guide decision making have been particularly prominent. Early models concentrated on increasing our understanding of the system with limited impact on decision making, but derived parameter estimates necessary for later models, which informed further field studies to refine key parameters. Successive models, which have generally included stochasticity, have since played a more explicit role in supporting decision making.

RECOMMENDATIONS

Below we describe a series of recommendations borne out of our experience of data analysis and modeling largely in relation to the badger/bTB system. Our recommendations relate first to themes for future models of bTB in badgers, and second to the presentation of model outputs to decision makers.

Future Models of bTB in Badgers

The following themes could be usefully explored in future models of bTB in badgers, but may also apply to other wildlife disease systems.

1. Recent models suggest that vaccination is a useful tool for controlling bTB in badgers, with the potential to be applied as an exit strategy from culling. Hence, more detailed investigations of vaccination strategies are required. Field and experimental evidence indicate that the current vaccine (BCG) does not provide complete protection from infection (75), but may confer partial protection, or slow down disease progression. To date most models assume that it confers lifetime protection from infection to a given proportion of the vaccinated population. Technically, these models place vaccinated badgers in a different category that has no increased mortality and no ability to infect others. Therefore, these individuals could become infected, and even react to various live tests, but fail to transmit infection, so the models do not actually assume complete protection, but an inability to become infectious. The available empirical data cannot easily distinguish between a proportion of vaccinated animals being very well protected, and all vaccinated animals experiencing slower disease progression. Such partial protection would lead to a reduced efficacy of disease control and requires further investigation in the field and through modeling. Further evidence is also required to determine the duration of protection (whether complete or partial).
2. Intervention duration and frequency have received little attention in models, and could usefully be explored in more detail. Most models assume either continuous or annual application of management, but recent evidence suggests that breaks in treatment may be possible without significant detrimental effects (54). This is important because even short breaks in management of a single year at a time may reduce overall cost and thus improve the economic outcome.
3. Social perturbation in culled badger populations has so far been simulated using a fixed effect, or by pattern-matching model output with field data. Modeling suggests that the presence of perturbation can be pivotal in determining whether a culling strategy is worth pursuing, but perturbation has only been modeled as an on/off effect. Further empirical evidence on the magnitude of perturbation effects encountered under different conditions, and refined model parameterization are vital to more accurately assess likely outcomes of different culling strategies and allow comparison with other approaches.
4. Within-individual level effects have not been explored in badger models. Where animals are tested, or subjected to management interventions (e.g., vaccination) in stochastic models, independence in outcome is assumed. This means that repeated testing (or repeated vaccination), will eventually detect (or sero-convert) every individual. Instead, it may

be that some individuals can never produce a positive test result (or be successfully vaccinated) due to a physiological process/characteristic. This would cause repeated (e.g., annual) management strategies to be less effective, but it is not clear how large such an effect may be.

5. Between-individual effects have not been explored. Most models assume all individuals are the same in terms of their physiological and behavioral responses, although there is clear empirical evidence to the contrary. Social network analyses have revealed individuals occupying different network positions, with associated variation in infection exposure and transmission potential (76). Models that account for individual heterogeneity in transmission rates (within and between species) may be worth investigating with a view to assessing the potential impacts on disease dynamics of removing key individuals in targeted management interventions.
6. Recent interest in selective removal strategies has raised the issue of test performance. In a model the infection status of each individual is perfectly known, whereas test performance determines sensitivity (all infected animals that test negative, regardless of whether latent, infected or infectious). For bTB there is no gold standard test, and thus no way to map an individual onto a simulated categorical state. Thus, test performance is determined globally on the population, and not for each disease state in a model, although empirical evidence suggests some tests have a differential sensitivity according to the stage of disease progression (77, 78). Also, novel probabilistic approaches to describing infection status may help us to incorporate uncertainty in test outcomes and provide a more meaningful way to categorize individuals (79).
7. Theoretical studies have suggested that fertility control may be a useful tool for disease control, particularly in combination with other approaches, but it has yet to be simulated for specific bTB control strategies. Suitable agents are currently available to induce immunocontraception that may last a number of years from a single dose (80) and these are under investigation for badgers, which are unusual in having delayed implantation (http://sciencesearch.defra.gov.uk/Default.aspx?Menu=Menu&Module=More&Location=None&Completed=0&ProjectID=17952).
8. There still appears to be a disconnect between the calculation of R_0 (close to 1.0), and the high level and lengthy duration of control required to achieve disease eradication in stochastic models. The duration of control is not technically a problem, since R_0 indicates the level of control required and tells us nothing about the duration. So this disconnect may be because model simulations are not of sufficient duration, or a result of other issues such as the spatial distribution of animals and disease.

Presenting Model Outputs to Decision Makers

It is clear from our experience that some modeling is more informative to decision makers than others. Below we suggest steps to help improve the relevance of modeling to decision makers.

1. It is important to know whether the purpose of the model is to help inform decision making, or to explore the system under study. In the former, the question to be investigated needs to be clearly articulated, ideally with the involvement of decision makers. The question should be specific, with an example graph or table in mind as the output, which allows both parties to agree on the output metric.
2. What the model does and does not include should be agreed with the decision maker. For example, it should be established whether a wildlife bTB model should include cattle so as to estimate changes in herd breakdown rates, or social perturbation arising from the intervention. The model should include all those components that the decision maker regards as important if they are to trust the output, or demonstrate that such components have very limited effect on the output.
3. Models that are well described and identify their assumptions and limitations, are given more weight by decision makers. Mathematical descriptions of model processes may be required for scientific publication, but flow charts are easier to follow. There are also guidelines to present the description of complex individual based models (81, 82).
4. Model description should include details of verification and validation, and some level of sensitivity and uncertainty analysis. Verification is the process of checking that the model does what is expected, and validation is the process of checking output against real world data (where possible). Sensitivity or uncertainty analysis can be used to demonstrate that a decision should be robust to the parameter uncertainty.
5. Model output is often best described in terms of the potential decision, rather than as a prediction of future trends. Models are simplifications, and are unable to capture the future variability of the real world. However, the performance of two modeled strategies will suffer to the same degree from these issues, and so can provide valuable information on their likely relative benefits and hence inform decision making. For the purposes of comparison it may be useful to determine how often one strategy outperforms another, as this will increase confidence in any selection.

These recommendations have applications beyond the bTB/badger system. Specific themes such as those relating to vaccination efficacy, the potential for management interventions to change host behavior and influence disease dynamics in counter-productive ways, and the performance of diagnostic tests are broadly applicable. This illustrates how the body of work on modeling bTB has contributed to our general understanding of the dynamics and management of disease in wildlife hosts and demonstrated how to model these systems.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

PERSPECTIVE
published: 07 December 2018
doi: 10.3389/fvets.2018.00310

Can We Breed Cattle for Lower Bovine TB Infectivity?

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Host resistance and infectivity are genetic traits affecting infectious disease transmission. This Perspective discusses the potential exploitation of genetic variation in cattle infectivity, in addition to resistance, to reduce the risk, and prevalence of bovine tuberculosis (bTB). In bTB, variability in *M. bovis* shedding has been previously reported in cattle and wildlife hosts (badgers and wild boars), but the observed differences were attributed to dose and route of infection, rather than host genetics. This article addresses the extent to which cattle infectivity may play a role in bTB transmission, and discusses the feasibility, and potential benefits from incorporating infectivity into breeding programmes. The underlying hypothesis is that bTB infectivity, like resistance, is partly controlled by genetics. Identifying and reducing the number of cattle with high genetic infectivity, could reduce further a major risk factor for herds exposed to bTB. We outline evidence in support of this hypothesis and describe methodologies for detecting and estimating genetic parameters for infectivity. Using genetic-epidemiological prediction models we discuss the potential benefits of selection for reduced infectivity and increased resistance in terms of practical field measures of epidemic risk and severity. Simulations predict that adding infectivity to the breeding programme could enhance and accelerate the reduction in breakdown risk compared to selection on resistance alone. Therefore, given the recent launch of genetic evaluations for bTB resistance and the UK government's goal to eradicate bTB, it is timely to consider the potential of integrating infectivity into breeding schemes.

Keywords: disease resistance, disease control, animal breeding, infectivity, bovine Tuberculosis

OPEN ACCESS

Edited by:
Flavie Vial,
Animal and Plant Health Agency,
United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *22 August 2018*
Accepted: *22 November 2018*
Published: *07 December 2018*

Citation:
Tsairidou S, Allen A, Banos G, Coffey M, Anacleto O, Byrne AW, Skuce RA, Glass EJ, Woolliams JA and Doeschl-Wilson AB (2018) Can We Breed Cattle for Lower Bovine TB Infectivity? Front. Vet. Sci. 5:310. doi: 10.3389/fvets.2018.00310

INTRODUCTION

Bovine tuberculosis (bTB) is a zoonotic disease, which can compromise both human health and international livestock trade. Zoonotic TB caused by *Mycobacterium bovis*, is responsible for an estimated 10–15% of human TB cases (1) and was estimated in 2016 as causing 12,500 deaths globally (2, 3). Addressing bTB infection in humans has been embedded within the United Nations Sustainable Development Goals 2016–2030 and World Health Organisation's (WHO) End TB Strategy framework, which employs a "One Health" approach aiming to end the global TB epidemic by 2030 (2–4).

In the UK, bTB has been the most pressing animal health problem, with financial losses amounting to over £175 m per annum (5). Tackling bTB has been a persistent challenge for the livestock industry, veterinary profession and policy-makers, and also the research community. The current national bTB eradication strategy involves the systematic testing of herds to identify and then remove infected cattle, and uses the Single Intradermal Comparative Cervical Test (SICCT), complemented by abattoir carcass inspections and, with increasing frequency, interferon-gamma testing. This surveillance regime has been successful in reducing disease spread in areas where bTB is prevalent and many EU countries and regions, including Scotland, have achieved Officially bTB Free (OTF) status (6). However, bTB persists in several regions (7) and herd incidence has increased in Wales, and also in High Risk and Edge areas in England (March 2018), despite the decrease in the overall herd incidence in England (8). Therefore, the continuing difficulties in eradicating bTB necessitate further exploration of additional disease control interventions that can complement existing strategies.

Selective breeding can complement classic disease control strategies, reducing the requirement for biosecurity measures and movement restrictions which have a major economic impact for herds undergoing a bTB breakdown (9, 10). Within the last few decades, breeding programmes (genetic and genomic selection) in livestock have achieved a remarkable improvement in production, e.g., milk yield in dairy cattle (11), and fitness traits such as fertility (12). Expanding the breeding objectives to include health and welfare traits offers new opportunities for disease control (10). The focus of genetic disease control so far has been on selection for improved resistance to becoming infected or diseased after exposure to pathogens. For example, by exploiting heritable genetic variation in disease resistance it has been possible to reduce mastitis incidence in cattle (13, 14) and mortality caused by infectious pancreatic necrosis in Atlantic salmon (15). Many studies have presented overwhelming evidence for genetic variation in resistance to bTB in cattle (16–19), which supports inclusion of bTB resistance in cattle breeding objectives in countries where bTB is prevalent. Recent efforts to combine national bTB surveillance and genetic data have enabled the publication of cattle evaluations for resistance to bTB in the UK (TB Advantage), which are currently used by farmers on a voluntary basis (20).

Veterinarians and epidemiologists have long considered reducing host infectiousness as an effective means to decrease disease transmission (21, 22). Infectiousness can be defined as the product of the contact rate between the infected individual and non-infected individuals, the propensity to transmit infection once infected (termed "infectivity" herein), and the duration of the infectious period (23, 24). For bTB, the contact rate between infected and non-infected herds is reduced by the movement restrictions imposed on herds with a bTB breakdown status. The duration of the infectious period is reduced by the test-and-cull policy which removes detectable infected animals, albeit with moderate animal-level sensitivity. In principle, infectivity can be reduced by vaccination, however, currently there are no vaccines (or subsequent tests) commercially available that allow differentiating between naturally infected and vaccinated individuals (i.e., a DIVA test) and would hence enable the safe use of vaccination for bTB control. Phenotypic variation in infectiousness is supported by numerous epidemiological studies showing that the Pareto principle commonly applies in epidemics, such that 20% of individuals are responsible for 80% of transmission events (22, 25–28). The individual differences in disease transmission are often attributed to different shedding patterns which may indicate phenotypic variation in host infectivity.

Emerging evidence suggests that infectivity can be, at least to some extent, under host genetic control (21, 29–33). Resistance and infectivity are thus two potentially distinct host genetic traits affecting disease transmission (see **Table 1** for definitions and statistical and mechanistic distinctions between resistance and infectivity). Hence, if genetic variation in infectivity exists, can be estimated reliably, and has no significant impact on other desired traits, reduced infectivity could be a target for genetic improvement, in addition to disease resistance. Several authors have previously proposed (29, 34, 35) or demonstrated theoretically (36–39), that breeding livestock for both resistance and reduced infectivity can be an effective approach to reduce disease risk and prevalence.

In this Perspectives article, we (a) review existing evidence that cattle may genetically differ in their bTB infectivity, (b) outline data and methodology requirements for estimating genetic infectivity for bTB, (c) discuss the benefits from considering infectivity in genetic evaluations, and (d) identify key challenges and future research opportunities for incorporating infectivity, in addition to resistance, in cattle breeding programmes aiming to reduce bTB prevalence.

Emerging Evidence That Infectivity Is Genetically Controlled

In bTB, differences in shedding patterns of *M. bovis* have been reported in various studies, but those have been mostly attributed to phenotypic variation rather than host genetics. For example, the number and frequency of episodes of shedding of *M. bovis* in cattle, were found to be dose- and infection route-dependent (40). Even amongst controlled experimentally infected calves, significant variation in shedding patterns have been described amongst individuals when presented with the same dose and infection route (41). In wild boars, the intensity and shedding of mycobacteria from the *M. tuberculosis* complex were found to affect the probability of new infections, while shedding intensity was shedding-route-dependant (42). In badgers, heterogeneity in shedding was found between different social groups (43) which may indicate family, and hence genetic, differences in shedding. Other studies found that the type of tuberculous lesions developed can affect the potential of infected individuals for transmitting infection (44), while evidence suggests that cattle with and without confirmed lesions may constitute, at least to some extent, genetically different subpopulations (45). Heterogeneity in lesion formation and stability of infected individuals suggests variation

TABLE 1 | Mechanistic and statistical distinction between resistance and infectivity in the context of bTB.

	Resistance	Infectivity
Definition (generic)	Propensity of an individual to become infected, given exposure	Propensity of an individual, once infected, to transmit infection to non-infected group members
Interpretation (bTB context)	For a given uniform level of exposure, a more resistant cow has lower risk of becoming *M. bovis* infected than a cow with low resistance	Given uniform contact rates and duration of infectious period, group members exposed to an infected cow with high infectivity have a greater risk of becoming *M. bovis* infected than when exposed to an infected cow with low infectivity
Disease phenotypes used in statistical models to infer trait estimates	Individuals' bTB infection status, based on ante-mortem test results, measured at multiple time points throughout a breakdown, possibly combined with post-mortem test results	
Trait contribution to disease phenotype	Only affects a cow's own infection status (direct effect on own disease phenotype)	Can only affect the infection status of group members (indirect effect on disease phenotype of group member)
Underlying mechanisms	Unknown; Speculated to be related to mechanisms affecting bacterial entry, establishment and within-host replication	Unknown; Speculated to be related to mechanisms controlling bacterial shedding patterns

in mechanisms underlying infectivity rather than resistance, as less stable lesions are more prone to breaking open and thus to higher bacterial shedding. Human tuberculosis epidemiology is consistent with the existence of *M. tuberculosis* super-spreaders (46, 47), which may indicate the existence of individuals with high infectivity. In bTB epidemiological studies, the best model fit has been observed when accounting for *M. bovis* super-spreaders (48, 49), and super-spreading has been proposed for badgers and other wildlife species (7, 50, 51). However, there remains a controversy about the existence of super-spreaders in bTB (52).

In other diseases, genetic variation in infectivity was found to manifest itself in various ways, such as through genetic differences in the potential for, quantity and type of infectious material shed by infected hosts. For example, genetic variation was found in the fecal egg count of sheep artificially infected with the same gastro-intestinal parasite strain and dose (53, 54). Furthermore, in cases of hosts infected with more than one genotype of the same pathogen, host immune response can affect pathogen strain competition and diversity with subsequent effect on host infectivity (55). More direct evidence for genetic differences in host infectivity has been recently obtained from transmission experiments of viral and protozoal infections in fish (31, 33). In these studies fish were found to differ in their probability of becoming diseased depending on the family or genotype of the initially infected fish that seeded the infection.

In summary, phenotypic variation in host infectivity is a common phenomenon, and for some diseases, this was shown to encompass genetic variation. In bTB, phenotypic variation in *M. bovis* shedding has been demonstrated by a few studies, but the extent to which this variation is due to cattle genetics is currently unknown. It is possible that host disease resistance and infectivity share some common genetic pathways controlling pathogen replication and consequently shedding (**Table 1**). This raises the question as to what extent bTB infectivity and resistance are genetically correlated, and how combined resistance and infectivity can affect bTB transmission.

Data and Methodology Requirements for Estimating Genetic Effects for bTB Infectivity

Infectivity, referring to an individual's ability to transmit infection (**Table 1**), is difficult to measure directly from field data where transmission routes (who-infects-whom) are difficult to trace and many transmissions are not observed or detected. Infectivity phenotypes can be obtained by measuring individual shedding rates (56). Measuring shedding has only been practical in special cases, e.g., fecal egg count for nematodes, and is very challenging if carried out routinely on the scale of sample sizes needed to inform breeding programmes.

However, shedding is not the only phenotype that can be used to track infectivity. Instead, it is possible to estimate genetic variation in infectivity by monitoring the progression of infection, i.e., the infection status of individuals, in different herds over time. Recently, novel inference methods have been developed to simultaneously estimate and untangle genetic effects for resistance and infectivity from longitudinal data of individual infection status (**Table 1**) (34, 36, 57, 58).

Common requirements for estimating genetic variation in infectivity using these novel methods are that (i) genetically related individuals are spread over different epidemics (herds/breakdowns), (ii) individual epidemics occur in "closed" groups with minimum between-group transmission, and (iii) individual infection times differ, and are known or can be inferred. These requirements appear to be satisfied by bTB. The UK national bTB eradication scheme has generated systematic repeated records of SICCT test results for a large number of herds containing related animals. In addition, due to movement restrictions imposed on herds undergoing a breakdown, herds can be considered as closed groups during the breakdown period, and data collected can be used to infer infectivity. Although the exact time of cattle infection with *M. bovis* is unknown, the repeated SICCT testing during this period provides longitudinal measurements of individuals' infection status, from which infection times can be inferred using Bayesian inference and data augmentation methods (34, 58).

It remains to be tested with field bTB data, how various sources of uncertainty affect genetic infectivity estimates. For example, these methods assume knowledge of the true infection status of an individual, which raises the question whether SICCT and other monitoring records are appropriate for this purpose. Of these, SICCT is the most commonly available measurement but its relatively poor sensitivity is well documented, i.e., its ability to correctly identify infected individuals; published sensitivity estimates range from 26 to 91% (59–63). Whether the test result reflects the true infection status of an animal is under on-going investigation within the bTB research community. Nevertheless, the positive predictive value of the test, i.e., the proportion of individuals that test positively and truly have the disease, is sufficiently high that false positives are likely to be few amongst the observed reactors. The specificity of SICCT in officially tuberculosis free herds has been estimated to be 99.98% (64). Therefore, already recorded SICCT phenotypes can provide information to search for genetic effects associated with infectivity. Including information from culling associated with SICCT testing has proven adequate for obtaining sufficiently accurate estimated breeding values (EBVs) for bTB resistance in the current bTB genetic evaluations (20). Expanding these evaluations to consider both resistance and infectivity would be expected to be beneficial primarily in high bTB risk areas, where the positive predictive value of SICCT is higher due to the higher disease prevalence (65).

Expected Benefits From Implementing Infectivity as an Additional Disease Phenotype in Genetic Evaluations for bTB Control

bTB has been a seemingly intractable problem in the UK in recent decades and understanding how cattle genetics influences bTB spread is important for eradication. Under the hypothesis that some cattle infected with bTB are genetically more infectious than others, reducing the occurrence of cattle with higher genetic infectivity through selective breeding would (i) reduce bTB transmission between cattle by removing highly infectious individuals comprising a major risk factor for herds, (ii) reduce shedding of *M. bovis* and hence reduce a major source of infection for the environment (30) and wildlife vectors, e.g., indirectly reduce bTB spill over to badgers. Badgers are susceptible to *M. bovis* infection and reducing infectivity in cattle should also reduce the pathogen burden in the environment (shedding e.g., in milk, feces, air, etc.).

If we were to estimate infectivity effects, it would enable breeders and farmers to select bulls whose offspring are not only expected to be less likely to become infected (more resistant), but also less likely to transmit bTB infection, if infected. Selection on breeding values for resistance and infectivity is expected to reduce the population R_0 (37, 39), i.e., the expected number of secondary cases produced by a typical infectious individual in a completely susceptible population (66), hence contributing to disease control. However, bTB transmission occurs within and across different species. Hence the overall bTB R_0 is composed of $R_{0_{cattle}}$, $R_{0_{badgers}}$, $R_{0_{cattle-to-badgers}}$, and $R_{0_{badgers-to-cattle}}$ (67, 68), where the cross-species relationships warrant further investigation. Reducing cattle infectivity would be expected to reduce $R_{0_{cattle}}$ and $R_{0_{cattle-to-badgers}}$, as well as the infection feedback loop from $R_{0_{badgers-to-cattle}}$. A small reduction in each component may suffice to bring the overall R_0 to below 1 and make the risk of new breakdowns negligible (10, 69). Investigating individual differences in infectivity might shed light on the variation observed in herd bTB prevalence and the relationship of infectivity with detectable bTB status, and why in some herds bTB persists with recurrent/chronic breakdowns, while other herds appear to be able to rapidly clear infection. Investigating variation in infectivity would also shed light on the weather bTB super-spreaders exist, as animals at the tail of the distribution would be "super spreaders" relative to all others, and what is their role in bTB spread.

A genetic-epidemiological simulation model can be used to assess the relative benefits of using a selection index that includes both resistance and infectivity compared to selecting on resistance alone. For this purpose we extended a stochastic epidemiological Susceptible-Latent-Infectious-Test sensitive model for bTB that originally assumed genetic variation only in resistance (70) with parameter values from the British genetic evaluations for bTB resistance (20) to incorporate hypothetical additional variation in infectivity (34, 38). We then used this model to simulate bTB spread in each herd and predict the impact of selection on breakdown risk, defined by the proportion of simulated bTB epidemics where infected index cases generated secondary cases. This is pertinent as field characteristics of epidemics often show curvilinear responses to control strategies. We found that when adding infectivity alongside resistance to the breeding objective, the reduction of the risk of a bTB breakdown was substantial and more pronounced in the early generations (**Figure 1**) (34, 38). For example, assuming 50% selection on sires, moderate heritabilities and prediction accuracies for resistance and infectivity, and zero correlation between resistance and infectivity, the relative epidemic risk at generation 5 was $\sim$0.2 with selection for resistance alone, but $<$0.1 for combined selection for both resistance and infectivity, even when external sources of infection were included (**Figure 1**). These simulations designed as proof-of-principle, provide a crude estimate of the predicted effects which will depend on e.g., the magnitude of the genetic variance in the objective traits and various demographic factors. However, these findings are indicative that by targeting both resistance and infectivity in combination, disease control benefits can be of larger magnitude (i.e., more effective) and more responsive (i.e., quicker to see results) (38).

The epidemiological benefits and additional gain expected from adding infectivity to the breeding goal depends on its genetic correlation with other traits of economic interest. A classic example of the impact of adverse correlated responses, is the reduction of cattle fertility following selection on milk yield, due to adverse genetic correlation with milk yield (12). Based on estimated genetic correlations among traits, genetic selection for enhanced bTB resistance is not expected to adversely affect other traits in the breeding goal (16, 20), and was found to be unlikely to change the probability of correctly identifying non-infected

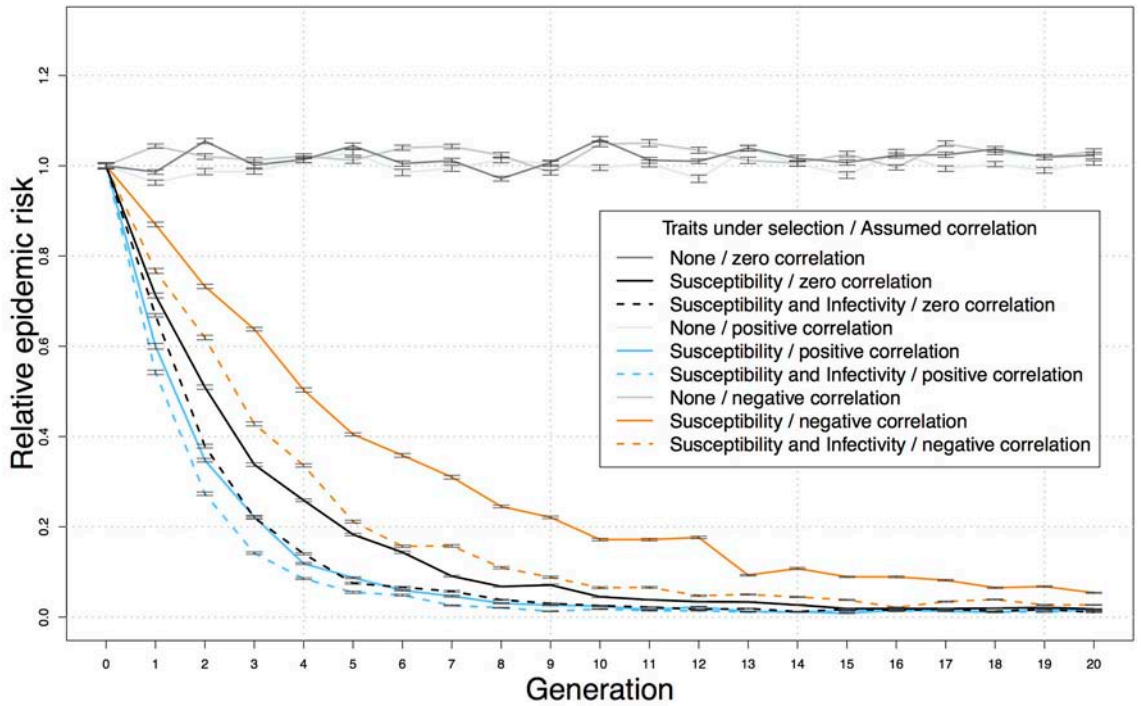

FIGURE 1 | Reduction in the predicted relative risk of bTB breakdown in a herd, following introduction of an infected cow, over 20 generations of selection for resistance and lower infectivity, or for resistance alone. Predictions from a stochastic genetic-epidemiological simulation model incorporating genetic variation in resistance and infectivity (38), comprising populations of 10,000 half-sib individuals randomly distributed into 100 herds of the same size. Means and standard errors were obtained over 50 replicates. The parameter values were based on the British genetic evaluations for bTB resistance (20) and a previous genetic-epidemiological model (70) as follows: bTB testing intervals of 60 days, SICCT sensitivity of 60%, 50% selection on the sires, accuracy of 0.5 and latent heritability of 0.6 [corresponding to heritability for the observed indicator traits of below 0.2 (70)] for both resistance and infectivity, economic values of one for both traits, and external force of infection of 5×10^{-5} (70). Each breakdown was initiated by one infectious individual, and variance and accuracy were assumed constant over generations. The correlation between resistance and infectivity was assumed to be zero, 0.5 or −0.5.

animals via the SICCT diagnostic test (71). However, genetic correlations between resistance and infectivity may affect the outcome of genetic bTB control. Indeed, based on the genetic-epidemiological bTB model described above (38) the strongest benefit of adding infectivity into the selection criterion compared to selecting on resistance alone is observed in the case of an adverse genetic correlation between the traits (**Figure 1**), and this is because the progress achieved by breeding schemes targeting only resistance would be delayed due to an indirect increase in infectivity. Considering breeding values for infectivity can help alleviate this delay and accelerate progress toward disease eradication (38).

Future Opportunities and Challenges

In principle, bTB surveillance schemes such as those implemented in the UK, RoI, and NZ, would permit researchers to pioneer the estimation of infectivity genetic effects without the need to collect new data. Current genetic evaluations for bTB resistance (20) use phenotype and pedigree information, with increasing amounts of genomic data. As the incidence of bTB reduces, the information obtained from pedigrees will also reduce. Therefore, genomic information becomes increasingly vital, and high-density genomic data can now be obtained cost-effectively by genotype imputation (72, 73). It has been shown that genomic prediction for bTB resistance using genomic information is feasible (18), and prediction accuracies can be improved by using larger training sets of genotyped animals and genome sequence information. This is pertinent for infectivity, as it has been shown in simulation studies that the prediction accuracy for infectivity is expected, at least initially, to be modest (34). This genomic information also allows investigation of the genetic architecture of bTB infectivity and the search for causal variants.

Challenges arising in the analysis of bTB data to uncover genetic infectivity include accounting for multiple and poorly understood transmission routes of *M. bovis*, and obtaining more reliable disease phenotypes. Separating the effects of the infectious dose from host response is extremely challenging in field situations where exposure may not be uniform (74). However, sophisticated Bayesian inference methods, coupled with phylodynamics and *M. bovis* genome sequence information, can help to infer transmission routes and obtain information on the networks of who-infects-whom (49, 75–78), which is useful for predicting infectivity (79). More reliable disease phenotypes could be obtained by quality control on individual tester performance to improve the consistency of data recording on the

farms (80), and by developing improved diagnostics. Machine learning techniques (Deep Learning) hold the promise that sufficiently accurate disease phenotypes can be obtained in a cost-effective manner for large sample sizes using routinely collected mid infra-red spectral data from milk recording (Coffey M. personal communication October 2018). Together, continuous development of improved diagnostic and modeling tools provide promising prospects for genetic bTB control.

CONCLUSION

Host infectivity is an important trait for disease transmission and emerging evidence suggests that it may be under genetic control to some extent; however, the role of genetic infectivity of cattle in bTB spread remains to be explored. Infectivity might be difficult to capture from noisy field data; however, the UK bTB surveillance database and newly developed statistical methods provide the opportunity to estimate genetic effects for infectivity. Exploiting genetic variation in infectivity as a complementary bTB control method is a low-investment high-return approach, as it can be developed at minimal cost using data already available. Simulation studies suggest that breeding for both disease resistance and infectivity can complement and substantially enhance current disease control approaches toward bTB eradication. Using UK data to determine genetic regulation of disease transmission can create a platform for controlling bTB in other countries and for controlling other infectious diseases.

AUTHOR CONTRIBUTIONS

ST, AA, RS, GB, JW, and AD-W conceived the perspectives. ST drafted the manuscript, designed and carried out the simulations and participated in the interpretation of findings. JW and AD-W contributed to the initial draft and simulation designs. All authors contributed to later versions of the manuscript, read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was carried out with funding from the Biotechnology and Biological Sciences Research Council Institute Strategic Programme grants BB/J004235/1 (ISP1) and BB/P013740/1 (ISP2) (OA, AD-W, GB and JW), and the European Union FP7 project FISHBOOST (KBBE - 7-613611) (ST). GB was also supported by the Rural and Environment Science and Analytical Services Division of the Scottish Government.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 01 October 2018
doi: 10.3389/fvets.2018.00237

Impact of Genetic Selection for Increased Cattle Resistance to Bovine Tuberculosis on Disease Transmission Dynamics

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Edited by:
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Reviewed by:
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Joseph Crispell, University College Dublin, Ireland

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *17 May 2018*
Accepted: *10 September 2018*
Published: *01 October 2018*

Citation:
Raphaka K, Sánchez-Molano E, Tsairidou S, Anacleto O, Glass EJ, Woolliams JA, Doeschl-Wilson A and Banos G (2018) Impact of Genetic Selection for Increased Cattle Resistance to Bovine Tuberculosis on Disease Transmission Dynamics. Front. Vet. Sci. 5:237. doi: 10.3389/fvets.2018.00237

Bovine tuberculosis (bTB) poses a challenge to animal health and welfare worldwide. Presence of genetic variation in host resistance to *Mycobacterium bovis* infection makes the trait amenable to improvement with genetic selection. Genetic evaluations for resistance to infection in dairy cattle are currently available in the United Kingdom (UK), enabling genetic selection of more resistant animals. However, the extent to which genetic selection could contribute to bTB eradication is unknown. The objective of this study was to quantify the impact of genetic selection for bTB resistance on cattle-to-cattle disease transmission dynamics and prevalence by developing a stochastic genetic epidemiological model. The model was used to implement genetic selection in a simulated cattle population. The model considered various levels of selection intensity over 20 generations assuming genetic heterogeneity in host resistance to infection. Our model attempted to represent the dairy cattle population structure and current bTB control strategies in the UK, and was informed by genetic and epidemiological parameters inferred from data collected from UK bTB infected dairy herds. The risk of a bTB breakdown was modeled as the percentage of herds where initially infected cows (index cases) generated secondary cases by infecting herd-mates. The model predicted that this risk would be reduced by half after 4, 6, 9, and 15 generations for selection intensities corresponding to genetic selection of the 10, 25, 50, and 70% most resistant sires, respectively. In herds undergoing bTB breakdowns, genetic selection reduced the severity of breakdowns over generations by reducing both the percentage of secondary cases and the duration over which new secondary cases were detected. Selection of the 10, 25, 50, and 70% most resistant sires reduced the percentage of secondary cases to <1% in 4, 5, 7, and 11 generations, respectively. Similarly, the proportion of long breakdowns (breakdowns in which secondary cases were detected for more than 365 days) was reduced by half in 2, 2, 3, and 4 generations, respectively. Collectively, results suggest that genetic selection could be a viable tool that can complement existing management and surveillance methods to control and ultimately eradicate bTB.

Keywords: bovine tuberculosis, resistance, susceptibility, epidemiological model, genetic selection, prevalence

INTRODUCTION

Bovine tuberculosis (bTB) is an infectious zoonotic disease of cattle caused by *Mycobacterium bovis* (*M. bovis*) that is endemic in many parts of the world (1). Notably, bTB continues to be a challenge in the United Kingdom (UK) despite a national eradication programme being in place for over five decades (2). In the UK, bTB control is mainly based on the culling of cattle that react positively to the single intradermal comparative cervical tuberculin test, commonly known as the skin test. When at least one positive reactor to the skin test is detected in a herd during routine testing, a "breakdown" status is declared, and animal movement restrictions are imposed on that herd. The herd is then systematically tested every 2 months and animals reacting positively to the skin test are sent to slaughter. When all animals test negative to two consecutive tests the breakdown officially ends and the herd re-enters routine surveillance (3).

In addition to herds being subjected to compulsory regular testing, other control measures are applied in relation to biosecurity (2, 4). However, so far, the existing control strategies have proven insufficient to eradicate the disease. This may be partially attributed to the low sensitivity of the skin test, potentially leading to undetected infected animals that contribute to the recurrence of breakdowns (5). Another contributing factor is the existence of wildlife reservoirs of *M. bovis* (for example, the Eurasian badger in the UK) (6). The problem persists and there is no clear evidence for a decline (7), despite the UK government spending over £175 million annually in the control of the disease (8). While Scotland was declared officially bTB free (OTF) in 2009, the governments of England and Wales have set a goal to become OTF by 2038 (4, 9). Thus, genetic selection for increased resistance of cattle to bTB may provide a potential complementary strategy (10) to achieve this goal.

Quantitative genetic studies have shown that there is genetic variation in cattle resistance to bTB (11–15). Therefore, it would be feasible to reduce disease prevalence and breakdown severity through selectively breeding for enhanced host resistance to the disease. In the UK, genetic evaluations of individual dairy cattle for resistance to bTB have been available since 2016. Availability of genetic evaluations enables the bovine industry to select sires based on their inherent capacity to produce more resistant progeny (16). However, before embarking on intense selection for enhanced resistance to bTB, it is important to understand the impact of such a selection process on disease risk and prevalence (17).

Genetic epidemiological models have been used to evaluate the role of genetic selection in populations undergoing an epidemic (17–19). Such models have been applied to a variety of diseases in farm animals including sea lice infection in the Atlantic salmon (20), bacterial (21, 22), and nematode (23) infestations in sheep, and Marek's disease in chickens (24). These studies estimated the impact of host genetic variation and genetic selection for increased host resistance on disease prevalence and spread. Several epidemiological models specific to bTB in cattle have been proposed (5, 25–31). None of them, however, has accounted for genetic variation in host resistance or considered genetic selection as a potential control option. In the present study, we propose an epidemiological model which, unlike previous models for bTB, incorporates genetic variation of disease resistance in the host, and models genetic selection.

Disease progression in previous epidemiological bTB models has been typically assumed to follow transition from the state of susceptible (*S*) to exposed (*E*), to test-sensitive (diagnosable; *T*), and finally to Infectious (*I*; *SETI* model). Typically, a susceptible animal becomes infectious only after going through the exposed and test-sensitive states (5, 27, 28, 30, 31). Pathogen transmission in the *SETI* model is such that infected animals that are test-sensitive and react positively to the skin test are removed before they become infectious. If this is the case, identification of infected animals through frequent comprehensive testing and immediate removal of test-positive animals as being currently carried out in the UK should substantially reduce bTB prevalence. However, given the current gap of knowledge about the relationship between *M. bovis* excretion and skin test response, and considering the persistence and general increase in bTB incidence over the past decade in the UK (7), other models of disease transmission dynamics need to be explored.

In the present study we considered a *SEIT* model where an animal becomes infectious (*I*) before infection can be detected by the skin test (*T*). This model implies that infected cattle may become infectious before they can be diagnosed and removed. Compared to the *SETI* model, *SEIT* represents the "worst case" scenario in terms of bTB transmission. The model follows the suggestion that all tuberculous cattle with lesions, particularly in the respiratory tract, should be considered as potential excretors of *M. bovis*, thus constituting sources of infection for other animals both within and across herds (32, 33).

The aim of the present study was to determine the impact of genetic selection for enhanced host resistance to bTB on cattle-to-cattle transmission dynamics and bTB prevalence using a *SEIT* epidemiological model.

MATERIAL AND METHODS

The impact of selection for increased resistance to bTB on the risk and severity of bTB breakdowns were investigated using a simulated, genetically heterogeneous cattle population. The proposed genetic epidemiological model was designed to simulate *M. bovis* infection dynamics in closed herds within the current UK bTB testing policy, firstly in the absence of selection and secondly following genetic selection for enhanced host resistance (reduced susceptibility) over 20 generations, with different selection intensities.

Simulated Populations

Non-overlapping generations of a dairy cattle population ($N = 20{,}000$) were generated comprising 50% males and 50% females. A founder generation was created, where sires and dams were randomly chosen and mated to create the base population. This base population was generated assuming a sire-to-offspring ratio of 1:50, thus being consistent with the national policy in reporting genetic evaluations for bTB in the UK (R. Mrode, personal communication, 2017). Large half-sib families were thus created, reflecting a realistic dairy cattle population structure where, with

the extensive use of artificial insemination, sires tend to have large numbers of progeny (daughters). Given that genetic selection of the best sires is the key component of selective breeding programmes in dairy cattle, selection was carried out based on estimated breeding values of sires generated as outlined below. This is also consistent with the current industry practice to only consider sire bTB genetic evaluations in selection.

Incorporating Genetic Variation in Host Susceptibility

Cattle susceptibility to bTB was modeled as a polygenic trait consistent with an infinitesimal model assuming presence of many loci each with a small additive effect on the trait (15, 34). More specifically, genetic variation for susceptibility was assumed to follow a normal distribution in the log scale, since previous studies suggested that disease traits are usually skewed (20, 35–37) and a log transformation is commonly used to achieve data normality (38). Considering that genetic evaluation methods may not capture all the additive genetic variance (σ_a^2) associated with a trait, therefore, both the true genetic value of an individual (TBV) for susceptibility and the corresponding estimated breeding value (EBV) were simulated drawing from normal distributions $N(0,\ \sigma_a^2)$ and $N(0,\ r^2\sigma_a^2)$, respectively, where r was the accuracy of the estimate. Thus, in the founder population, TBVs and EBVs were simulated from a multivariate normal distribution $MVN(0, \mathbf{G})$, where $\mathbf{G}$ corresponded to the genetic variance-covariance matrix. The covariance between TBVs and EBVs was derived as $cov_{TBV,EBV} = \mathrm{r}*\sqrt{\sigma_a^2}*\sqrt{\sigma_a^2 r^2}$. An additional term, the prediction error (PE) for each animal was computed as the difference between TBV and EBV.

In further generations, TBVs of the offspring of two selected animals were equal to the average TBV of the parents plus an individual Mendelian sampling (MS) term reflecting the random sampling and combination of parental alleles. This latter term followed a normal distribution $N(0,\ 0.5(1-\overline{F})\sigma_a^2)$, where $\overline{F}$ corresponded to the average inbreeding coefficient of the parents. In a similar way, the TBVs of the offspring were decomposed into EBV and PE, both being computed as the average of the respective parental values plus the corresponding MS terms, which were now drawn from normal distributions $N(0,\ 0.5(1-\overline{F})\sigma_{EBV}^2)$ and $N(0,\ 0.5(1-\overline{F})\sigma_{PE}^2)$, respectively. Therefore, simulated TBVs, EBVs, and PEs were computed for each offspring as:

$$\begin{aligned} EBV_{offspring} &= \overline{EBV}_{parents} + MS_{EBV} \\ PE_{offspring} &= \overline{PE}_{parents} + MS_{PE} \\ TBV_{offspring} &= EBV_{offspring} + PE_{offspring} \end{aligned}$$

In all generations, environmental effects were generated from a normal distribution $N(0,\ \sigma_e^2)$, where σ_e^2 corresponded to the environmental variance and was kept constant through all generations. Finally, the individual phenotypic value for underlying susceptibility to bTB i.e., g_i of each individual animal i was computed as the sum of the animal's TBV plus the corresponding environmental effect E, i.e., $g_i = TBV_i + E_i$.

Distribution of Animals Into Individual Herds

Currently, genetic evaluations for bTB in the UK assess the resistance of sires based on disease incidence of their daughters as described in Banos et al. (39). Therefore, breakdowns were simulated here based only on female offspring produced in each generation; the latter corresponds to 2–4 years in dairy cattle. A pool of selected sires was created, and female offspring were randomly allocated into 100 herds comprising 100 individuals each. Every selected sire contributed at least one daughter into one herd. Breakdowns were then simulated within each herd as outlined below.

The Epidemic Within Herd Transmission Model

A stochastic within-herd bTB transmission model was developed to simulate bTB spread in each herd and provide estimates of severity and duration of bTB breakdowns (**Supplementary Figure 1**). In particular, a compartmental *SEIT* model was assumed in which susceptible cows progress between the four infection states: (1) Susceptible state (S), where the animal is not infected but susceptible to infection; (2) Exposed state (E), where the animal is infected but not infectious and is undetectable by the skin test; (3) Infectious state (I), where the animal is able to infect others but is still undetectable by the skin test; (4) Test-sensitive state (T), where the infectious animal is now detectable by the skin test. Furthermore, the model incorporated the current UK policy of a 60 days routine skin test performed on all animals following the onset of a breakdown. At the specific test-days, infected animals at detectable state T may be diagnosed as reactors assuming a test sensitivity of Ω. Cows that reacted positively to the skin test were removed from the herd, in line with the UK official test-and-cull procedure (**Supplementary Figure 1**).

Infection (transition from state S to E) was modeled as a Poisson distribution process with time dependent average infection rate $\lambda(t) = \alpha + \beta(I(t) + T(t))$, where $I(t)$ and $T(t)$ were the number of animals in the herd at the I and T states at time t, respectively, and the parameters α and β represented transmission coefficients for external sources of infection (aggregate of all potential sources of external infection including wildlife, infected move-in cattle and infected cattle from contiguous farms) and for within-herd cattle-to-cattle transmission, respectively (**Supplementary Figure 1**) (30, 31). A density dependent mode of transmission was assumed as herd size is known to be correlated to bTB incidence and persistence (40–42). Progression of infected cows from E to I state and from I to T state occurred at average rates σ and γ, respectively (**Supplementary Figure 1**).

Individual variation in susceptibility was incorporated into the model through each individual's log-normally distributed susceptibility phenotype calculated as outlined above. The individual infection rate of individual i at time t was then defined as $\lambda_i(t) = e^{g_i}(\alpha + \beta(I(t) + T(t)))$, where g_i refers to the normally distributed susceptibility value specified by the genetic model above. In contrast to the population averages for α, β

σ, and γ, which were kept constant over successive generations, the average susceptibility g changed over generations because of genetic selection.

To generate a sufficient number of herds experiencing breakdowns in the first generation, the epidemic in each herd was started by two randomly chosen infectious individuals in state I, termed "index cases." Two individuals were chosen here instead of one to ensure that breakdowns did not die out within the first 60 days of duration. This editing step allowed us to generate enough data to test the various genetic selection practices described below.

Disease progression within each herd was then simulated as a series of random independent events representing the transition of an animal between two successive states in the compartmental *SEIT* model. The time to the next event (inter-event time), the corresponding event type (for example, transition from S to E), and the corresponding individual experiencing the transition were determined using Gillespie's direct algorithm adapted to heterogeneous populations as outlined in Lipschutz-Powell et al. (35).

Possible events in our model were the infection of a susceptible animal (transition from S to E), an exposed animal becoming infectious (transition from E to I), an infectious animal becoming test-sensitive (transition from I to T) and a test-sensitive animal being removed from the herd after testing positive to the skin test (transition from T to R). However, the latter event was modeled separately at time intervals of 60 days according to the official skin test schedule. For the other events the inter-event time was sampled from an exponential distribution with rate equal to the sum of all process rates calculated as $R_{total} = \sum_{i=1}^{N_S} e^{g_i}(\alpha + \beta(I+T)) + \sigma N_E + \gamma N_I$, where Nx is the total number of animals in each x state within the herd. In other words, the time to the next event was estimated as $-\ln(y)/R_{total}$, where $y \sim U(0, 1)$. The specific event type e that occurs at that particular time was sampled by drawing a random variable from a distinct distribution with probability $\frac{p(e)=R_e}{R_{total}}$. R_e is the rate of occurrence of the specific event. The individual in the particular event was then chosen randomly, and in the case of infection (S to E) it was weighted by the individual's susceptibility phenotype.

In line with the current bTB control strategy, the epidemic in each herd was simulated until the end of a bTB breakdown, defined by two consecutive negative skin tests for all herd members (3). During the epidemic, the number of individuals in each disease state together with the corresponding times was recorded, and based on these, the total number of reactors and the duration of each epidemic (i.e., the time from beginning to end of a breakdown) were derived.

Model Parameterization and Validation

Input parameters for the epidemiological bTB model illustrated in **Supplementary Figure 1** were based on real field data used for national genetic evaluations for bTB in the UK. These data consisted of 1,210,652 cow records from 10,589 herds where breakdowns had been declared between the years 2000 and 2014. The mean number of animals per herd in the dataset was 114, and the recorded number of infected animals referred to reactors diagnosed by the skin test. Based on the latest bTB epidemiological study in the UK (31) the value of the external rate of infection α in the simulation (**Supplementary Figure 1**) was set to 5×10^{-7} days^{-1}. Furthermore, a skin test sensitivity (Ω) of 0.60 was used as in Banos et al. (39), which is the value considered in the current official UK genetic evaluation for bTB resistance. To determine the remaining parameter values of the *SEIT* model (β, σ, γ, as well as genetic and environmental variances for underlying susceptibility), multiple parameter combinations were tested and the corresponding model output was compared to the following characteristics derived from analyzing the field data: mean percentage of skin-test reactors per breakdown (8.5%), mean duration of breakdown from official onset to end (366 days), and genetic variance (0.0032) and heritability (0.10) of the observed bTB phenotype indicating presence (reactor) or absence (non-reactor) of bTB. We derived these estimates from the analysis of the above-mentioned field data using the model described in Banos et al. (39).

The bTB susceptibility phenotype g in the *SEIT* model (**Supplementary Figure 1**) corresponds to the underlying scale of the binary presence or absence of the disease trait in the data analyses (39) (observed scale). To make the model results concordant with the observed scale, a range of different genetic and environmental variance estimates for the underlying scale in the base population were explored and the corresponding heritability and genetic variance estimates on the observed scale were calculated. The final genetic and environmental variances chosen for the simulated data on the observed scale and used to generate the base population were those that were closest to the real field data estimates on the observed scale.

In order to study the impact of variation in epidemiological parameters on disease epidemic and genetic selection, two additional simulation scenarios were run, one assuming a 10-fold increase in the rate of external infection ($\alpha = 5 \times 10^{-6}$ days^{-1}) and another considering a lower sensitivity of the skin test ($\Omega = 0.30$); the latter is similar to the lower credible interval obtained in the meta-analysis of skin-test sensitivity by Nuñez-Garcia et al. (43).

Genetic Selection Process and Impact

Firstly, epidemics were simulated for 20 generations without any genetic selection (100% of sires used for breeding) in order to establish the baseline of bTB transmission dynamics. Subsequently, truncation selection of genetically resistant sires was simulated for 20 generations. Sires were selected for breeding based on their underlying susceptibility EBVs. Different levels of selection intensity were explored by selecting the 10, 25, 50, and 70% most resistant (least susceptible) sires. These reflect different potential selection strategies against the disease. Selected sires were randomly mated with cows. Dams were randomly selected in each generation. Population size and sex ratios were kept constant across generations. The female offspring of these sires then formed the next generation of individuals for which bTB epidemics were simulated.

The impact of genetic selection on bTB prevalence was assessed in each generation by estimating the mean underlying susceptibility to *M. bovis* infection in the population as well as the risk and severity of breakdowns. A breakdown was assumed to have occurred when at least one secondary case was produced from the index cases within a herd. Otherwise, in the absence of a secondary case a "no breakdown" was declared and duration equal to 0 days was assigned. Therefore, the risk of a breakdown (probability of a breakdown occurring) was defined as the proportion of simulated epidemics that resulted in at least one secondary case (infected cow other than the index cases that seeded the epidemic). The severity of a breakdown was then assessed by estimating the percentage of secondary cases and the duration of their occurrence within the breakdown (duration of secondary cases). Breakdowns were categorized as mild, moderate, and severe based on mean percentage of secondary cases being less or equal to 3% (only 1 secondary case), 3–10%, and above 10% (10% equating 50% of breakdowns in the distribution) respectively. Breakdowns were also categorized as short, medium and long depending on whether the duration of secondary cases was less than or equal to 180 days, between 180 and 365 days, and above 365 days, respectively.

Finally, to assess the impact of the *SEIT* model assumption that animals become first infectious and then test-sensitive, the same simulations were run separately assuming a *SETI* epidemiological model. In the latter, infected animals were test-sensitive, hence detectable, before they became infectious. The same parameters were used as for the *SEIT* model.

In all cases, each selection scenario reflecting one of the four selection intensities described above was replicated 50 times. Results were averaged across all herds and replicates for each generation.

RESULTS

Parameter Values and Model Fit to Real Data

Parameter values were identified to ensure that simulated and real bTB breakdowns shared similar characteristics with respect to the distributions of mean percentage of reactors per breakdown, total duration of breakdown, genetic and phenotypic variance and heritability of susceptibility on the observed scale (**Figure 1**; **Table 1**). The distributions of both the mean percentage of reactors per breakdown and the total duration of breakdown were more long-tailed in real data compared to simulated data (**Figure 1**), probably because real data were affected by more extreme and unpredictable environmental conditions than those modeled in the simulation. Significant correlations ($p < 0.001$) were found between mean percentage of infected individuals per breakdown and mean duration of breakdown in both datasets; however, the correlation was smaller in real data (0.43) than in simulated data (0.85), for the same reason as stated above.

The rate of progression from the E to I state, σ, corresponded to an exposed state duration ($1/\sigma$) of 25 days (**Table 1**). The rate of progression γ from I to T state suggested that, once a cow becomes infectious, she is expected to respond to the skin test within ($1/\gamma$) 2 days.

Impact of Genetic Selection on Underlying Susceptibility

Genetic selection resulted in a reduction in the mean underlying susceptibility to bTB and the corresponding genetic variance (**Supplementary Figure 2**). The initial underlying susceptibility phenotype in the base population was simulated with a mean of zero, hence the decrease in susceptibility due to selection is depicted by negative values in **Supplementary Figure 2B**. Greater reduction was observed for higher selection intensities. As expected, no change in genetic variance and mean susceptibility was observed over generations in absence of selection.

Impact of Genetic Selection on Epidemic Profiles

Figure 2 shows the *SEIT* profiles (proportions of individuals in different states of the *SEIT* model) over successive generations for different selection intensities. The proportion of infected animals, including those in the exposed, infectious and test-sensitive states, was high before selection and significantly reduced after implementation of selection. As expected, there was no significant reduction in the number of infected individuals and duration of the epidemic over generations when no selection was performed (**Figure 2A**). Selection noticeably affected both the epidemic risk (illustrated here by the decreasing number of epidemic profiles over successive generations in **Figures 2B–E**) and severity (illustrated here by the number of infected (*E*, *T,* and *I* states) individuals and epidemic duration). As expected, the higher the selection intensity, the stronger was the impact on the epidemic profile (**Figures 2B–E**).

Impact of Genetic Selection on Risk of a Breakdown to Occur

Figure 3 shows a decrease in the probability of a breakdown occurring with increasing selection intensity. Prior to selection, the mean probability of occurrence of a breakdown was 81.8%. When higher selection intensities were applied corresponding to selection of the 10 and 25% most resistant sires, this probability was halved after 4 and 6 generations, respectively. A similar result was achieved for lower selection intensities (50 and 70% most resistant sires) after 9 and 15 generations, respectively.

Impact of Genetic Selection on Percentage of Secondary Cases and Duration of Their Occurrence Within Breakdowns

Genetic selection led to a decline in the percentage of secondary cases per breakdown (**Figure 4A**). To reduce the percentage of secondary cases per breakdown to <1%, 4, 5, 7, and 11 generations of selection were required when 10, 25, 50, and 70% most resistant sires were selected, respectively. The corresponding duration of secondary case occurrence within a breakdown in these generations was reduced by more than half to 114.9, 125.5, 139.9, and 141.8 days for the four selection intensities, respectively, compared to 326.1 days before selection was introduced (**Figure 4B**). Furthermore, selection for 12 and 17 generations was required to eliminate the epidemics (occurrence of secondary cases less than or equal to 0.1%) when 10 and

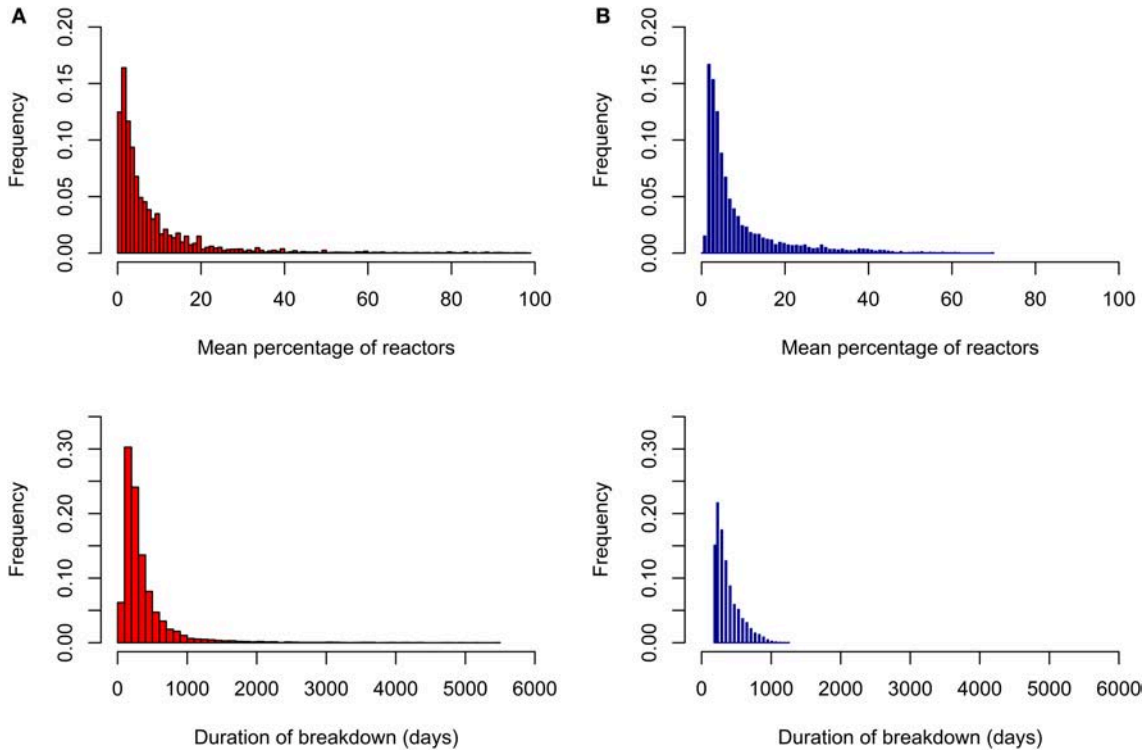

FIGURE 1 | Distribution of percentage of reactors to the skin test per breakdown and duration of breakdown. Results from real data are given in red **(A)** and from simulated data in blue **(B)**.

25% most resistant sires were selected, respectively. However, elimination of bTB was not possible with lower selection intensities (greater proportion of sires selected) during the simulated selection period of 20 generations. In the absence of selection, the percentage of secondary cases and time for induction of secondary cases fluctuated around the initial mean (**Figures 4A,B**).

The effects of genetic selection when breakdowns were categorized according to severity are illustrated in **Figure 5** and **Supplementary Figure 3**. Prior to selection, the proportion of mild, moderate and severe breakdowns was 0.46, 0.32, and 0.22, respectively. During selection, the overall severity of breakdowns decreased across generations (**Figure 5**). When high selection intensities were applied (selection of the 10 or 25% most resistant sires), almost all breakdowns became mild by generation 10 (**Figure 5A**). However, it was only when selection of the 10% most resistant sires was implemented that breakdowns became short at the end of selection (**Supplementary Figure 3A**). Proportion of long breakdowns was reduced by more than 50% after 2, 2, 3, and 4 generations for selection of 10, 25, 50, and 70% most resistant sires, respectively (**Supplementary Figure 3B**). In the absence of selection, severity of breakdowns remained constant, with slight fluctuations across generations (**Figure 5**; **Supplementary Figure 3**).

The above results collectively demonstrate how genetic selection has the potential to reduce the probability of a breakdown occurring and the severity of the breakdowns that do eventually occur.

Impact of Variation in Epidemiological Parameters

Scenarios with a 10-fold increase in the external rate of infection ($\alpha = 5 \times 10^{-6}$ days^{-1} instead of 5×10^{-7} days^{-1}) are shown in **Supplementary Data Sheet 1**. All other parameters being the same, this increase led to a small non-significant tendency toward more severe breakdowns in early generations but did not influence the impact of genetic selection on disease epidemic, probability of breakdown occurrence and severity of breakdowns.

The reduction of the skin test sensitivity to 0.30 from 0.60 led to an increase in the severity of breakdowns in terms of number of secondary cases and duration but did not affect the probability of a breakdown to occur (**Supplementary Data Sheet 2**). Importantly, the impact of genetic selection on the disease transmission dynamics was similarly demonstrable in the case of reduced sensitivity of the skin test.

Comparison Between SEIT and SETI Models

The impact of genetic selection on the risk and severity of breakdowns under the two models were very similar (**Supplementary Figure 4**). For the same parameter values, slightly more secondary cases per breakdown were generated

TABLE 1 | Epidemiological and genetic parameters of bovine tuberculosis in simulated and real (field) data.

	Simulated data	Real data
PERCENTAGE OF REACTORS TO THE SKIN-TEST (%)		
Average	8.7	8.5
Range (min–max)	0.0–70	0.08–98.0
3rd Quartile	10.0	9.5
Standard deviation	9.5	12.4
DURATION OF BREAKDOWN (NO. DAYS)		
Average	365.9	365.7
Range (min–max)	180.0–1,260	60.0–5,457
3rd Quartile	420.0	409.0
Standard deviation	174.7	395.1
EPIDEMIOLOGICAL PARAMETERS		
Rate of external infection (α) [days^{-1}]	5×10^{-7}	
Transmission coefficient (β)	0.012	
Rate from exposed to infectious state (σ) [days^{-1}]	0.04	
Rate from infectious to test-sensitive state (γ) [days^{-1}]	0.5	
Rate of detection (Ω)	0.60	
GENETIC PARAMETERS OF SUSCEPTIBILITY		
Underlying scale		
Genetic variance	0.3	
Environmental variance	0.3	
Accuracy of selection	0.63	
Observed scale		
Genetic variance	0.0034	0.0032
Phenotypic variance	0.032	0.031
Heritability	0.106	0.103

with the *SEIT* (6.8%) compared to the *SETI* (5.8%) model in the base population (unselected population). The same number of generations was required in either model to reduce the probability (risk) of a breakdown to occur by half. Similarly, the difference in time required to achieve a certain percentage of reduction (e.g., 50%) in secondary cases or time for induction of secondary cases between the two models was always less than one generation (**Supplementary Figure 4**).

DISCUSSION

Considerable advances in infectious disease control may be achieved by selective breeding programmes that include disease resistance of animals in the breeding goal (44). In this context, a breeding programme that exploits existing genetic variation in host susceptibility to bTB could form an important part of the national bTB eradication strategy (11–13, 15, 39). However, quantitative genetics theory alone cannot predict how genetic gain in disease resistance translates into reduction of bTB breakdown risk and severity. The novelty of the present study lies in (i) the development of a genetic epidemiological model that combines for the first time quantitative genetics and epidemiological dynamics of bTB, and (ii) the ability of this model to assess the consequences of genetic selection for enhanced host resistance on bTB prevalence and dynamics.

Our choice of model parameter values was informed by previous literature estimates (5, 27–31, 45) and bTB field data in order to represent UK field conditions. Similarities between model and field or experimental data are essential for drawing reliable conclusions from model predictions (46). In the present study, real data were somewhat more variable than simulated data as manifested by a wider range and greater standard deviation. Otherwise, the simulated model outputs, including mean values and genetic parameters, were similar to results obtained from field data analysis. The distributions of percentage of reactors to the skin test in both real and simulated data were characteristically skewed to the right and correlated with breakdown duration. Skewness in the distribution of disease traits may be attributed to between animal genetic variation (20) and also environmental effects (47). In the real data, other factors such as differences in herd size, management, badger prevalence and climatic conditions are likely to contribute to the diversity observed in epidemic characteristics (42, 48, 49). Many of these factors are recorded in practice, and can be captured by statistical models and accounted for in the genetic evaluation. Other, non-systematic sources of variation would constitute noise in the statistical models. Increasing model complexity by including various systematic or non-systematic effects into the simulation model may increase variability in the model predictions, but would not affect selection response.

Although the bTB model in the present study differs from previous epidemiological bTB models that did not incorporate genetic variation in the host, the estimated population average transmission coefficient β was within the range of transmission coefficients (0.006–0.014 days^{-1}) previously reported (5, 27, 29, 31, 50). The duration of the exposed state (E) in our model was 25 days, thus slightly higher than the 20 days estimated by O'Hare et al. (31) using UK data and a *SETI* model. In our study, an animal that became infectious was expected to become detectable within 2 days. This short time interval may be sufficient for some additional infected animals to infect others prior to their own diagnosis and subsequent removal from the herd. This may partly explain the persistence of bTB in the UK despite the on-going regime of skin testing and slaughtering of positive reactors. The 2 days between the I and T states in the present study is comparable to the 1.8 days estimated by Conlan et al. (5), where early infectiousness was assumed (considering animals in both E and T states in the *SETI* model to be infectious). In their model the E state was referred to as the occult state to denote that, although infectious, animals were not detectable by the skin test (5). These estimates would imply that, once animals are infectious a relatively short time is required before they can be detected by the skin test.

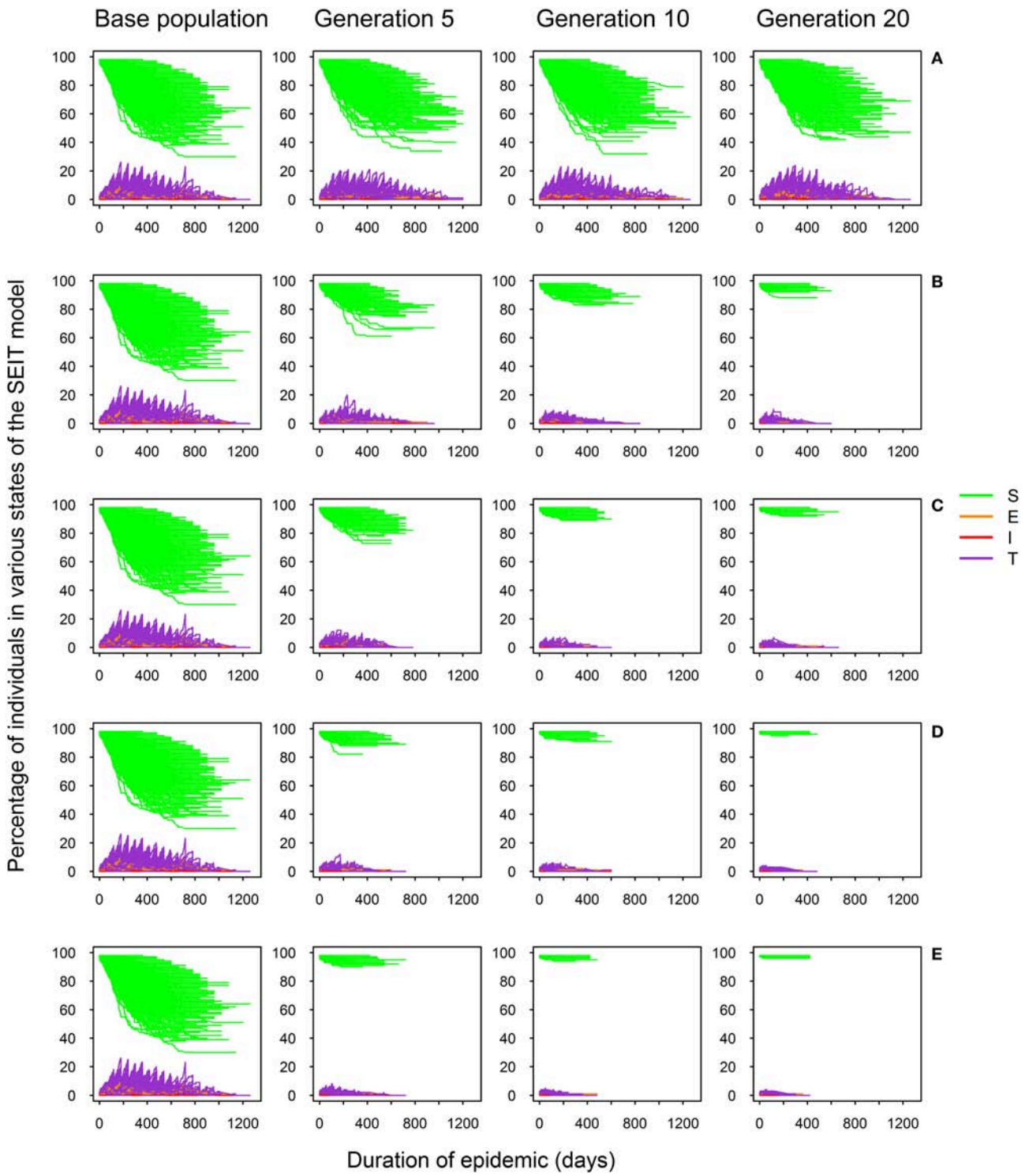

FIGURE 2 | *SEIT* model profiles across 20 generations for five selection intensities defined by the percentage of selected sires: 100% (no selection; **A**), 70% **(B)**, 50% **(C)**, 25% **(D),** and 10% **(E)**; proportion of susceptible (*S*), exposed (*E*), infectious (*I*), and test-sensitive (*T*) individuals during the course of the epidemic.

Several important implications arise from our results as far as interpretation of bTB transmission and evaluation of control strategies are concerned, particularly with regards to the implementation of genetic selection for increased host disease resistance. Although the potential of the latter as a complementary strategy for disease control has been recognized

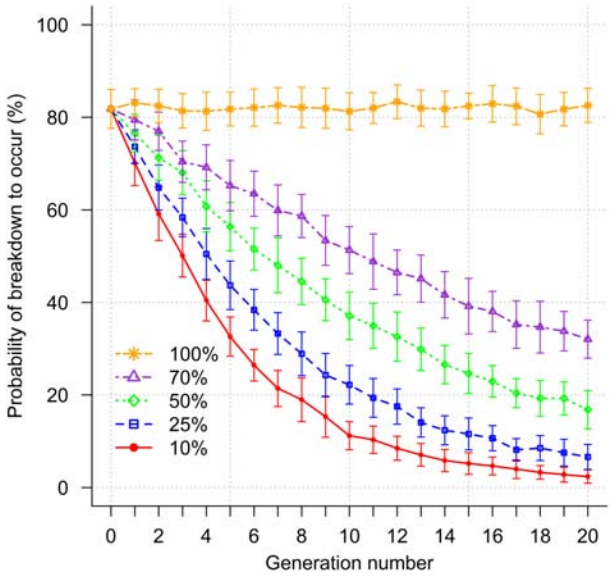

FIGURE 3 | Impact of genetic selection on risk of breakdown (probability of a breakdown to occur). Selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires.

(10), its utility in terms of reducing disease risk, prevalence, and severity has not been previously assessed.

Susceptibility on the underlying scale affects the probability of an individual to become infected. Therefore, as animals become more resistant, the expectation is for them to become less likely to be infected. Our results demonstrate how reduction in individual infection probability as a result of genetic selection for host resistance to bTB relates to the probability of breakdowns to happen in the first place. Equally important, even when a breakdown was to occur, it would be less severe in terms of number of infected individuals and duration compared to a no selection scenario. Thus, our results are in agreement with previous studies that demonstrated that selection can reduce both the risk and severity of epidemics for other diseases in livestock and fish (17, 20, 21, 51, 52). This is expected to lead to a reduction, not only in frequency of future breakdowns but also in economic losses, as prolonged breakdowns consume substantial resources. Furthermore, as selection reduces the number of reactors during a bTB breakdown, it is also expected to reduce the risk of recurrence (53, 54). Recurrence has been found to be high in the UK, where 23% (38%) of breakdowns recur within 12 (24) months despite the on-going testing regime (55).

We explored the amount of genetic progress in bTB resistance when sires were selected at different levels of selection intensity. Simulating different selection intensities provides insights into future options for breeders. In all cases, our model predicted that most benefits would emerge within the first 5–10 generations of selection. The lowest selection intensity considered here, corresponding to selection of the 70% genetically most resistant sires, reflects a conservative approach that may be taken by breeders regarding novel traits in the breeding programme (G. Banos, unpublished data available upon request). Our results suggest that with such low selection intensity, genetic selection alone would not eradicate bTB by the time England and Wales are set to achieve OTF status (year 2038, which would correspond to 4–5 generations in conventional breeding programmes or about 2–2.5 generations in genomic breeding programmes). Thus, it would be tempting to consider medium to high selection intensities in the breeding programme. However, care must be taken when higher selection intensities are opted for because of possible antagonistic genetic correlations between bTB and other important dairy traits (56) in the breeding goal. Antagonism would imply that genetically improving one trait compromises the other and may be dealt with using an optimized selection index of multiple traits.

Selection could be applied complementarily to other interventions including existing measures in order to expedite the eradication process. In the context of the genetic-epidemiological model described here, this would include continued efforts to reduce the external source of infection, referring to wildlife-to-cattle, and neighboring and incoming cattle-to-local cattle transmission. Furthermore, improvement of sensitivity of major bTB diagnostic tools such as the skin test and abattoir inspection could translate into an increased removal rate of infected cattle and, hence, reduce the average herd infectivity; further research would be needed to quantify such possible benefits. Other options not included in our model such as selecting for increased resistance in dams in addition to sires, genetic selection to reduce infectivity in addition to susceptibility (57), and genomic selection could also be explored. The latter has a potential to considerably shorten the generation interval and expedite genetic gains (58, 59).

Given the global importance of bTB, a large number of epidemiological models for bTB transmission have been published in the scientific literature (5, 25, 30, 31, 45, 50, 60). The models differ widely in their scope and purpose, although the majority of models focus on estimating transmission parameters and transmission routes from epidemiological data, or explore the impact of different surveillance or control options on bTB prevalence. To the best of our knowledge, this is the first model that incorporates genetic disease control strategies.

To model within-herd transmission dynamics, the epidemiological bTB model in the present study adopted a similar compartmental approach as in recently published stochastic epidemiological bTB models that have been fitted to UK bTB data (5, 30, 31). However, to assess the impact of genetic selection on bTB prevalence and dynamics, we adopted the *SEIT* transmission model, while a more optimistic *SETI* model in terms of transmission has been previously used in the majority of epidemiological studies. Information about the suitability of *SEIT* or *SETI* models for bovine tuberculosis is non-existent. In other diseases, both *SETI* and *SEIT* models have proven to be biologically reasonable. Diseases in humans such as HIV or hepatitis C show epidemiological processes concordant with the *SEIT* model, with window periods between infection and detection when the infected individuals are also infectious (61). Furthermore, in case of human tuberculosis, the window period for the Mantoux test (a skin test based in the presence of immune response against tuberculin) is

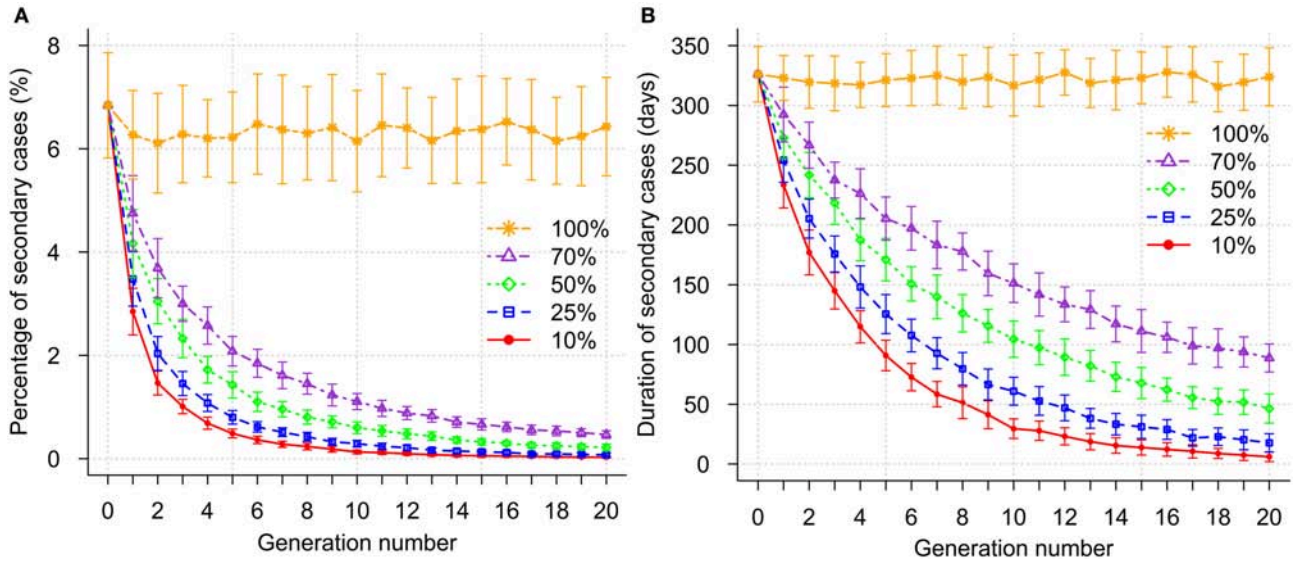

FIGURE 4 | Impact of genetic selection on percentage of secondary cases **(A)** and duration of secondary case occurrence **(B)** within a breakdown. Selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires.

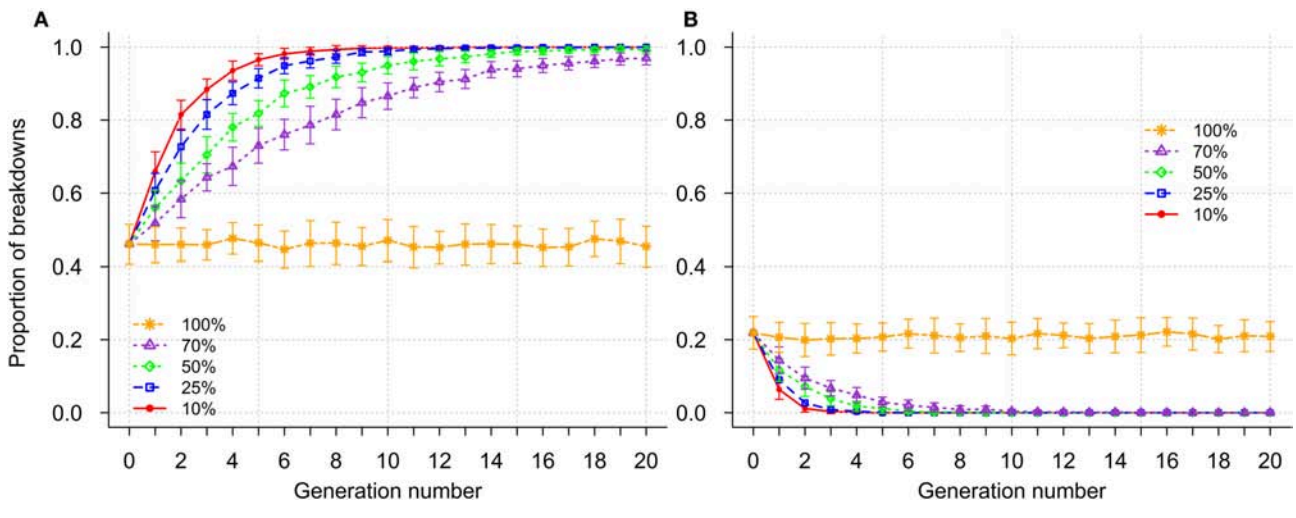

FIGURE 5 | Impact of genetic selection on the percentage of secondary case(s) occurrence within a breakdown; mild (≤3% secondary cases - **A**) and severe (>10% secondary cases - **B**); selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires.

between 2 and 6 weeks (62), with an incubation period for the disease of 2–12 weeks, thus potentially allowing enough time for individuals to become infectious before the window period closes. This is particularly true when the individual has a slow immune response that delays detection. While the onset of infectiousness in relation to reactivity to the skin test is currently not known, inference based approaches have demonstrated an equally good model fit to empirical data if cattle were assumed to become infectious without epidemiological latency, i.e., before entering the detectable state (5). Results from the present study demonstrated that the *SEIT* model indeed represented the "worst" case scenario resulting in more secondary cases per breakdown than the *SETI* model. The number of secondary cases increased in the *SEIT* model because animals became infectious and could infect others before being detected and removed. However, despite the difference between the models in terms of bTB transmission, the present study showed that the impact of genetic selection tended not to differ much between the two models. The similarity between the models may be partly attributed to the relatively short time interval of 2 days estimated between the I and T states. Differences between the model predictions might have been more pronounced if this time interval was longer and the contribution of the external force of infection (α) higher.

Some important assumptions warrant further discussion. In the present study, the external source of infection (α) was kept constant across generations. However, selection is expected to reduce external infection because as animals become more resistant and the number of infectious cows declines, cattle-to-cattle and cattle-to-wildlife-to-cattle transmissions are expected to reduce over time. Therefore, keeping the external source of infection constant in the simulations depicts a somewhat conservative approach regarding the favorable impact of genetic selection. Similarly, the accuracy of selection was kept constant in the simulations, but may also decline as bTB outbreaks decrease across generations and genetically resistant cows become harder to identify. Lower accuracies could slow down response to selection. However, continuous bTB field data collection combined with optimized bTB genetic evaluation methods would counter the effect of reduction in disease prevalence and maintain accuracy of selection over generations. A common concern about genetic control strategies is the impact of selection for host resistance on potential pathogen evolution, which may slow down the predicted genetic gain in host resistance. However, in the case of bTB, the relatively low genetic variability of *M. bovis strains* within cattle populations (63), combined with the evidence from quantitative genetics studies incorporated in the model that host resistance is controlled by many genes, implies that this risk can be considered as negligible (64).

Even though the model aimed to mimic the overall population structure of UK dairy herds, demographic characteristics were not explicitly included in the present study. Not including specific demographic characteristics would particularly affect the estimates of breakdown risk, which are conditional on the introduction of infected cows in each herd. It should be noted that whilst these estimates are useful means to quantify and compare selection response, they differ from the absolute risk of a bTB breakdown, which also depends on the probability of index cases to occur in the first place and on various additional factors not considered in the model, such as cattle movement across herds of different sizes, or different management characteristics and exposure to wildlife (40, 42, 49).

Furthermore, the parameters used in the present study were obtained from literature estimates and statistical comparison of simulated with real disease data. Whilst this approach is very common for epidemiological prediction models (20, 21, 23, 25, 27), it cannot be guaranteed that alternative sets of parameter values would not provide a better model fit to the data. To test this, more sophisticated statistical inference techniques (30, 31, 37) would be required. Thus, future modeling studies may build on our work, including explicit descriptions of additional risk factors associated with bTB prevalence combined with statistical inference techniques for parameter estimation.

Apart from genetic variation in cattle resistance to bTB, no other sources of genetic or individual variation in the model parameters were included in the model. This is in line with standard animal breeding approaches, which focus primarily on selection for disease resistance. Although it is possible that cows may also vary genetically in the duration of the exposed or infectious state, or even in their skin test sensitivity, including genetic variation in the corresponding epidemiological model parameters may affect epidemiological characteristics within each generation (19, 22), but will not affect the predicted responses to selection for disease resistance. Also, within the context of the above assumptions, changing the values of some key epidemiological parameters did not seem to affect the impact of genetic selection on disease transmission dynamics manifested by probability of breakdown occurrence and severity of breakdowns. However, these parameters would largely determine the dynamics of a bTB epidemic, especially when genetic selection is not taken into account. Specifically, our analyses revealed that a decreased sensitivity of the skin test would lead to more severe breakdowns, affecting both the number of secondary cases and the duration of breakdowns. Therefore, the development of diagnostics with high sensitivity that would allow early and accurate detection of infected individual is strongly encouraged.

In the present study, the purpose of some simplifications was to allow a clear demonstration of the predicted effects of genetic selection for enhanced host resistance against the disease on the evolution and dynamics of epidemics. We maintain that the predicted impact of selection is still relevant when such simplifications are lifted. For example, we assumed that all herds in the simulation had the same size, which was similar to the average herd size in the UK dairy cattle population. In reality, herd size varies implying possibly different individual profiles of epidemics in larger vs. smaller herds. However, at population level, the overall epidemic profile will reflect that of the average-sized herd. Furthermore, sire distribution across herds is independent of herd size meaning the overall accuracy of genetic evaluation and selection would not be very close to what was simulated here.

The genetic-epidemiological model developed in the present study provides the first quantitative estimates of the impact of selection for increased resistance on bTB prevalence. In all cases, selection for increased resistance translates into noticeable epidemiological benefits. Strong selection intensities on bTB resistance would particularly benefit high risk geographic areas where the disease is highly prevalent and highly resistant sires are required. The prospects of assimilating bTB resistance into the national selection programme are convincing despite the moderate heritability of the trait. For example, while heritability of clinical mastitis in dairy cattle is low and unfavorably correlated with milk production traits, mastitis is nonetheless included in selective breeding programmes in several countries (65, 66).

CONCLUSIONS

We developed a genetic epidemiological model to investigate the impact of genetic selection for enhanced bTB resistance on disease prevalence and dynamics. Results demonstrated that genetic selection could substantially reduce bTB prevalence and severity of breakdowns over generations of selection. Our study also highlights the importance of considering genetic selection as an additional control tool that can complement existing strategies. Considering genetic selection is pertinent, especially

with the view of accelerating the control and eradication of bTB to achieve the national goal of OTF status by 2038 as planned in England and Wales. Future work could consider additional genetic selection strategies such as selection for resistant dams and selection for reduced individual animal infectivity.

AVAILABILITY OF DATA

Data generated from the present study will be made available on request to qualified researchers.

AUTHOR CONTRIBUTIONS

KR, AD-W, EG, JW, and GB designed the study. KR and ES-M performed the analysis. KR, ES-M, ST, OA, AD-W, and GB interpreted the results. KR prepared the manuscript. KR, ES-M, ST, OA, EG, JW, AD-W, and GB revised the manuscript and improved its content.

FUNDING

The research was funded by Biotechnology and Biological Sciences Research Council (BBSRC Institute Strategic Programme Grants BB/J004235/1, BBS/E/D/20002172 (ISP2.1) and BBS/E/D/30002275 (IPS3.1), the UK Commonwealth Scholarship Commission (KR), and the Rural and Environment Science and Analytical Services Division of the Scottish Government (GB).

ACKNOWLEDGMENTS

Field data used in the present study for comparisons were availed by Edinburgh Genetic Evaluation Services (EGENES) within the Scotland's Rural College.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00237/full#supplementary-material

Supplementary Data Sheet 1 | Simulated scenario with increased rate of external infection ($\alpha = 5 \times 10^{-6}$ instead of 5×10^{-7}), all other parameters remaining constant. Figures shown are: *SEIT* model profiles across 20 generations for five selection intensities defined by the percentage of selected sires: 100% (no selection; **A**), 70% **(B)**, 50% **(C)**, 25% **(D),** and 10% **(E)**; proportion of susceptible (*S*), exposed (*E*), infectious (*I*), and test-sensitive (*T*) individuals during the course of the epidemic. Impact of genetic selection on risk of breakdown (probability of a breakdown to occur). Selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires. Impact of genetic selection on percentage of secondary cases **(A)** and duration of secondary case occurrence **(B)** within a breakdown. Selection intensities correspond to selection of the 10, 25, 50, 70 and 100% (no selection) most resistant sires. Impact of genetic selection on the percentage of secondary case(s) occurrence within a breakdown; mild (≤3% secondary cases—**A**) and severe (>10% secondary cases—**B**); selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires. Impact of genetic selection on the duration of secondary case(s) within a breakdown; short (≤180 days - **A**) and long (>365 days - **B**); selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires.

Supplementary Data Sheet 2 | Simulated scenario with decreased sensitivity of the skin test (0.30 instead of 0.60), all other parameters remaining constant. Figures shown are: *SEIT* model profiles across 20 generations for five selection intensities defined by the percentage of selected sires: 100% (no selection; **A**), 70% **(B)**, 50% **(C)**, 25% **(D)**, and 10% **(E)**; proportion of susceptible (*S*), exposed (*E*), infectious (*I*), and test-sensitive (*T*) individuals during the course of the epidemic. Impact of genetic selection on risk of breakdown (probability of a breakdown to occur). Selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires. Impact of genetic selection on percentage of secondary cases **(A)** and duration of secondary case occurrence **(B)** within a breakdown. Selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires. Impact of genetic selection on the percentage of secondary case(s) occurrence within a breakdown; mild (≤3% secondary cases - **A**) and severe (>10% secondary cases - **B**); selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires. Impact of genetic selection on the duration of secondary case(s) within a breakdown; short (≤180 days - **A**) and long (>365 days - **B**); selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires.

Supplementary Figure 1 | Scheme of the compartmental genetic-epidemiological bTB model. The compartments depict the transition between different animal disease states [Susceptible, Exposed (latent), Infectious and Test-sensitive (detectable)] in the adopted *SEIT* model with assumed heterogeneity in underlying host susceptibility to bTB. Once cows in the Test-sensitive state are diagnosed, they are removed from the herd (Removed compartment). The transition between the compartments depends on the background infection (B), the population average values for the epidemiological parameters: transmission coefficient, β; rate of infection from external sources, α; force of infection from herd-mates, λ; progression rate from Exposed to Infectious state, σ; progression rate from Infectious to Test-sensitive state, γ; skin test sensitivity, Ω; and the distribution of the underlying susceptibility of cattle to bTB (g). Genetic selection affects the g and, thus the individual and average rates of progression from Susceptible to the subsequent states.

Supplementary Figure 2 | Impact of genetic selection on the host underlying susceptibility to bovine tuberculosis. Changes in genetic variation **(A)** and mean susceptibility on the underlying scale **(B)**; selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant of sires.

Supplementary Figure 3 | Impact of genetic selection on the duration of breakdown; short (≤180 days - **A**) and long (>365 days - **B**); selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires.

Supplementary Figure 4 | Impact of genetic selection on average risk of breakdown, and percentage and duration of secondary case(s) occurrence within breakdown in the *SEIT* **(A)** and *SETI* **(B)** models. Selection intensities correspond to selection of the 10, 25, 50, 70, and 100% (no selection) most resistant sires. The dashed horizontal lines represent reduction by 50%.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 30 October 2018
doi: 10.3389/fvets.2018.00253

Whole Genome Sequencing of *Mycobacterium bovis* Isolated From Livestock in the United States, 1989–2018

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 29 June 2018
Accepted: 24 September 2018
Published: 30 October 2018

Citation:
Orloski K, Robbe-Austerman S, Stuber T, Hench B and Schoenbaum M (2018) Whole Genome Sequencing of Mycobacterium bovis Isolated From Livestock in the United States, 1989–2018. Front. Vet. Sci. 5:253. doi: 10.3389/fvets.2018.00253

The United States official bovine tuberculosis (bTB) eradication program has utilized genotyping for *Mycobacterium bovis* isolates since 2000 and whole genome sequencing was implemented in 2013. The program has been highly successful, yet as bTB prevalence has reached historic lows, a small number of new bTB-affected cattle herds occur annually. Therefore, understanding the epidemiology of bTB transmission is critically important, in order to target limited resources for surveillance and achieve eradication. This evaluation described the diversity and epidemiology of *M. bovis* isolates identified in the USA livestock. Isolates from animals within the bTB endemic area of Michigan were excluded. Broad diversity was found among 1,248 isolates, collected from affected cattle and farmed cervids herds and fed cattle during 1989–2018. Nearly 70% of isolates from 109 herds/cases during 1999–2018 were European clonal complex 1 and 30% were European clonal complex 2. The sources of infection based on the herd investigation were known for 41% of herds/cases and 59% were not epidemiologically linked to another USA origin herd. Whole genome sequencing results were consistent with the investigation findings and previously unrecognized links between herds and cases were disclosed. For herds/cases with an unknown source of infection, WGS results suggested several possible sources, including undocumented cattle movement, imported cattle and humans. The use of WGS in new cases has reduced the time and costs associated with epidemiological investigations. Within herd SNP diversity was evaluated by examining 18 herds with 10 or more isolates sequenced. Forty percent of isolates had not diverged or accumulated any SNPs, and 86% of the isolates had accumulated 3 or fewer SNPs. The results of WGS does not support a bTB reservoir in USA cattle. The bTB eradication program appears to be highly effective as the majority of herds/cases in the USA are unique strains with limited herd to herd transmission.

Keywords: *Mycobacterium bovis*, bovine tuberculosis, genotyping, whole genome sequencing, bovine, cervid

INTRODUCTION

Mycobacterium bovis (bTB) has a broad host range, causing economic loss to beef and dairy production and infecting humans and wildlife. Therefore, most developed countries and many developing countries have national bovine tuberculosis eradication programs in livestock. The United States (USA) began a national eradication program for *M. bovis* infection in cattle in 1917. At the program's inception, the apparent prevalence of bTB was 5% of cattle, as estimated by positive responses to the caudal fold tuberculin skin test (CFT) (1). The program's history has been documented elsewhere, including the reduction of prevalence in cattle to <0.005% of cattle herds today (2, 3).

During 2001 to 2011, 92 U.S. cattle herds were infected with *M. bovis*, in an estimated cattle population of 87 million head on 913,000 operations (3). During 1991–2004, there were 41 bTB-affected farmed cervid herds (4, 5). State and Federal veterinarians conduct extensive investigations when bTB is detected; routinely investigating animals that arrived and left the herd within the last 5 years. The program's cornerstone activities are national surveillance for cattle, bison and farmed cervids and quarantine of bTB-affected herds until the infection is no longer detected in individual animals. Despite these efforts, each year there are 2–15 affected cattle herds (3). Affected farmed cervid herds occur sporadically, with the most recent occurrence in 2009. As the USA bTB prevalence has reached historic lows, understanding the epidemiology of bTB transmission is critically important, in order to target limited resources for surveillance and achieve eradication. Challenges to the final eradication of bTB in the USA include a wildlife reservoir in white-tailed deer in northeastern Michigan, sporadic occurrences in dairies and beef herds, bTB in imported feeder cattle, and limitations in the ability to trace animals (3, 6).

Genotyping of *M. bovis* isolates has been in use since 2000 in the official USA bTB eradication program, beginning with IS*6110* based restriction fragment length polymorphism analysis, then adding spoligotyping in 2004 and multiple loci variable number tandem repeat analysis in 2008. These results showed that strains in the USA were highly diverse in both genotypes and geographical locations, with overlap in strains between USA origin and Mexican origin cattle. However, the low resolution of these genotyping methods failed to identify transmission paths (7). Whole genome sequencing is useful at elucidating sources of infection, resolving indistinguishable genotypes identified by other methods and potentially estimating when a new strain was introduced (8, 9). Whole genome sequencing (WGS) was implemented at NVSL on an experimental basis in 2012 and for official program use in January 2013, when WGS replaced traditional spoligotyping and VNTR. The laboratory was able to provide WGS results within the same time frame as traditional genotyping (typically within 4–6 weeks from tissue submission), which was then used to inform the field investigation. Several training programs and webinars were done to prepare the staff for interpreting results (10).

The objectives of this paper are to characterize *M. bovis* isolates identified in the USA from livestock and captive/farmed wildlife, and describe the molecular epidemiology of *M. bovis* in bTB-affected cattle herds in the USA. This information will assist animal health officials and the cattle industry in understanding the transmission of *M. bovis* and use this information for disease prevention.

METHODS

Isolate Selection

Because all official bTB eradication program laboratory diagnostics were performed at the United States Department of Agriculture (USDA), National Veterinary Services Laboratories (NVSL), all isolates archived and maintained at that facility up to May 2018 were sequenced. Prior to 2000, there were no procedures in place to permanently archive isolates, consequently a limited number of isolates prior to 2000 were available. After 2000, nearly all *M. bovis* isolates that were obtained through official bTB program activities from livestock and other animals residing on premises with bTB-infected cattle or farmed cervids were available. In addition, *M. bovis* and *M. caprae* isolates originating from clinical specimens that were submitted to the NVSL were included. Clinical specimens are defined as those originating from diagnostic submissions from animals that are not legally covered by official bTB eradication program regulations. For example, animals in zoological collections or laboratory animals. These isolates originated from other domestic and captive animals residing in the USA that were not under bTB eradication program regulatory authority. Official USDA records detailing the epidemiological investigations that occurred during federal fiscal years 1999 through June 2018 were correlated with the corresponding sequenced isolates. Because a complete list of confirmed bTB-affected herds was not available prior to 1999, results for isolates identified before 1999 were analyzed separately within this paper. Also included in the paper were five reference isolates, AF2122/97 (Biosample: ERS1462286), Ravenel (Biosample: SAMN04448492), BCG (Biosample: SAMN06847294), AN5 (Biosample: SAMN04448491), and 94-1MIDNRdeerAlp (Biosample: SAMN04386752) (the index Michigan deer isolate). See **Supplemental File 1** for isolate metadata. All samples were collected and tested under the authority of the Code of Federal Regulations (CFR) enacted to guide the State-Federal Cooperative bovine tuberculosis eradication program as outlined in 9 CFR part 49, 50.

Official Program Standards

Official program activities include ongoing slaughter surveillance, live animal testing, and investigations of bTB-affected herds. These activities are described in the USDA, Bovine Tuberculosis Eradication, Uniform Methods and Rules (11), and are summarized elsewhere (8, 9, 12, 13). Briefly, antemortem testing is performed on cattle, bison and farmed cervids for a number of reasons, such as entry to a show or sale, state entry requirements, and as part of bTB-affected herd investigations. The CFT is the primary test for cattle and bison, and the single cervical tuberculin skin test (SCT) and the Dual Path Platform (DPP®) are the primary tests in

farmed cervids. Secondary tests are administered to responders. Slaughter surveillance in cattle and bison consists of standardized carcass inspection conducted at federally inspected slaughter establishments (14).

When bTB is suspected, an official investigation is conducted by state and federal animal health regulatory officials (11). This investigation collects information about the premises where the animal resided (city, county, state) and its movements prior to confirming infection. The investigation includes adjacent, contact and possible source herds for the affected herd. Bovine tuberculosis affected herds are classified as epidemiologically linked based on investigative evidence. Investigative evidence includes but is not limited to slaughter establishment records, records of animal movement, such as a bill of sale or certificate of veterinary inspection or other official documents. These records are used to determine where an infected animal resided over time, identify potentially exposed animals and herds and look for the source of infection. Herds exposed through animal movements from a bTB-affected herd are tested, and exposed animals are removed, necropsied and sampled.

Case Definitions

Adult cattle were defined as sexually intact animals >2 years of age, whereas fed cattle are defined as castrated or spayed animals without regard to age that are raised for the purpose of beef production. Another type of cattle are those animals used for roping and other performance events. Castration status has precedence over age, for example, a 4-year-old castrated steer is classified as a fed animal. Slaughter surveillance targets culled adult cattle because these are more likely to exhibit lesions suspicious of bTB (15). bTB-affected herds were classified by production type, including beef, dairy, mixed (beef and dairy), event cattle (roping, rodeo animals), farmed cervids, and unknown.

The case definition used for classifying an animal as confirmed infected with *M. bovis* was either a histologic diagnosis of compatible for mycobacteriosis with a positive polymerase chain reaction (PCR) test performed on formalin fixed tissue using primers for IS*6110* to identify *M. tuberculosis* complex, or bacteriological isolation of *M. bovis*. Affected herds are confirmed when an animal from within the herd is confirmed with bTB. When this criterion cannot be met, the singleton animal (generally found as a result of slaughter surveillance) was defined as a case. The source of infection for affected herds was based on the results of epidemiological investigations as being either unknown, or another USA herd. The latter classification was applied when there was documented animal movement or the potential for fence line or other direct contact. Outbreaks were defined as two or more bTB-affected herds or cases with a documented exposure, such as animal movement between premises.

To identify the likely source, (likely external to the USA or internal transmission within USA) we conservatively estimated that a USA origin strain would not be tlikely to be exported and established in another country after the USA's national bTB prevalence was below 0.5%, which occurred around 1960. Consequently herds that could have shared a common ancestor within the last 60 years would more likely be internal transmission rather than importation. Using the average reported SNP mutation rate of 0.3 SNP/year (16), suggests a reasonable cutoff point of 20 SNPs. Consequently, we considered isolates that were within 20 SNPs of sharing a common ancestor with USA origin cattle to originate from USA and isolates that were within 20 SNPs of sharing a common ancestor with Mexican origin cattle isolates to have originated from Mexico. If more than 20 SNPs had accumulated since sharing a common ancestor with an isolate in the database, we considered the source unknown. Isolates from Michigan cattle within the known endemic area and all Michigan wildlife were excluded.

Laboratory Methods

During slaughter inspection or when bTB test positive animals are euthanized and necropsied, granulomatous-appearing lesions are collected and submitted to the laboratory for histologic examination, PCR testing and mycobacterial culture (17). For some bTB test positive animals, if no visible lesions are observed during necropsy, representative head, abdominal, and thoracic lymph nodes are collected and tested (9, 11, 13). For herds with many bTB infected animals, generally at least 10 isolates were collected and sequenced and in some herds many more were sequenced when sufficient resources existed.

To obtain the WGS, isolates were sequenced on a MiSeq instrument (Illumina, San Diego, CA, United States) using 2 × 250 paired-end chemistry and the Nextera XT library preparation kit (Illumina, San Diego, CA, United States). FASTQ files were put through the NVSL in-house pipeline (https://github.com/USDA-VS). Reads were aligned to the reference genome AF2122/97, NCBI accession number NC_002945 (18), using BWA (19) and post-processing of the alignment was done using Samtools (20). BAM files were processed based on Genome Analysis Toolkit (GATK)'s "best practice" workflow (21, 22). SNPs were called using GATK's HaplotypeCaller with ploidy set to 2, outputting SNPs to variant call format (VCF) files. PPE-PGRS and repeat regions were filtered as well as SNPs that uniformly had QUAL scores < 150 across isolates. To identify SNP calls that were heterozygous, a SNP with an allele call of AC = 1 was relabeled using the International Union of Pure and Applied Chemistry guidelines for ambiguous calls. In order to manage and more accurately analyze this large and diverse dataset, a small number of isolates representing the diversity of the entire dataset were ran through the pipeline. High quality SNPs were identified that clustered the isolates into smaller more manageable groups. Groups were created based on the number of isolates as well as the evolutionary distance. Because these groupings were based on convenience for analysis purposes, they were not necessarily similar in evolutionary distance. For example, group 23 and 24 are very closely related, but because there were so many isolates in that clustered closely together, 2 groups were made. Individual SNPs tables and phylogenetic trees were then created for each group after removing all uninformative SNPs that were homogeneous between the grouped isolates. Because of this process, the reference AF2122, worked as the outgroup isolate for all individual groups. SNPs were then further verified manually

using Integrative Genomics Viewer (23) and additional filtering of problematic SNPs was performed on a group by group basis using the SNP tables. Phylogenetic trees were created using RAxML (24) and the GTRCATI model with default settings and accuracy of the phylogenetic tree was confirmed using the manually validated SNP table.

Data Analysis

We examined WGS results for *M. bovis* isolates from cattle and farmed cervid breeding herds and individual animal cases detected through surveillance in the USA, and described the diversity of the isolates within herds and between herds. We identified the most recent common ancestor (MRCA) in the herd. Then, the most closely related isolate was selected from the NVSL WGS database, based on having the fewest number of single nucleotide polymorphisms (SNP) differences, when compared to the herd or case isolate. A pairwise comparison of SNPs to the MRCA was recorded. For herds with *M. bovis* isolates from multiple bTB confirmed infected animals, the tip with the shortest branch length to the tree root was used for the comparison. The most closely related isolates were grouped based on information about the animal or human from which the isolate was obtained. For example, whether the isolate was from imported cattle or a confirmed bTB-affected herd in the USA.

RESULTS

Sequencing was performed on 1,248 isolates, this included 154 that were published previously (8, 9). Of these, 185 isolates were collected during 1989–1998, and 1,063 were collected during 1999–2018. The *M. bovis* isolates separated into 24 major phylogenetic groups (**Figure 1**). Twelve *M. bovis* isolates were obtained from clinical specimens submitted to NVSL (four non-human primates, one jaguar, one elephant, one domestic cat, one brocket deer, 4 unknown species from zoological collections). There were four *M. caprae* isolates from three non-human primates and one rhinoceros residing in zoological collections.

bTB-Affected Herds and Slaughter Cases in Culled Adult Cattle

There were 83 bTB-affected herds, and 26 cases in infected adult domestic cattle during 1999–2018 (**Supplemental Table 1**). An isolate was not available for two additional cattle herds that are not included in this analysis. The production types included 54 beef herds/cases (49.5%), 37 dairies (33.9%), 3 event/rodeo (2.8%), 3 mixed purpose (2.8%), 11 farmed cervids (10.1%), and one herd of an unknown production type. The herds/cases were located in 21 States (Arizona, California, Colorado, Indiana, Iowa, Kansas, Kentucky, Michigan, Minnesota, Mississippi, North Dakota, Nebraska, New Mexico, New York, Ohio, Oklahoma, Oregon, South Dakota, Texas, Washington, Wisconsin).

There were 563 bTB isolates from these 109 herds/cases (**Supplemental Table 1**). No herds were identified with more than one strain. Seventy-six (69.7%) of the herds/cases were European clonal complex 1 (EU1) strains, 32 (29.4%) were European clonal complex 2 (EU2) strains, and one herd was infected with a strain not identified within a clonal complex (group 8) (**Supplemental Table 1**, **Table 1**). Groups 8 and 14 contain isolates associated with outbreaks in USA and Canada farmed cervid herds (4, 5, 25, 26). The number of isolates per herd ranged from one to 48, (median 2, mean 5), and 52/83 herds (62.7%) had more than one isolate (**Supplemental Table 1**).

Within herd SNP diversity was evaluated by examining the 18 herds with 10 or more isolates sequenced, identifying the common ancestor, or index sequence, and then counting the number of SNPs accumulated from the index sequence (**Table 2**). Forty percent of isolates had not diverged or accumulated any SNPs, 86% of the isolates had accumulated 3 or fewer SNPs and no cattle herds contained isolates that had accumulated more than 6 SNPs (**Table 2**). These accumulated SNPs may be unique to a single isolate or found in a cluster of isolates within a herd. The highest diversity occurred in two herds, a 2009 farmed cervid herd with 28 isolates and 35 SNPs, and a 2017 cattle herd with 25 SNPs among 13 isolates. Based on the epidemiologic investigation, both herds were likely infected for several years.

Despite all of the retrospective data and epidemiological investigations that have been conducted in the USA, we were able to attribute multiple transmission events to a single cow only one time. In this instance, a cow with disseminated TB lesions was sold into a feedlot and exposed a group of cows for <30 days. Within 90 days of exposure, all exposed cows were slaughtered and 6 were identified with lesions. The isolate sequence recovered from a pooled sample of the lesions from the index cow along with the 6 isolate sequences recovered from exposed cows are shown in **Table 3**. In this event, four different SNP profiles were transmitted to these 6 cows.

The sources of infection based on the investigation results were known for 45/109 (41.3%) of herds/cases (epidemiologically linked via animal movement or adjacent premises contact) and 64 were not epidemiologically linked to another USA origin herd. There was only one documented case of transmission from Mexico, in a roping steer newly imported from Mexico that was found to be infected during a herd test for interstate movement. These 45 herds with a known infection source were associated with 14 outbreaks. Eight outbreaks involved two herds each, two outbreaks involved three herds, one outbreak involved five herds (13), one involved six herds (five herds occurred before 1999) and one outbreak in Minnesota involved 12 herds (8).

The source of infection could not be determined epidemiologically for 64 herds/cases.The most recent common ancestor was within 20 SNPs of a Mexican origin animal for 22 (34.9%) of USA herds/cases suggesting Mexico may be the source (**Supplemental Table 1**). Twelve cases/herds were within 20 SNPs of unknown origin fed cattle and 20 were within 20 SNPs other USA herds/cases. Isolates from the remaining nine herds/cases, were >20 SNPs from the most recent common ancestor, therefore, no conclusive linkage was found by genetic sequencing or epidemiology. Interestingly, only one of these 64 herds/cases were within 5 SNPs of Mexican origin fed cattle slaughtered in the USA. One additional isolate from a dairy cow was indistinguishable from a worker in the same dairy that was initially diagnosed with bTB, and the dairy was subsequently tested (27).

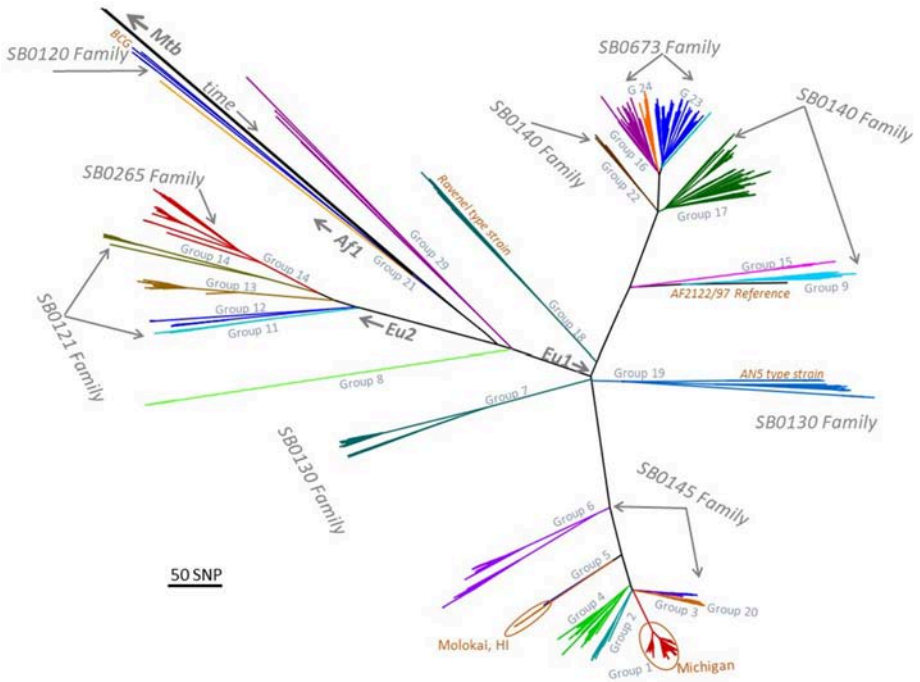

FIGURE 1 | Low resolution phylogenetic tree representing 1,248 *Mycobacterium bovis* isolates and major spoligotyping families. MTBC, M. tuberculosis complex; EU1, European clonal complex 1; EU2, European clonal complex 2; Af1, African complex 1; SNP, single nucleotide polymorphism; BCG, bacille Calmette-Guerin; HI, Hawaii; AN5, *M. bovis* AN5 strain.

There was one extensive outbreak involving two closely related clusters within phylogenetic group 14. Group 14 consists of USA farmed cervids and cattle and Canada farmed cervids (4, 5, 25, 26). The first cluster involved 1 farmed cervid herd (Nebraska), three cattle herds [Nebraska, South Dakota (2)], and 1 cattle case in Nebraska during 2009–2013. The cattle herds and case were epidemiologically linked by animal movement or fence line contact and the source herd was a bTB-affected cervid herd [(26), USDA Veterinary Services, unpublished information]. The second cluster, when limited to known animal movement or fence line contact consisted of three cervid (Indiana) and two cattle herds during 2009-2017 (Indiana, Michigan). One of the three Indiana cervid herds (2009) was the source herd. However, three additional cattle herds (Indiana (2), Kentucky) and 3 cases (Indiana (2), Arizona) occurred during 2009-2017, but did not have documented links to the second cluster. Isolates from these six herds/cases are either indistinguishable or have 1-2 SNPs from the 2009 index farmed cervid herd and other group 14 isolates, indicating undocumented animal movements or contacts occurred.

During 1989–1998, there were 50 bTB isolates obtained from 24 affected herds, representing 12 cattle and 12 farmed cervid herds (**Supplemental Table 1**). These isolates were from 11 States, including Hawaii, Missouri, Montana, New Mexico, New York, Oklahoma, Puerto Rico, Texas, Virginia, Vermont, and Wisconsin. One hundred bTB-affected herds occurred during this time; therefore, isolates were not available for 76 herds. Existing isolates from 1989–1998 separated into 10 major phylogenetic groups (groups 5, 6, 8, 9, 13, 14, 16, 19, 21, 24). Seven of these groups contained isolates recovered from Mexico origin cattle (groups 6, 9, 13, 14, 16, 19, 24), while three did not (groups 5, 8, 21). Four groups were not represented after 1998 (groups 5, 9, 21, 24). The Hawaii isolate was obtained from a beef herd in 1997. The most closely related isolate to the beef herd were from feral swine, obtained during wildlife surveillance efforts there during 2007–2009.

Group 7, subgroup B, provides examples of WGS results (**Supplemental File 2**). Substantial diversity exists among the 108 isolates, which is typical of the phylogenetic groups. There are two outbreaks, one involving three South Dakota beef herds, and the second involving five Colorado beef and dairy herds [an isolate was not available for one herd, (13)]. For the Colorado outbreak, 12 isolates were sequenced with two unique SNPs; however, the majority of nearly 90 isolates from the index dairy herd were not sequenced due to resource limitations. For the South Dakota outbreak, 30 isolates from the index herd (designated 17-A in the isolate name) were sequenced, with 20 unique SNPs. Isolates from three additional animals sold to other herds (herds 17-B and 17-C) from the index herd had the same SNP pattern as isolates from animals in the index herd. In addition to the two outbreaks, Group 7 includes a single beef herd infection and several single animal cases. The single beef herd occurred in Oklahoma in 2007, for which one fed steer from this herd was found in a Kansas feedlot. There is one case in an adult beef cow found through slaughter surveillance that traced to New Mexico in 2004. This case could not be linked to a bTB-affected herd. The isolate from this animal is only one SNP different

TABLE 1 | Whole genome sequencing group for 83 bTB-affected herds and 26 cases during 1999-2018.

Whole genome sequencing group	Number of herds and cases	Percent of total
EUROPEAN CLONAL COMPLEX 1		
2	2	1.8
3	1	0.9
4	1	0.9
6	12	11.0
7	11	10.1
16	8	7.3
17	21	19.3
19	1	0.9
20	3	2.8
22	2	1.8
23	13	11.9
No associated clonal complex		
8	1	0.9
EUROPEAN CLONAL COMPLEX 2		
11	1	0.9
12	2	1.8
13	6	5.5
14	24	22.0
Total	109	100.0

from the 2007 Oklahoma herd. It is hypothesized the 2004 case originated from the Oklahoma herd; however, investigative information is no longer available. A second, unrelated case found through slaughter surveillance occurred during 2005 in a Nebraska beef cow, in which bTB could not be confirmed in the herd of origin. The most closely related isolates to this beef cow are from unknown (1992) and Mexican (2012) origin fed cattle slaughtered in the US (10 and 12 SNPs, respectively). Other group 7 isolates include 36 cases in fed cattle from 1991–2012; many of these were imported from Mexico and slaughtered in Texas. Finally, there are 18 results for isolates from cattle in Mexico.

Slaughter Cases in fed Cattle

There were a total of 521 confirmed bTB cases in fed cattle found through slaughter inspection during 1990–2018 (**Figure 2**). These isolates separated into 19 phylogenetic groups (**Table 4**). One isolate had a mixed infection. When considered by country of origin, 276 (53.0%) were from Mexico, the country of origin could not be determined for 223 (42.8%) and 22 (4.2%) occurred in USA origin cattle. An additional two cases in fed cattle from Canada slaughtered in Washington State were classified in group 23 (data not shown). Twenty of the USA origin cases separated into six phylogenetic groups and originated from six known bTB-affected herds. One case was untraceable and one case was under investigation at the time of this report. The country of origin could not be determined for 223 isolates from fed cattle. The most common reason that country of origin cannot be determined is because official animal identification was not available and the infected animal had been comingled with both USA and Mexican origin cattle in pastures or feedlots prior to slaughter (VS unpublished). Twenty-four isolates (4.6%) were from cases that occurred during 1989–1993, representing a small fraction of 1,504 bTB feedlot investigations that were reported during 1989–1993 (28).

Four groups (9, 15, 18, 24) were represented in fed cattle cases but not bTB-affected herds and cases, and one group was found in a farmed cervid herd but not in fed cattle (group 8). The five largest groups are 7, 13, 16, 17, and 23, and contain 72.3% of isolates from fed cattle. In comparison, the five largest groups are 6, 7, 14, 17, and 23, containing 65.4% of affected herds and cases during 1989–2018.

Spoligotyping

A comparison of spoligotyping and whole genome sequencing results are shown in **Figure 1**. There are six spoligotyping families that occur in the USA and each WGS group falls within one spoligotype family. Spoligotyping family SB0673 contains WGS Groups 16, 23, and 24; SB0120 contains Group 21; SB0121 contains Groups 11-14; SB0140 contains Groups 9, 17, 17 and 22; SB0130 contains Groups 7 and 19; and, SB0145 contains Groups 1-6 (**Figure 1**).

DISCUSSION

Consistent with previous reports, broad diversity exists among USA bTB isolates detected in cattle and farmed cervids (7). Not unexpectedly, many of the fed cattle isolates were from imported cattle, and contain even more diversity. WGS results do not support a bTB reservoir in USA cattle. The bTB eradication program appears to be highly effective as the majority of herds/cases in the USA are unique strains with limited herd to herd transmission. Two major exceptions occurred outside the endemic area of Michigan: the first in farmed cervid herds that subsequently spilled into cattle herds (15 herds/cases from 2009 to 2018) and the second in Minnesota where bTB spilled over into the local white-tailed deer and 12 cattle herds were affected (8). Farmed cervids are subject to official bTB program requirements including surveillance, and no bTB-affected cervid herds have occurred since 2009.

In all cases, WGS results corroborated investigative evidence of herd-to-herd transmission events. Not unexpectedly, there was less SNP diversity between epidemiologically linked herds, almost half these herds had no unique SNPs and the maximum number of unique SNPs was four (**Supplemental Table 1**). Nearly half of the herd to herd transmission events had a SNP genotype that had been found in the source herd.

WGS results also discovered previously unrecognized links between herds and cases. Isolates from 12 herds/cases with an unknown source of infection were within three SNPs of other USA herds/cases during 2009–2018, including six herds/cases clustering within the group 14 outbreak. In another example, the isolate from a 2010 bTB-infected Holstein cow from an Ohio

TABLE 2 | The number of single nucleotide polymorphisms (SNPs) from the common ancestor genotypes among bTB-affected herds with >10 isolates, United States, 1999–2018.

	Number of SNPs								
Herd name	0	1	2	3	4	5	6	7	8
2001 TX beef (%)	100	0	0	0	0	0	0	0	0
2002 TX dairy (%)	45	27	0	27	0	0	0	0	0
2003 CA dairy (%)	54	23	8	15	0	0	0	0	0
2007 NM dairy (%)	64	29	7	0	0	0	0	0	0
2009 IN cervid A (%)	0	17	0	0	33	33	17	0	0
2009 NE cervid (%)	0	37	11	4	11	15	19	0	4
2010 CO dairy A (%)	83	17	0	0	0	0	0	0	0
2012 CA dairy B (%)	75	25	0	0	0	0	0	0	0
2013 CA dairy (%)	47	27	20	7	0	0	0	0	0
2013 MI Dairy (%)	23	6	64	6	0	0	0	0	0
2014 TX dairy (%)	4	78	6	10	2	0	0	0	0
2015 TX organic dairy (%)	36	28	23	4	6	2	0	0	0
2016 IN beef (%)	13	0	4	29	38	13	4	0	0
2016 IN Longhorn (%)	23	8	31	8	23	0	8	0	0
2016 NM dairy A (%)	19	29	38	10	5	0	0	0	0
2017 NM Dairy A (%)	82	18	0	0	0	0	0	0	0
2017 SD beef A (%)	39	21	6	21	9	3	0	0	0
2017 SD beef D (%)	14	52	29	5	0	0	0	0	0
(%)	**40**	**24**	**14**	**8**	**7**	**4**	**3**	**0**	**0**

Green, the number of unique SNPs did not occur; Light to dark red, the proportion of isolates that had 0–8 unique SNPs (lower to higher proportion).

TABLE 3 | Example of accumulated single nucleotide polymorphisms (SNPs) resulting from multiple transmission events from one animal, 2018.

Genome position based on the reference NC_002945.4	**1999500**	**1021045**	**241655**	**1147922**
Reference call	**C**	**C**	**C**	**C**
18-0522_SD_IA_Fed-Cow-Index	S*	C	C	C
18-1919_SD_IA_Fed-Cow	**G**	C	C	C
18-1930_SD_IA_Fed-Cow	**G**	C	C	C
18-1932_SD_IA_Fed-Cow	**G**	**T**	C	C
18-1927_SD_IA_Fed-Cow	**G**	**T**	C	C
18-1922_SD_IA_Fed-Cow	C	C	**T**	C
18-1904_SD_IA_Fed-Cow	C	C	C	**T**

**S Designates a heterogenous SNP call containing both cytosine and guanine. The colors indicate a unique SNP from the reference call.*

dairy had one additional SNP from a single isolate obtained from a 2008 New Mexico dairy cow (**Supplemental Table 1**). Investigative evidence indicated the 2010 Ohio dairy cow originated from New Mexico though no direct links could be found to the 2008 herd. These findings raise the possibility of undocumented animal movements or exposure. A pathways analysis of 12 bTB-affected California dairy herds based predominantly on herd investigations, concluded that with one exception, *M. bovis* occurred because of independent introductions from sources outside the system (12).

Most of the isolates recovered from imported fed cattle are not closely related to isolates from USA herds, with only one of the 109 herds/cases within 5 SNPs of an imported fed steer, despite having nearly complete representation of fed cattle and affected herds during 1999–2018. This suggests there may be other vectors transmitting bTB to the USA national herd such as humans or even imported dairy products (29, 30). It may also be possible to have undetected residual strains from historical cases. In one example, 11 years elapsed between bTB detection in epidemiologically linked California dairy herds (12). Alternatively, a limitation of this analysis are missing isolates from bTB-infected animals not detected through surveillance activities. Slaughter surveillance in the USA has an estimated sensitivity of detecting a bTB affected herd in 1 year of 3.2% in small beef herds (1-49 head), to 50.6% in large dairies (>500 head) (31). A Bayesian molecular clock phylogenetic analysis of the Minnesota outbreak reported the median time to the most recent common ancestor was 1999 (range 1991, 2005) for isolates from the Minnesota outbreak (3 SNPs) and its mostly closely related isolate, a 2012 Texas beef herd (8 SNPs) (8). We were unable to determine if there were two introductions into the USA of a closely related genotype from Mexico, or if common ancestors were present in the USA, possibly as early as 1991, but were not present in the NVSL database.

The most closely related isolates to some affected herds are from fed cattle that occurred earlier in time than the affected herds, many prior to 1999. We hypothesized that if imported cattle were the source of infection for affected herds, we would find closely related isolates among imported fed cattle and

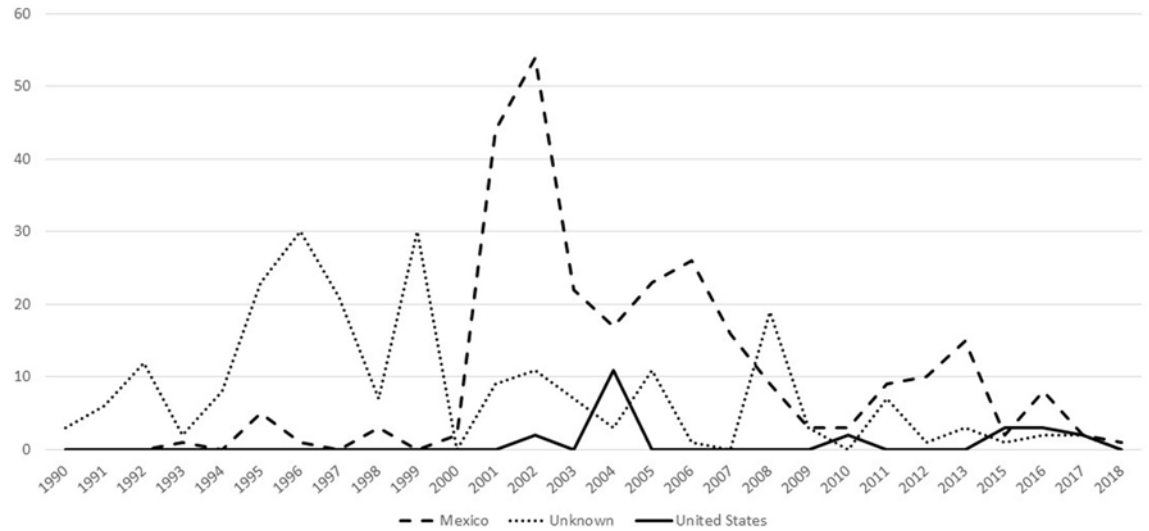

FIGURE 2 | Number of bTB cases that have whole genome sequencing results, by year and country of animal's origin for fed cattle slaughtered in the U.S, federal fiscal years 1990-2018 ($n = 521$).

TABLE 4 | Number of isolates by whole genome sequencing group and country of animal's origin for fed cattle slaughtered in the U.S, 1990–2018.

Group	Mexico	Unknown	USA	Total
Mixed	0	1	0	1
2	6	3	0	9
3	1	0	0	1
4	7	7	0	14
6	15	10	1	26
7	24	21	0	45
9	7	1	0	8
11	1	0	0	1
12	3	2	0	5
13	17	23	1	41
14	8	3	6	17
15	5	9	0	14
16	17	12	4	33
17	49	69	0	118
18	2	0	0	2
19	4	1	0	5
20	1	2	0	3
22	9	7	0	16
23	83	46	10	139
24	17	6	0	23
Total	276	223	22	521

affected herds/cases during 1999–2018. That pre-1999 fed cattle cases are the closest match to some post-1999 affected herds/cases is noteworthy, because the pre-1999 isolates from fed cattle represent only a small fraction of the cases that occurred (28). There was a substantially higher risk of bTB introduction from Mexican origin cattle during 1983–1993, compared to today (28).

Limited diversity occurred within most herds, with the majority of isolates having three or fewer SNPs. This may be useful in guiding epidemiologic investigations. For example, additional unique SNPs may indicate that an intermediary herd exists. The estimated SNP occurrence per genome per year ranges from 0.147 (32) to 0.53 (2.5% lower 0.22, 97.5% upper 0.94, (16). Applying these to the time frames and SNPs reported here may indicate a common exposure for the isolates with five or fewer SNPs, while separate introduction events may be more likely for isolates with >5 SNPs. The unique SNPs observed among animals within a herd may represent strain variation caused by animal to animal transmission, or an actual mutation. In the one example we observed, four different SNP profiles were transmitted to six cows exposed for 30 days to a single bTB-infected beef cow. Thacker et al. (33) reported that a single SNP genotype was recovered in 80% of affected tissues from experimentally infected white tailed deer. The inoculum and isolates from the remaining animals contained different WGS genotypes, some with the same SNP, and it was hypothesized that the SNP was not a mutation but was present though undetected in the original inoculation.

The use of WGS in new cases has focused epidemiological investigations and significantly reduced time and costs associated with these investigations and reduced the burden on livestock producers. Whole genome sequencing results for a 2013 case in a California Holstein heifer without animal identification enabled resources to focus on testing an epidemiologically linked herd (**Supplemental Table 1**). If these results had not been available, as many as 60 herds that had contributed cattle to the slaughter lot of the bTB infected case would have been administered a whole herd test. However, care must also be taken that the scope of the investigation not be prematurely limited by WGS results, at the risk of missing cases. WGS results provided critical information that enabled the successful detection of bTB spread

to cattle from the 2009 NE infected farmed cervid herd (26). In another case, whole genome sequencing evidence linked three bTB-affected cattle operations to the index herd in the absence of cattle movements between the premises (9).

bTB prevalence in dairies is approximately twice that of beef herds (3). The risk factors for disease transmission among beef and dairy herds in the USA are unknown. The WGS results reported here may be useful in guiding future work to identify risk factors for bTB transmission. General trends in the beef and dairy industries during the study period include a decrease in the number of beef and dairy cattle and cattle operations during 1993–2008, while the average herd size has increased (34–36). The magnitude of this change was greater in dairy operations, where the number of operations declined 58.4% during 1991–2006, while the average dairy herd size doubled from 54 to 122 cows (36). Larger dairies continue to increase in herd size, and almost 30% of dairy operations introduced new cattle. Similarly, almost 35% of cow-calf beef operations introduced new cattle, most commonly weaned beef bulls and weaned steers (34).

Transmission from human workers to cattle has been hypothesized, especially for dairy cattle operations because of intensive management practices (12). In 2013, a USA dairy worker was diagnosed with bTB. The dairy herd was tested and bTB was confirmed in three animals (27). One animal was infected with the identical strain as the worker. While the direction of transmission could not be determined, this case suggests the possibility of human to animal transmission, as no other sources of exposure for the cattle could be identified. An evaluation of human and cattle *M. bovis* isolates from Baja California, Mexico found that 155 isolates from cattle and 17 from humans clustered into seven major groups (37). The human isolates were interspersed among the cattle isolates, and cheese is the suspected source of exposure of *M. bovis* for humans. In these examples from the USA and Mexico, direct or indirect contact between the human and animal subjects occurred; however, as noted previously, closely related isolates should not be used to imply transmission events, in the absence of epidemiologic linkages.

All isolates were sequenced for some herds, particularly those with a small number of bTB infected animals, but because of resource limitations, a smaller proportion of available isolates were sequenced for larger herds with dozens or hundreds of bTB infected animals. This limitation may bias these results by potentially under reporting the number of SNPs in herds with large numbers of infected animals, or conversely, fail to document if limited SNPs are present despite extensive within herd transmission.

CONCLUSION

Whole genome sequencing has become a cost effective, essential tool for the USA official bTB eradication program, providing information that increases the success and efficiency of the extensive investigations that occur when bTB is confirmed in cattle and farmed cervids. The isolates that occur among USA livestock are diverse, and the lack of diversity within herds support that a reservoir does not exist in the USA cattle population, although transmission of one strain has continued to occur from farmed cervids to cattle herds. The source of infection is unknown for approximately half of bTB-affected herds, and WGS results suggest several possible sources, including undocumented cattle movement, imported cattle, as well as humans or unpasteurized dairy products.

AUTHOR CONTRIBUTIONS

SR-A and TS performed WGS and provided summarized results. KO and SR-A compiled and analyzed the summarized WGS results. KO and SR-A wrote the paper. BH and MS provided epidemiological information. BH and MS reviewed/edited the paper.

ACKNOWLEDGMENTS

We thank Drs. Pauline Nol and Michael Carter for reviewing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00253/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AD and the handling Editor declared their shared affiliation.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 30 October 2018
doi: 10.3389/fvets.2018.00272

Whole Genome Sequencing for Determining the Source of *Mycobacterium bovis* Infections in Livestock Herds and Wildlife in New Zealand

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OPEN ACCESS

Edited by:
Andrew William Byrne, Agri-Food and Biosciences Institute (AFBI), United Kingdom

Reviewed by:
Pauline Kamath, University of Maine, United States
José Patané, Universidade de São Paulo, Brazil

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *04 July 2018*
Accepted: *11 October 2018*
Published: *30 October 2018*

Citation:
Price-Carter M, Brauning R, de Lisle GW, Livingstone P, Neill M, Sinclair J, Paterson B, Atkinson G, Knowles G, Crews K, Crispell J, Kao R, Robbe-Austerman S, Stuber T, Parkhill J, Wood J, Harris S and Collins DM (2018) Whole Genome Sequencing for Determining the Source of Mycobacterium bovis Infections in Livestock Herds and Wildlife in New Zealand. Front. Vet. Sci. 5:272. doi: 10.3389/fvets.2018.00272

The ability to DNA fingerprint *Mycobacterium bovis* isolates helped to define the role of wildlife in the persistence of bovine tuberculosis in New Zealand. DNA fingerprinting results currently help to guide wildlife control measures and also aid in tracing the source of infections that result from movement of livestock. During the last 5 years we have developed the ability to distinguish New Zealand (NZ) *M. bovis* isolates by comparing the sequences of whole genome sequenced (WGS) *M. bovis* samples. WGS provides much higher resolution than our other established typing methods and greatly improves the definition of the regional localization of NZ *M. bovis* types. Three outbreak investigations are described and results demonstrate how WGS analysis has led to the confirmation of epidemiological sourcing of infection, to better definition of new sources of infection by ruling out other possible sources, and has revealed probable wildlife infection in an area considered to be free of infected wildlife. The routine use of WGS analyses for sourcing new *M. bovis* infections will be an important component of the strategy employed to eradicate bovine TB from NZ livestock and wildlife.

Keywords: *Mycobacterium bovis*, molecular fingerprint, whole genome sequencing, New Zealand, bovine tuberculosis control, epidemiology

INTRODUCTION

Efforts to control bovine tuberculosis (TB) in domestic livestock in New Zealand (NZ) are driven by the zoonotic risk of the causative agent *Mycobacterium bovis* and its possible impacts on international trade (1, 2). Although in many countries bovine TB has been controlled successfully with test and slaughter strategies and movement restriction, control

is particularly challenging in countries like NZ in which there is a wildlife reservoir of infection (1, 3). In Britain and Ireland the Eurasian badger harbors and spreads *M. bovis*, in France the wild boar, in Michigan and Minnesota in the USA, deer, and in NZ the brush-tail possum, [reviewed in (4)]. Effective control under these circumstances involves not only test and slaughter and movement control but also knowledge and control of the infection status in wildlife (3, 5). The challenges imposed in different parts of the world by these varied sources have been reviewed (6). Despite the challenging circumstances imposed by its wildlife reservoir, the control of bovine TB in NZ has recently been re-evaluated and there are now ambitious goals of achieving TB free livestock and wildlife by 2026 and 2040, respectively (2, 7).

Molecular methods provide a means to detect and characterize the spread of pathogens in both domestic livestock and in wildlife populations (3–5, 8). Studies in NZ that employed an early DNA fingerprinting assay that compared the restriction pattern of DNA digests (Restriction Endonuclease Analysis REA typing) of *M. bovis* isolates, demonstrated that livestock and wildlife in the same regions tended to share the same types and thus helped to define the role of wildlife in the spread of bovine TB in New Zealand (1, 9, 10). REA typing was used routinely for over 20 years to efficiently guide wildlife control measures and to aid in tracing the sources of infections that resulted from movement of livestock (10). In other parts of the world, comparison of the direct repeat region of the *M. bovis* chromosome by a process called spoligotyping, and a more sensitive PCR based method that compares repeated sequences at different sites in *M. bovis* genomes, [Variable Number Tandem Repeat (VNTR)] have been used for monitoring the genotypes of isolates from wildlife and livestock, providing insight into the types and spread of *M. bovis* (10–14). Because VNTR was simpler to perform and interpret than REA and was almost as discriminating, the REA method was replaced in NZ by VNTR in 2012 (15). VNTR fingerprint typing is routinely employed in NZ to determine the source of new livestock infections and the types carried by wildlife. In many cases VNTR clearly identifies the regional source of new infections, but it is of less use in cases where the same type is widespread in one or more regions of the country.

Recent advances in DNA sequencing have facilitated the routine comparisons of entire bacterial genomes [whole genome sequencing (WGS)] for determining the source of bacterial infections and this technology shows promise in aiding bovine TB control including situations that are complicated by wildlife reservoirs (4, 16–22). The single nucleotide polymorphism (SNP) lineages that result from WGS are far superior to the "types" that come from comparing a small number of sites in Spoligo or VNTR typing analyses. There are typically tens to hundreds of SNPs common to a major branch, and 10 s of SNPs common to sub-clusters in each branch. Because so many more similarities and differences are considered in comparisons of lineages, there is less chance for misinterpretation of the relationship between isolates than when typing by VNTR (18, 23). In addition, these lineages provide information about shared common ancestors that is not always obvious by VNTR and spoligotyping. When coupled with knowledge of how quickly these bacteria accumulate new SNPs, this information can provide temporal clues about the arrival and divergence of types, which can greatly aid epidemiological investigations.

The rigor of WGS for elucidating phylogenetic relationships in NZ cycles was demonstrated with an analysis (24) performed on 296 NZ genomes that were available at the time. Four clades were identified and shown to have significant clustering by both REA type and by region but to lack significant clustering by host. These results verified the regional localization of types and rapid switching between wildlife and livestock hosts that was suggested by REA typing. With the extra resolution provided by WGS there were numerous instances where isolates that had identical REA types could now be distinguished. Analysis by a Bayesian approach (Bayesian Evolutionary Analysis by Sampling Trees BEAST) (25) on a subgroup for which there were an adequate number of wildlife and livestock isolates from one clade, that were spread over time, indicated that although there was significant variation, *M. bovis* in infected animals in NZ was accumulating mutations in a clocklike manner at a rate of 0.53 (2.5% Lower: 0.22, 97.5% Upper: 0.94) events per genome per year. The most recent common ancestor (MRCA) to this group was estimated to have been circulating in 1859 (2.5% Lower 1525 97.5% Upper 1936) which agreed with the time when *M. bovis* was likely to have been introduced into NZ in cattle imported directly and indirectly (via Australia) from the UK (26). This study provided convincing evidence that the enhanced resolution from WGS had potential to aid in more precisely determining whether new infections were from persistence or the introduction of infection into NZ livestock and wildlife populations.

Through partnership and contracted work with TBfree and collaborations with Wellcome Sanger Institute, the Wellcome Trust University of Glasgow, United States Department of Agriculture (USDA), Animal Health and Veterinary Laboratories Agency (AHVLA), Landcare Research and Massey University, we have developed a database with over 700 WGS entries of important NZ *M. bovis* types, and a data processing method that identifies robust SNPs that differ from a reference genome and compares these SNPs to those detected in other isolates. This information has helped to precisely define the lineage of NZ *M. bovis* types and has facilitated accurate determination of the source of new infections. Our WGS database has been enriched in recent years by characterizing additional isolates from recent herd breakdowns and outbreaks and the characterization by the WGS of REA and VNTR types that were once prevalent in NZ. Here we demonstrate the suitability of WGS for routine surveillance with three investigations into NZ. *M. bovis* outbreaks in which genetic relatedness of the isolates were determined by

Abbreviations: : *M. bovis*, *Mycobacterium bovis*; *M. tuberculosis*, *M. tuberculosis*; NZ, New Zealand; WGS, Whole Genome Sequencing; VNTR, Variable Number Tandem Repeat; REA, Restriction Enzyme Analysis; BEAST, Bayesian Evolutionary Analysis by Sampling Trees; AHVLA, Animal Health and Veterinary Laboratories Agency; USDA, United States Department of Agriculture; NZGL, New Zealand Genomics Limited; UK, United Kingdom; CTAB-N-cetyl-N,N,N-trimethyl ammonium bromide; BWA, Burrows, Wheeler Aligner; OTGO, Otago; CNI, Central North Island; WC, West Coast; PPD, purified protein derivative; Bo, bovine; Po, possum; Pi, pig; Ce, cervine; Fe, ferret; ML, Maximum Likelihood.

comparing these novel SNP lineages to others in the database. The benefits of WGS over REA and VNTR typing methods in each case are discussed.

MATERIALS AND METHODS

The AgResearch *M. bovis* archive has over 8000 NZ isolates that were cultured between 1985 and 2018, from livestock and wildlife suspected of *M. bovis* infection during the post-mortem examination performed as part of routine surveillance. Conventional microbiological tests [described in (27)] were used to positively identify *M. bovis* infection. The WGS database has been assembled by characterizing isolates from the archive selected to provide a representative sample of the *M. bovis* population circulating in cattle and wildlife across NZ between 1985 and 2018. Most isolates that were characterized by WGS were previously either REA typed (10) or VNTR typed (15) and in some cases were typed by both methods. Culture and DNA isolation was performed either at the AgResearch Wallaceville or the AgResearch Hopkirk sites in level 3 containment facilities, adhering to the biosafety guidelines for these procedures outlined in the AgResearch containment facility manual. A total of 783 isolates; 417 bovine, 112 ferret, 106 possum, 72 pig, 67 cervine, 3 feline, 2 stoat, 1 hedgehog, and 1 human isolate were characterized by WGS. Selected isolates were cultured in Tween albumin (TAB) media from frozen stock and DNA was prepared by CTAB extraction essentially as described in (28). DNA submitted for sequencing at the New Zealand Genomic Limited facility at Massey University in NZ (NZGL) and the United States Department of Agriculture (USDA) was additionally purified by digestion with 20 mg/ml RNAse after lysozyme treatment, and with a phenol chloroform isoamyl alcohol extraction after incubation in N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB). DNA library preparation and genome sequencing were performed either at the USDA facility in Ames Iowa USA, at the NZGL facility at Massey University in NZ, or at The Wellcome Trust Glasgow facility in the UK, on an IIlumina MiSeq instrument, with 2 X 250 bp paired-end reads or at the Wellcome Sanger Institute (Cambridge, UK) on an Illumina HiSeq instrument with 2 × 150 bp paired-end reads.

Raw genomic data were trimmed using the DynamicTrim algorithm (v2.0, default settings) in SolexaQA software (29) and mapped to the original UK reference genome (NC_002945.3, AF2122/97) (30) using the Burrows-Wheeler Aligner (BWA)-MEM algorithm (v0.7.9a-r786 with -M setting) in the BWA alignment tool (31). From the resulting alignments, reads that mapped to more than one location in the genome (SAM flags >= 256) were removed. Results were further processed with SAM tools software (32) (v0.1.19-44428cd with settings view -q 30; then rmdup -S) to remove low quality mappings and PCR duplicates. Indels and non A/C/T/G reference alleles were ignored, bcftools (v0.1.19-44428cd, with setting view -N -I -cvg). Subsequently a minimum alignment quality of 80, a minimum total depth per SNP of 10, a maximum reference allele count per SNP of 2, a maximum FQ value of −55, and a reference to alternative allele ratio of at least 0.9 was enforced. Multiinter from the bedtools suite (v2.17.0) was used to generate a final list of potential genomic differences. SNPs detected in regions that are not well characterized by this methodology, (33) such as PE PGRS regions, IS elements, and poorly covered regions were excluded from the analyses via VCF software (34) (v0.1.12b). Poorly covered regions were defined by comparing 344 genomes with an average coverage of 45X or higher. Regions from which SNPs were excluded and also individual SNPs that were excluded from all of the genomes because they were poorly covered in some of the genomes are listed in **Supplementary File 1**. SNPs that were determined to be of high quality when detected in genomes with 45X or higher coverage and detected but filtered from more poorly covered genomes were added back to the filtered VCF files of the poorly covered genomes. The remaining core SNPs were processed together to produce concatenated alignments, which were compared in order to define the phylogenetic relationship of the isolates. A *Mycobacterium caprae* genome (strain 09-0454) is included as an out-group to root these comparisons. Average coverage and *in silico* spoligotyping were determined with vSNP software / https://github.com/USDA-VS/vSNP using the recently amended UK reference NC_002945.4 (35).

The relationship between isolates that are shown here were determined by BioNJ phylogenetic trees with 100 replicates, using a Jukes and Cantor model with SeaView 4 software (36) and also with RAxML software (37) (version raxmlHPC-PTHREADS-SSE3) with 1000 replicates using a GTRGAMMAI model. BioNJ and RAxML Phylogenetic trees were compared side by side using Phylo.io software (38) and were displayed and colored for other Figures using FigTree v1.4.2 software (39). Distance matrices were generated from concatenated SNP sequences with the Muscle Aligner (40) in the Geneious software package and were colored using the color scale formatter in Excel or alternatively with an R script that uses R's Gplot package to create heat maps via the heatmap.2() function. Global distributions of the four major NZ spoligotypes were obtained with the similarity search tool at the Pasteur-Guadeloupe website http://www.pasteur-guadeloupe.fr:8081/SpolSimilaritySearch/ (41).

RESULTS

A total of 782 *M. bovis* genomes were used here as a basis for comparison of NZ breakdowns and outbreaks. The alignment length for this selection of isolates was 8261 sites. Metadata, coverage statistics and the *in silico* spoligotyping results for these isolates are listed in the spreadsheet in **Supplementary File 2**. The time span for these isolates is 30 years from 1988 to 2018 and includes representatives of important types from throughout the North and South Island. The relationship of prevalent NZ *M. bovis* types is illustrated by the SNP phylogeny that was generated by maximum likelihood analysis in **Figure 1** and is compared to a phylogeny determined by the BioNJ distance method in **Supplementary File 3**. Several genomes from overseas *M. bovis* isolates, including the PPD

strain AN5, and three isolates from the USDA elite collection (05_8628, 94_5053, and 12_1874) are included to aid with these comparisons. The 4 distinct branches that were initially detected (24) were also evident in this larger group, and each clade was shown here to share more recent common ancestors with overseas isolates than with other NZ isolates. The same relationship was evident by a BioNJ distance analysis, perhaps reflecting the clonal, primarily non-recombining nature of NZ *M. bovis* evolution (see **Supplementary File 3a**). As was seen in the initial characterization (24) of a subset of these isolates, WGS results for this larger group of isolates corroborate findings from previous REA and VNTR typing studies which revealed that distinct types predominate in different parts of NZ (9, 10, 15).

In silico spoligotyping revealed that most of the NZ isolates in the database have spoligotypes that were common in the UK when cattle were imported into NZ in late 1860s (42), 34% were SB0130 and 41% were SB0140. Two other prevalent spoligotypes that were characterized extensively were SB1504, (121 isolates 15%) a type that is endemic in the Marlborough North Canterbury region of the South Island and SB1031 (23 isolates 3%) a type that is endemic in Southland in the South Island (see the map presented in **Figure 2B**) and was once prevalent in Australia (43). The Global distribution of these types is illustrated in the **Supplementary File 4**. Although SB0130, SB0140 and SB1031 have been isolated in other parts of the world (43), SB1504 has so far only been detected in NZ, suggesting either that it evolved from a different type just prior to becoming established in NZ or that other global sources of this type have not yet been discovered.

The phylogenetic relationship and geographical source of the three outbreaks investigations that will be discussed below are described in the phylogeny in **Figure 2A** and map in **Figure 2B**. Infections that were investigated were from (A) Mt. Cargill in Otago, (B) South Westland in the South Island, and (C) Waiuku in the Central North Island (CNI).

Mt. Cargill Outbreak

An investigation of isolates from the Mt. Cargill region of the South Island was carried out to aid in determining the source of this recent infection, which appeared to have spread throughout the Mt. Cargill region within 1–2 years. We analyzed three groups of isolates: (i) isolates from recently infected cattle (9 isolates), farmed deer (2 isolates) and wildlife (14 isolates) in the region; (ii) a selection of isolates (5 cattle, 5 wildlife) from the AgResearch strain archive that had come from sources within 15 km of the outbreak; and (iii) a group of recent cattle isolates with similar types (AgR1665 type VNTR104, AgR1689 type VNTR135 and AgR1669 VNTR27) to those found in the Mt. Cargill region that had come from outside Mt. Cargill. All isolates were characterized by WGS in order to determine if this outbreak was from local wildlife reinfection, or from introduction of a different type into the region. Also shown in the accompanying figures are the relationship of these outbreak investigation isolates to previously characterized Otago isolates (AgR96, AgR707, AgR703, AgR726, AgR734, AgR51, AgR76, and AgR53) in the WGS database.

Results of this investigation are shown in (a) SNP Table and (b) the Phylogram (C) the Map in **Figure 3**. When WGS data for Mt Cargill outbreak isolates was compared to WGS data for the other isolates that were characterized for this investigation, the Mt. Cargill outbreak isolates (boxed in purple in **Figure 3a**) shared their most recent common ancestors with livestock and wildlife isolates from more than 20 km north of Mt Cargill and were more distantly related to the other examined types that were prevalent in the nearest known wildlife/domestic stock cases from west of Mount Cargill. The closest known relative to the outbreak was a 2012 isolate (AgR1665) from Waikouaiti (see the SNP table in **Figure 3a**). AgR1665 was missing one SNP that was common to the outbreak isolates, a C to T change at position 4328907 in the reference genome, but had the 11 others that were common to the outbreak cluster. Using the mutation rate estimate from Crispell et al. [0.53 (2.5% Lower: 0.22, 97.5% Upper: 0.94)] this suggests that this likely precursor and the Mt. Cargill outbreak lineage diverged from a common ancestor approximately 2 (3–7) years prior to when the 2012 precursor isolate was detected. The next closest known relatives were several wildlife isolates from northern Otago (AgR707, AgR96, and AgR1673). Mt Cargill isolates shared 10 common SNPs with these wildlife isolates. The Mt. Cargill and North Otago wildlife isolates shared 2 SNPs with recently characterized isolates from Northern Otago (AgR1717 and AgR1666, boxed in yellow in the phylogenetic tree in **Figure 3b** and indicated by yellow symbols on the map in **Figure 3c**). Both groups had acquired numerous SNPs (>20) since diverging from their common ancestor. These results provide evidence to suggest that this type had prevailed in Northern Otago for many years. **Supplementary File 3b** compares the relationship of these isolates by maximum likelihood and BioNJ distance analyses, and the conclusions drawn about the relationship of these isolates was the same regardless of the phylogenetic method used for the analysis perhaps because of the many SNPs that were common to the outbreak isolates and their closest known relatives. Although these results did not rule out the possibility that the infection was circulating undetected in the Mt Cargill region previous to the outbreak, our WGS comparison of outbreak isolates to common wildlife and livestock types suggest that this infection is more likely to have moved into Mt. Cargill from wildlife or livestock from the north than to have come from local wildlife. A direction of the spread of this infection based on these data is indicated by the arrows on the map in **Figure 3c**.

Although in most cases VNTR types of these isolates correlated well with SNP sub-clusters, since the closest relative, a 2012 Waikouaiti cattle isolate (AgR1665) had a slightly variant VNTR type (see the VNTR104 types tabulated in **Figure 3b**), if the Mt. Cargill outbreak investigation was based solely on VNTR results the relevance of this isolate to the outbreak would be much less evident than it is from the SNP lineage. This tree also illustrates how the SNP lineage determined by WGS defines the relationship between early isolates that were originally characterized only by REA to later isolates that were originally

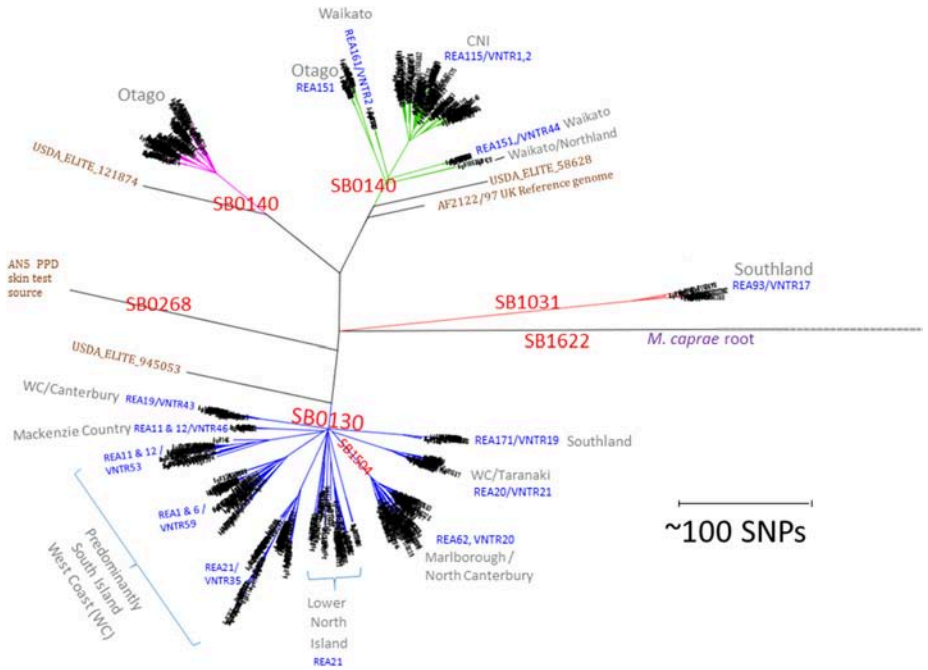

FIGURE 1 | NZ *M. bovis* types. Radial Maximum Likelihood (ML) Phylogram illustrating the relationship of NZ *M. bovis* isolates in the NZ WGS database. The scale bar indicates the approximate distance in SNPs between isolates. "SB" numbers labeled in red are internationally recognized spoligotypes based on differences in the DR/CRISPR region. The REA and VNTR types listed in blue text are the predominant REA type(s) and or VNTR types in the indicated cluster and they are predominant in regions listed in gray. Overseas isolates that are included for comparison are labeled in brown: the UK reference (AF2122/97), the UK strain commonly used as a source of PPD (AN5) and 3 USDA ELITE strains (58628, 945053, and 121874). The branches in the four NZ clusters are colored differently to highlight the distinction from other branches.

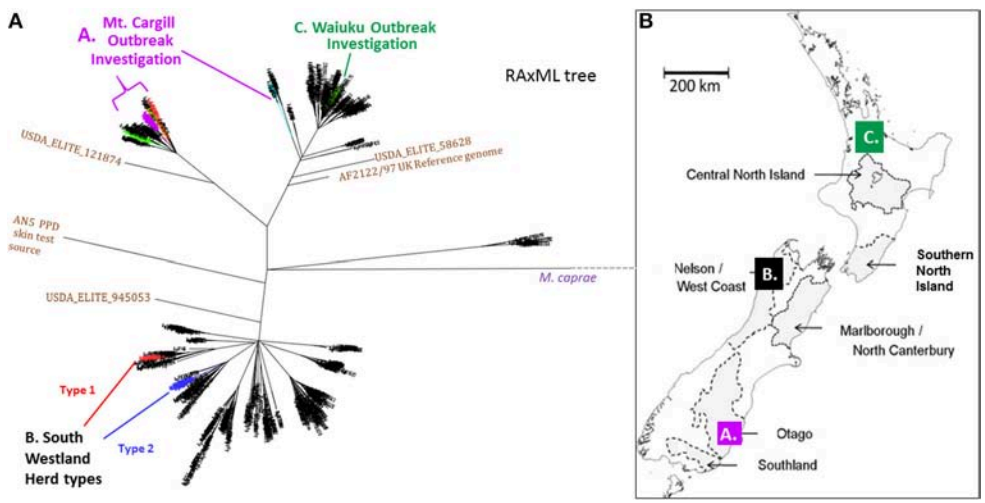

FIGURE 2 | (A) NZ *M. bovis* Radial ML Phylogram illustrating the Phylogenetic relationship of the isolates that will be discussed in more detail below. (A) Mt. Cargill investigation isolates-These isolates cluster in the Otago clade and are colored the same as in the boxed sub-groups in Phylogram in **Figure 3a**. (B) South Westland breakdown types- The two types isolated from the herd in South Westland and the closest relatives (shown in SNP tables in **Figure 4B**) are colored differently; the breakdown type 1 isolate clusters in the VNTR53 group and it and its closest known relatives are colored red, the breakdown type 2 isolates clusters in the VNTR59 subgroup and it and its closest known relatives are colored blue. (C) Waiuku outbreak- isolates cluster in the CNI clade and outbreak isolates described in the SNP table in **Figure 6B** are colored green. **(B)** Map of NZ indicating vector risk areas (shaded regions) and illustrating geographical source of the isolates from the three outbreak investigations described below. Colored squares on the map indicate the regions of the discussed outbreak investigations. The three different investigations that will be discussed below are indicated by letters and color in the Phylogram in **(A)** and the map in **(B)**.

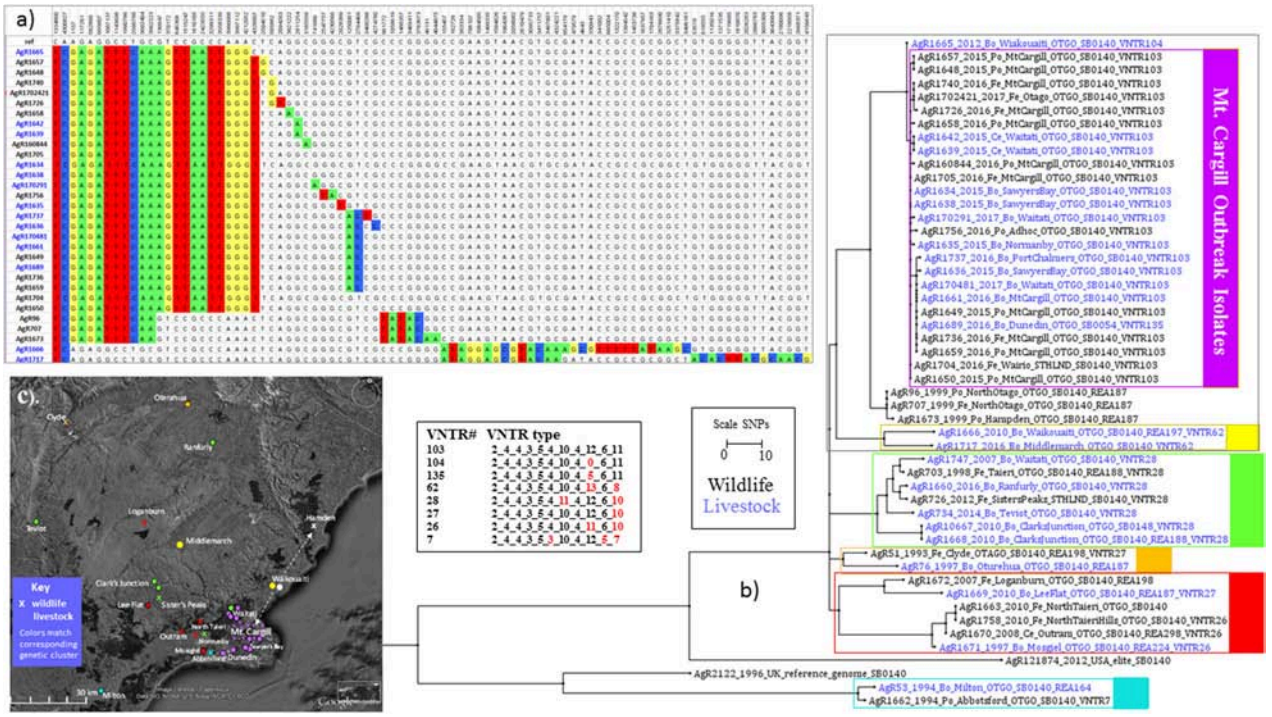

FIGURE 3 | Mt. Cargill Outbreak Investigation. Genetic and spatial relationship of *M. bovis* isolates from southeast Otago. **(a)** SNP table and Phylogram for selected isolates from the Otago cluster. The SNP table illustrates SNP differences in outbreak isolates and their closest known relatives. Chromosomal positions in genomic reference NC_002945.3 are listed across the top. The DNA base found at the indicated chromosomal position in the reference is listed in the next line. DNA bases in the table are colored to indicate differences from the reference genome. **(b)** Square ML Phylogram. The scale bar indicates the distance in SNPs between isolates displayed in the phylograph. Livestock metadata in the tree (_Bo_ for bovine _Ce_ for cervine) is colored blue and wildlife metadata (_Po_ for possum, _Fe_ for ferret), is colored black. The numbers for the listed VNTR types are the number of repeats at the 11 loci as described in Price-Carter et al. (15): Miru40_EtrD_EtrC_EtrE_NZ2_QUB18_QUB11a_QUB26_DR2_DR1_QUB3232. Red numbers in this VNTR table indicate differences from the outbreak type VNTR103. **(c)** Map of sources of isolates shown in the phylogenetic tree in **(b)**. Symbols on the map indicate the approximate regional sources and are colored to match the genetic cluster of the isolate as indicated by the boxes in **(b)**. The arrows on the map in **(c)** indicate the proposed direction of the spread of this infection based on WGS results.

characterized only by VNTR, which can be very helpful when trying to understand the source of new infections.

South Westland TB Infected Herd

Results from the investigation into the findings of TB cases in a previously disease free dairy herd located in South Westland, West Coast, South Island are shown in **Figures 4**, **5** and **Supplementary Files 3c,d,** and **5**). The herd had two separate findings of bovine tuberculosis approximately 5 months apart. There was a clear herd skin test between the two animals being identified at slaughter. Both TB cases were considered to be anergic animals (infected but not detectable through our standard testing procedures) as they were not identified as infected until they were inspected at slaughter, and both animals had been repeatedly TB skin tested before and after leaving their herd of origin.

These isolates were two distinct VNTR types; the first TB case (AgR738) was identified as type VNTR59, and the second case TB case (AgR744) was identified as type VNTR53 (see **Figures 4A,B** and **Supplementary File 5**). The Phylogram in **Figure 4A** illustrates the phylogenetic relationship of the isolates of these two types to others of these types in the database. This Phylogram was determined by ML analysis. The same relationship was evident by BioNJ analysis (see **Supplementary Files 3c,d**). The distinguishing SNPs in the SNP tables in **Figure 4B** provide a more detailed comparison of the differences between these TB isolates and their closest relatives. WGS clearly demonstrated the close relationship between the isolates and those from historic cases linked to the original locations of these animals. Animal movements were traced using information collected at the time of the epidemiological investigation. Animal identification and movement records were scrutinized as well as gathering information directly from farmers at that time. Although the two types that were detected in this herd could be distinguished from each other by VNTR assay, WGS analysis has allowed these to be compared to other isolates and confirm the most likely transmission pathway (see the square Phylograms in **Supplementary File 5**).

WGS clearly narrowed the list of likely suspects in each case. The isolate from the first TB case (AgR738) was identical or nearly identical by WGS to isolates from a recent outbreak up the coast in the Kowhitirangi and Arahura regions (see the SNP table **Figure 4B**). All of these outbreak isolates appeared to share

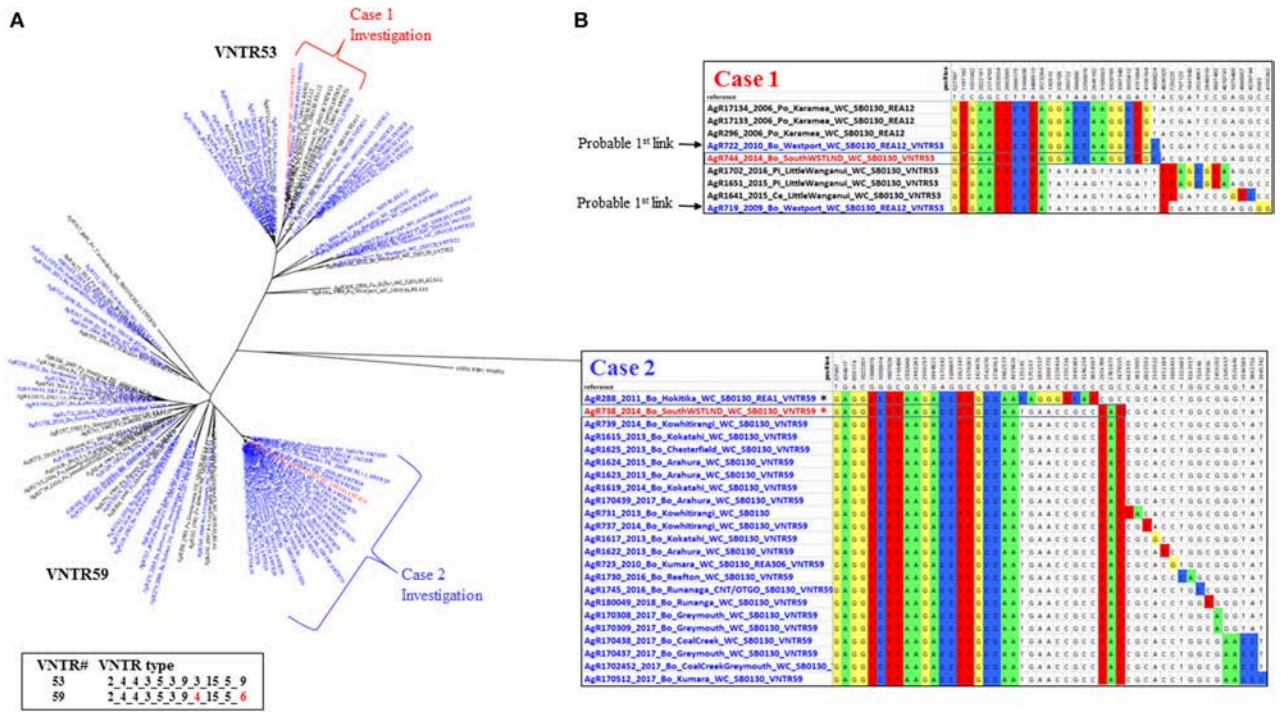

FIGURE 4 | Multiple South Westland herd infections. **(A)** Radial ML Phylogram illustrating the genetic relationship of the two types of *M. bovis* isolates detected during a South Westland breakdown investigation, to other type VNTR59/REA1/REA6 and VNTR53/REA11/REA12 *M. bovis* isolates in the database. Metadata for isolates from this herd are colored red, other livestock metadata are colored blue and wildlife metadata black. Brackets indicate close relatives of the breakdown isolates and are also described in the SNP table in **(B)**. Also shown are the two different VNTR types, with numbering as described in the legend for **Figure 3**. **(B)** SNP tables illustrating the relationship of each type to its closest relatives. The coloring and numbering in this tables is as described in **Figure 3**. SNPs detected in the case 1 and in case 2 isolates are boxed within the table. The asterisk in the case 2 table indicates an isolate (AgR288) that was ruled out as a possible source of infection by this investigation.

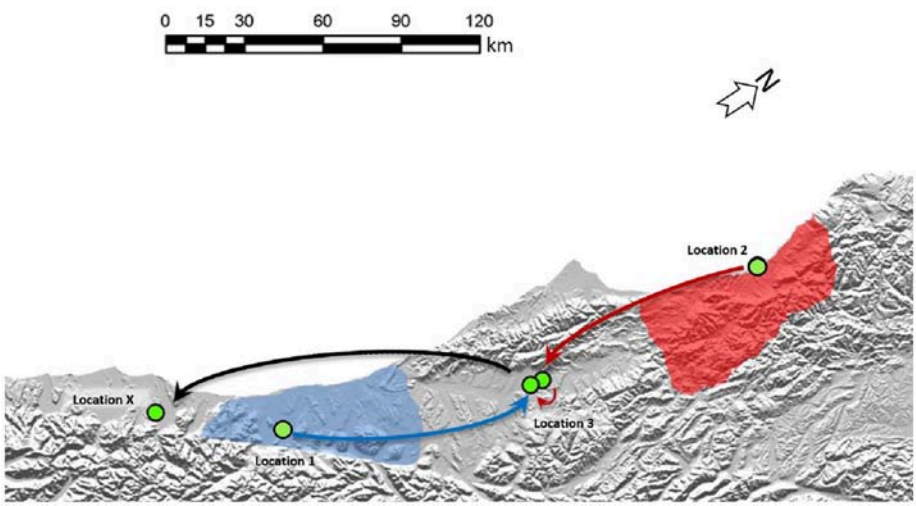

FIGURE 5 | Two separate origins of TB in a South Westland Herd. Cattle movements that led to these two types of infection in the South Westland herd are indicated by arrows. Green circles indicate approximate locations where these animals resided. Shaded areas on the map indicate regions where VNTR53/REA11/REA12 (red) and VNTR59/REA1/REA6 (blue) are endemic in wildlife populations. TB case 1 moved from Location 1 to location 3 before moving to Location X (from where it was identified as TB positive at routine slaughter). TB case 2 was moved from its herd of origin in location 2 to a farm near Location 3 before moving to location 3 and then on to Location X from where it was identified as being infected. The region described here is indicated by the black box in the map in **Figure 2A**.

a recent common ancestor with AgR288, a 2011 cattle beast isolate from a Hokitika farm, and this was thought to be a likely original source, but this isolate was ruled out as a source for the outbreak by WGS, since it was missing the 3 SNPs that are common to the outbreak and had additional SNPs not found in the outbreak isolates (**Figure 4B**). By WGS, the isolate from the second TB case (AgR744) was identical to AgR722, a livestock isolate from Westport, over 170 km from Location X and shared a recent common ancestor with several wildlife isolates (AgR296, AgR17133, AgR17134) from Karamea (location 2) which is over 250 km from Location X (see SNP tables in **figure 4B** and the map in **Figure 5**). Although all four locations on which the animals resided are within a formal Movement Control Area (MCA) where all stock over 12 months of age are to be tested annually AND all stock that are moved are to be TB tested within the 60 days preceding the movement, these results suggests that this infection has most likely resulted from the long distance movement of infected livestock.

Waiuku Outbreak Investigation

The investigation of the *M. bovis* outbreak that began in Waiuku, Central North Island clearly illustrated the close relationship of epidemiologically linked livestock isolates and demonstrated their more distant relationship to other types from the Central North Island (see the Phylogram in **Figure 6A**). The relationship of isolates in the green colored portion of the Phylogram in **Figure 5A** was compared by maximum likelihood and BioNJ methods (**Supplementary File 3e**) and found to be nearly identical by both methods.

There were two cycles of infection associated with this outbreak, the first occurred between 2007 and 2010 and the second in 2013 (see **Figure 7a**). When compared by WGS, there were 10 SNPs detected that were common to both early and later outbreak isolates (see the SNP table in **Figure 6B**) suggesting that both outbreaks were from the same source of infection rather than from two different types introduced into the area. This infection was spread to herds in other regions of the Central North Island (**Figure 7**). Although no infection was detected from the likely source of this spread (black box in **Figure 7a**), isolates from infected animals that had been moved from this herd shared the 10 SNPs that were common to this outbreak (6b).

The relationship of Waiuku outbreak isolates to the closest known wildlife isolates in the database, recent pig isolates from Hauturu (AgR16102 and AgR730),Tihoi (AgR17003411) and Hauhungaroa (AgR1704532) as well as a possum isolate from 2001 (AgR1795) from Taumaranui, are also shown in the SNP table in **Figure 6B**. These results indicate that these wildlife isolates share a common ancestor with the outbreak isolates but they do not have the 10 outbreak specific SNPs and have acquired 13-15 SNPs that were not detected in the outbreak isolate genomes indicating that they are not closely related.

By combining epidemiological investigation with SNP lineage comparisons, far more insight is gained than was possible by VNTR or REA typing. A good example of this is provided by a SNP detected in isolates from livestock in Waiuku that were not known to be linked by movement but were farmed within 4 km of one another (AgR508, AgR546, and AgR548, boxed in black in the SNP table in **Figure 6B** and circled in the transmission path diagram in **Figure 7**) suggesting perhaps that despite extensive surveillance, there may have been either a wildlife vector for this Waiuku infection, or alternatively that there was undocumented herd movement occurring.

Distance of Epidemiologically Linked Isolates

Genetic pairwise distances are the number of SNPs that differ when two isolates are compared. **Table 1** compares pairwise distances for the epidemiologically linked isolates discussed above and the heat map in **Figure 8** and in **Supplementary File 6** show pairwise distances for all of the isolates illustrated for the three discussed investigations. Mt. Cargill isolates were collected over a period of 5 years and differed from one another by 0-5 SNPs. AgR 738, the case 2 isolate from the South Westland herd and its 22 close relatives from the Kowhitirangi outbreak were collected over a period of approximately 5 years, and differed from one another by 0–7 SNPs. The 12 Waiuku outbreak isolates were collected over a period of 6 years and differed from one another by 0–9 SNPs. The New Zealand *M. bovis* mutation rate determined by Crispell et al. (24) (0.53 with a range 0.22–0.94) was closest to that estimated for human tuberculosis by Walker et al. (44) (0.5 with a range of 0.3–0.7), and the pairwise distances of epidemiologically linked isolates shown in **Table 1** are within the 12 SNP limit for epidemiological linkage determined by Walker and colleagues for *Mycobacterium tuberculosis*. These groups of isolates differ from unlinked isolates of the same types by 10's of SNPs and from isolates from other branches of the phylogenetic tree by hundreds of SNPs (see heat maps is **Figure 8** and the more detailed versions in **Supplementary File 6**).

DISCUSSION

Results of our current investigation demonstrated the same overall relationship of types described previously, since by WGS isolates cluster into the same groups that were determined by REA and VNTR analysis, but with the much finer resolution provided by WGS there is increased ability to rule out likely sources of infection. *In silico* spoligotyping confirmed that at least three of the four detected clades were likely to have been imported along with British sources of cattle in the middle to late 1800s. The regional clustering of types determined with REA and VNTR methods was corroborated since livestock and wildlife from the same region clustered. The Mt. Cargill and South Westland investigations illustrated how WGS leads to better definition of the source of new infections by ruling out potential sources, and all three investigations have led to the confirmation of epidemiological sourcing of infection. In addition, the Waiuku investigation indicated probable wildlife infection in an area considered to be free of infection.

The neighbor joining method is often considered useful for getting a quick approximate idea of genetic relationships because it is based principally on genetic distances, and does not incorporate the more accurate models of sequence evolution that are exploited in maximum likelihood analysis. The strikingly

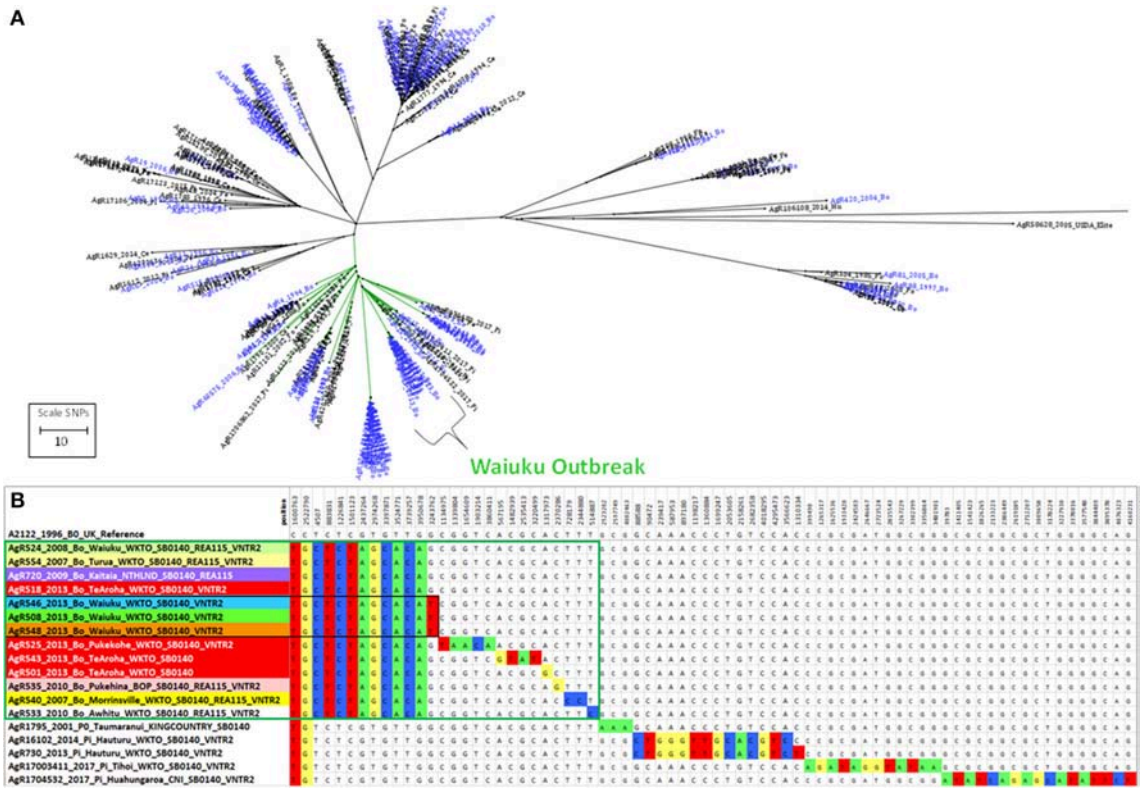

FIGURE 6 | Genetic relationship of Waiuku outbreak Isolates. **(A)** Radial ML Phylogram illustrating the genetic relationship of *M. bovis* isolates from the Waiuku outbreak to other livestock and wildlife isolates in the Central North Island cluster. Livestock metadata are colored blue and wildlife metadata black. Waiuku isolates are indicated by the bracket. The green colored branch indicates the isolates that are compared in **Supplementary File 3e**. **(B)** The relationship of isolates from the Waiuku outbreak is illustrated in a SNP table with DNA bases in the table colored to indicate differences from the reference genome. Metadata for isolates from different herds that were characterized by WGS are shaded differently. Waiuku outbreak isolates characterized for this investigation are boxed in green. Isolates that are boxed in black were not known to be linked by movement but were from farms within 4 km of one another.

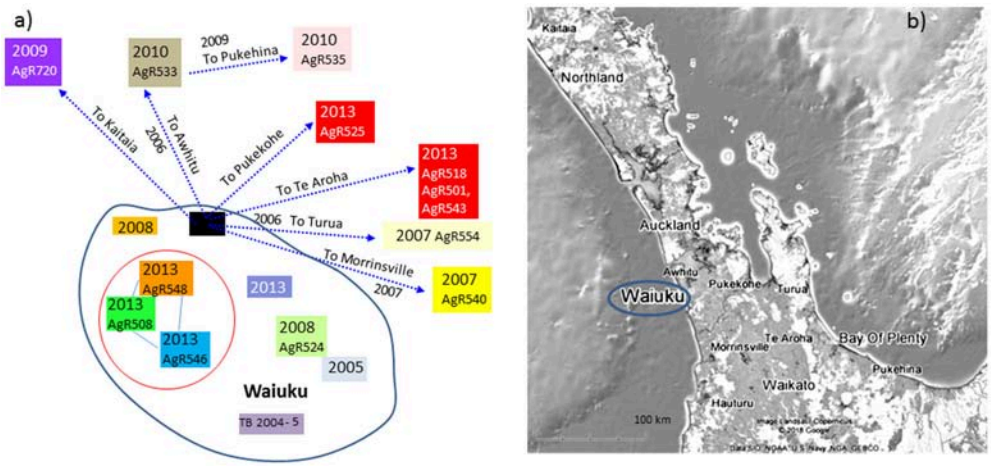

FIGURE 7 | Waiuku outbreak Transmission path. **(a)** The direction of spread deciphered from epidemiological investigation, **(b)** a map illustrating the geographical sources of the characterized isolates. Isolates that have been characterized by WGS are colored to match the genomic data shown in the SNP table in **Figure 6B**. Colored boxes without AgR numbers represent isolates that were not characterized by WGS. Isolates that are circled in red were not known to be linked by movement but were from farms within 4 km of one another.

TABLE 1 | Pairwise genomic distances of epidemiologically linked isolates.

Outbreak	Number of isolates	Time span	Approx. # years	Pairwise distance (SNPs)	Hosts	VNTR
Mt. Cargill	26	2012–2017	5–6	0–5	cattle, deer, possum	103
Kowhitirangi	23	2013–2018	5–6	0–7	cattle	59
Waiuku	12	2007–2013	6–7	0–9	cattle	2

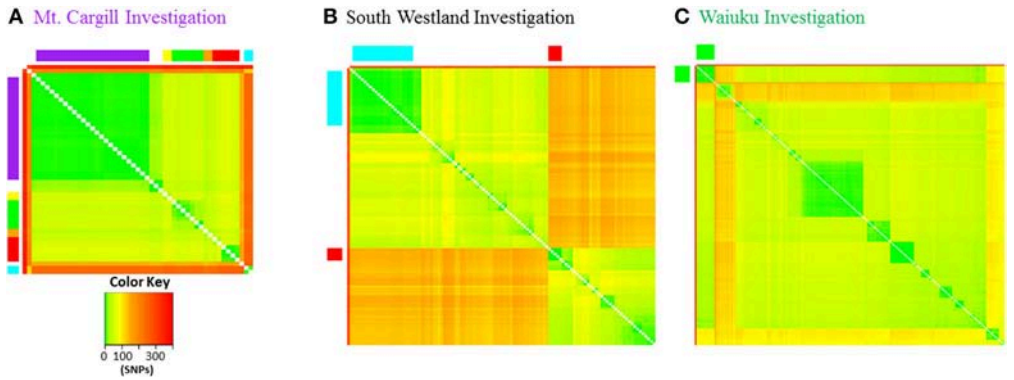

FIGURE 8 | Pairwise genetic distance heat maps**.** Distance in SNPs between pairs of isolates is illustrated by the different colors as indicated in the color key. **(A)** Mt. Cargill outbreak isolates are in the same order as in the Phylogram in **Figure 3** and colors along the outside of the plot correspond to those in the Phylogram and also to those in the more detailed distance plots in **Supplementary File 6**. **(B)** VNTR53 and VNTR59 isolates. Close relatives of South Westland breakdown type 1 are indicated by red squares and type 2 by the blue rectangles along the outside of the plot. Isolates are in the same order as in the more detailed distance plots in **Supplementary File 6**. **(C)** CNI branch isolates. Waiuku outbreak isolates are indicated by the green squares along the outside of the plot.

similar relationships determined for NZ *M. bovis* isolates by the maximum likelihood, neighbor joining distance methods and SNP tables, suggest that when analyzed by our WGS method, *M. bovis* in NZ cycles of animal infection appears to be evolving in a manner that is well described by incremental changes in genetic distance-clonal evolution. These results are in agreement with the evolutionary mechanism suggested in Smith et al. (45) where it was noted that these bacteria do not tend to carry or incorporate foreign DNA and that their genomes evolve primarily by deletions and the acquisition of SNPs.

The numerous SNPs that are shared by and distinguish members within and between groups give a much more robust indication of the relationship of isolates than our previous typing methods. In most cases the same REA and VNTR types tended to be grouped into one WGS cluster, but in several situations WGS revealed flaws in the apparent relationship of types determined by the other molecular methods. Several instances of homoplasy (the detection of the same REA or VNTR type in distantly related WGS clusters) were revealed during the course of our characterization of NZ types. For example, although most VNTR types tended to cluster into only one sub-group in the Otago branch, type VNTR27 isolates cluster in several different groups (see the phylogenetic tree in **Figure 3b**). This clustering of unrelated types has been described in other comparisons of WGS to VNTR typing (23, 46, 47). There were also several instances where types tended to switch back and forth within a subgroup, (see REA types 11 and 12 and REA types 1 and 6 in the tree in **Supplementary Figure 5**), as was observed by Trewby et al. (18).

Although WGS is far superior to our previous typing methods there are factors that limit the usefulness of WGS data for epidemiological investigations. *M. bovis* accumulates mutations in a clocklike manner, but the fixation of new changes into the population is slow and highly variable over short times. For an example see the SNP table in **Figure 6B**. Waiuku outbreak strains isolated within a year of one another varied by 0–5 SNPs. This variability has been noted in other *M. bovis* (16) and *M. tuberculosis* studies (48), and can make it difficult to determine whether the transmission is occurring within the herd or from local wildlife reinfection. This high variability over short times also makes it difficult to use Bayesian techniques such as BEAST for reconstructing recent local transmission pathways since there is not enough of a consistent temporal signal. Because the *M. bovis* lifestyle switches between an active systemic infection and a localized (difficult to detect) latent infection the time of infection is not necessarily close to the time of isolation. This makes it more difficult to determine when a new infection was introduced. When sampling disease with a wildlife reservoir the data may represent a low proportion of the total infection and it can therefore be difficult to draw valid conclusions about the direction of transmission. This influence of sampling bias was clearly illustrated in our previous work (24). In the current study, the finding of no close links between wildlife and Waiuku livestock may be because this type evolved in livestock populations and therefore there is no transmission linkage with

wildlife but it could also be because the wildlife source was not sampled. This same factor weakens the conclusion drawn in the Otago study; although our analyses seem to indicate the infection in Mt Cargill came into the area in infected livestock, because of uneven sampling we cannot be 100% certain that the infection had not spread from an undetected local wildlife source.

The phylogeny in **Figure 1** illustrates that NZ types share common ancestors with types isolated in other parts of the world [also see **Supplementary File 4** and (43)]. We noted previously (24) that NZ strains tended to accumulate mutations at a faster rate than their UK relatives and surmised that the enhanced mutation rate may be the result of the larger amount of bacterial growth in possums, the major wildlife reservoir. Teasing apart these types of differences may be helpful for understanding transmission pathways in other bovine TB cycles.

CONCLUSION

As the NZ epidemic diminishes, accuracy and high resolution becomes even more important for the identification of true sources. By ruling out possible sources of infection the enhanced resolution provided by WGS will likely reduce expenditure on the monitoring of herd infections and of wildlife monitoring and control. The routine use of WGS analyses for determining the source of *M. bovis* infections will be an important component of the strategy employed to eradicate bovine TB from NZ.

DATA AVAILABILITY

The datasets for this study have been deposited in the NCBI Sequence Read Archive (reference # SRP155672) and will be made publically available once this article has been accepted for publication.

AUTHOR CONTRIBUTIONS

MP-C coordinated sequencing and data processing and drafted and revised the manuscript. DC, MP-C, GdL, and PL designed the analysis. RB developed data processing python scripts for mapping filtering and labeling genomic data. RB and MN developed figures. MN and JS shared results of epidemiological investigations, MN drafted a section of the manuscript, DC, GdL, PL, MN, JS, GK, GA, and KC were involved with selection of the isolates and interpretation of results. DC, GD, MP-C, and BP were involved with typing of isolates. JP, SH, and JW provided 200 sequences from Wellcome Sanger. RK provided 100 sequences from Wellcome Trust Glasgow. SR-A and TS shared reference genomes, helped with data filtering and data presentation. TS helped with installing and running vSNP software. DC, GdL, RB, BP GA RK SR-A JP, and JC critically reviewed and helped revise drafts of the manuscript. JP and MN helped with responses to reviewers.

FUNDING

The bulk of this research has been funded by OSPRI TB free project R-30766. Wellcome Sanger funded the sequencing 200 genomes, Wellcome Trust Glasgow funded the sequencing 100 genomes and Massey funded the sequencing of 5 genomes and the AHVLA funded the sequencing of 3 genomes.

ACKNOWLEDGMENTS

We would like to acknowledge the efforts of Maree Joyce, Melissa Surry, Gary Yates, Brian Moonsamy, Rebecca Terry, and Kwang Subharat for help with re-culturing these isolates from the strain archive, Maree Joyce, Justine Jacobs, and Kwang Subharat for help with DNA extraction, Richard Fong from NZGL, Doug Harris from Wellcome Sanger, Julie Galbraith from Glasgow University and Patrick Camp from the USDA for DNA library preparation and sequencing, Hannah Trewby for help with data interpretation, Josephine Bryant for initial characterization of the Wellcome Trust Sanger isolates, Nigel French for funding the sequencing of 5 NZ *M. bovis* genomes and Noel Smith for sequencing 3 NZ *M. bovis* genomes and the reviewers of this manuscript for their very helpful suggestions for improvements and clarification.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00272/full#supplementary-material

Supplementary File 1 | SNPs excluded from the analysis. Numbers indicate regions or individual positions in UK reference version NC_002945.3 for which SNPs were excluded from all isolates.

Supplementary File 2 | Isolate metadata, coverage statistics, *in silico* Spoligotype data and metadata explanations.

Supplementary File 3 | Comparison of RaxML and BioNJ phylogenies. **(a)** NZ *M. bovis* tree. The four clades identified in **Figure 1** with differently colored branches are boxed in the same color in this Figure. **(b)** Mt. Cargill outbreak investigation- all isolates shown in the Phylogram in **Figure 3a** are compared. Outbreak isolates are boxed in red. **(c-d)** South Westland investigation isolates are indicated by the red arrow, **(c)** case 1. **(d)** case 2. **(e)** Waiuku investigation. The scale indicates the level of relatedness between plots.

Supplementary File 4 | Global distribution of NZ spoligotypes. Coloring in these plots reflects the relative abundance of this type in the indicated region.

Supplementary File 5 | Square ML Phylogram illustrating the relationship of West Coast types considered for the South Westland investigations. Breakdown isolate metadata is colored red, livestock metadata is colored blue and wildlife metadata is colored black.

Supplementary File 6 | Distance Matrices. Closely related isolates are colored green, more distant isolates yellow, then orange with the most distinct isolates colored red. Color bars indicate the corresponding cluster in **Figures 3**, **4** and **6**.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 26 October 2018
doi: 10.3389/fvets.2018.00259

Efficacy and Safety of BCG Vaccine for Control of Tuberculosis in Domestic Livestock and Wildlife

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Bovine tuberculosis (TB) continues to be an intractable problem in many countries, particularly where "test and slaughter" policies cannot be implemented or where wildlife reservoirs of *Mycobacterium bovis* infection serve as a recurrent source of infection for domestic livestock. Alternative control measures are urgently required and vaccination is a promising option. Although the *M. bovis* bacille Calmette-Guérin (BCG) vaccine has been used in humans for nearly a century, its use in animals has been limited, principally as protection against TB has been incomplete and vaccination may result in animals reacting in the tuberculin skin test. Valuable insights have been gained over the past 25 years to optimise protection induced by BCG vaccine in animals and in the development of tests to differentiate infected from vaccinated animals (DIVA). This review examines factors affecting the efficacy of BCG vaccine in cattle, recent field trials, use of DIVA tests and the effectiveness of BCG vaccine in other domestic livestock as well as in wildlife. Oral delivery of BCG vaccine to wildlife reservoirs of infection such as European badgers, brushtail possums, wild boar, and deer has been shown to induce protection against TB and could prove to be a practical means to vaccinate these species at scale. Testing of BCG vaccine in a wide range of animal species has indicated that it is safe and vaccination has the potential to be a valuable tool to assist in the control of TB in both domestic livestock and wildlife.

Keywords: BCG, cattle, diagnosis, goats, deer, tuberculosis, vaccination, wildlife

OPEN ACCESS

Edited by:
Srinand Sreevatsan, Michigan State University, United States

Reviewed by:
Catalina Picasso, University of Minnesota Twin Cities, United States
Mirinda Van Kleef, Agricultural Research Council of South Africa (ARC-SA), South Africa

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *29 July 2018*
Accepted: *01 October 2018*
Published: *26 October 2018*

Citation:
Buddle BM, Vordermeier HM, Chambers MA and de Klerk-Lorist L-M (2018) Efficacy and Safety of BCG Vaccine for Control of Tuberculosis in Domestic Livestock and Wildlife. Front. Vet. Sci. 5:259. doi: 10.3389/fvets.2018.00259

INTRODUCTION

Tuberculosis (TB) in domestic livestock and wildlife is caused by *Mycobacterium bovis, Mycobacterium caprae,* and other members of the *Mycobacterium tuberculosis* complex, including *M. tuberculosis* whose role in animal TB is being increasingly recognised, particularly in studies from Africa and Asia (1). Livestock TB continues to be a major economic animal health problem worldwide. It has been estimated that >50 million cattle are infected worldwide, costing US$3 billion annually (2). The disease is an important zoonosis, causing TB in humans, particularly through consumption of unpasteurised milk or through co-habitation with infected animals. The "test and slaughter" bovine TB control programmes introduced in many countries in the mid-twentieth century achieved dramatic results and a number of countries were able to eradicate this

disease. However, these control programmes have not been affordable or socially acceptable in many developing countries, and more than 94% of the world's population live in countries in which control of TB in cattle or buffaloes is limited or absent (3). Furthermore, a confounding factor in the control of bovine TB in a number of countries has been the existence of wildlife reservoirs of *M. bovis* infection.

Wildlife serving as maintenance hosts for *M. bovis* include the Australian brushtail possum (*Trichosurus vulpecula*) in New Zealand, the European badger (*Meles meles*) in United Kingdom (UK) and Ireland, white-tailed deer (*Odocoileus virginianus*) in Michigan, USA [reviewed in (4)] and Eurasian wild boar (*Sus scrofa*) in the Iberian Peninsula, Spain (5). In addition, red deer (*Cervus elaphus*) in several parts of Europe (6), African buffalo (*Syncerus caffer*) in South Africa (7), and wood bison (*Bison bison athabascae*) and wapiti (*Cervus elephus manitobensis*) in Canada (8) serve as maintenance hosts for infection in hunting estates and national parks. These various maintenance hosts act as sources of infection for domestic species, and in national parks, infection can spill over to other unique wildlife species including Iberian lynx (*Lynx pardinus*), lions (*Panthera leo*), leopard (*Panthera pardus*), and wild dogs (*Lycaon pictus*). Partial control has been achieved for some of these maintenance hosts by minimising contact with livestock, reducing the density of animals or banning artificial feeding that causes local high densities of animals (9–11). However, few if any of these control measures can be implemented for some protected species or where interference of a natural regulated ecosystem is deemed undesirable. For these reasons, the development and use of vaccines for control of TB in both domestic livestock and wild animals is very appealing.

Although no TB vaccines are currently registered for protection against TB in domestic livestock, there is renewed interest in their use from the realisation of the financial impact of bovine TB on animal health and trade, and due to the difficulty controlling the disease. In addition, the use of vaccines to control the TB in wildlife reservoirs of infection could be very valuable in limiting the spread of infection to domestic livestock and *M. bovis* bacille Calmette-Guérin (BCG) was registered for intramuscular administration to badgers in the UK in 2010. Evidence of the use of a vaccine to control a disease in wildlife has been shown from the success of using vaccination to control rabies in foxes in Europe (12). BCG vaccine is the only registered TB vaccine for humans and was developed by Calmette and Guérin from a strain of *M. bovis* originally isolated by Nocard from a case of tuberculous mastitis. Following serial passage of the strain on ox bile glycerine-potato medium for 230 passages, between 1908 and 1919, this variant strain was shown to be attenuated in animals and conferred resistance to challenge of animals with virulent *M. bovis* and *M. tuberculosis* [reviewed in (13)]. The strain was distributed to many countries in the 1920s and continuing passage in differing conditions produced a considerable number of daughter strains, with varying antigenic profiles (14).

Vaccination of humans commenced in 1921 and in a meta-analysis, vaccination of newborns and infants significantly reduced the risk of TB by an average of over 50%, although efficacy ranged from 0 to 80% (15). Many field trials of BCG vaccination of cattle were conducted in the first half of the twentieth century and the major caveats that restricted the use of TB vaccines in cattle were that protection was not complete and vaccination could sensitise animals to respond in traditional TB diagnostic tests. These problems can now be potentially overcome by using vaccination integrated with other control measures and use of diagnostic tests which can differentiate infected from vaccinated animals (DIVA tests). Currently, there is very large effort to develop improved TB vaccines for humans, by developing vaccines which may replace BCG or those that could boost immunity following initial vaccination with BCG (16). Research to develop improved TB vaccines for livestock is following a similar path. Information on the efficacy of BCG in animals can be of assistance in the development of these new generation vaccines for use in multiple species. For most wildlife species, however, vaccination efforts are restricted to the use of a single-shot vaccine since access to the same individuals in order to deliver a booster is unrealistic. The focus of this paper is to provide a review of the efficacy and safety of BCG vaccine in domestic livestock and wildlife to assist in optimising the use of BCG vaccine in animals as well as providing a guide for the development of improved TB vaccines. TB vaccines that do not use BCG are being studied for some applications, such as a heat-inactivated *M. bovis* whole-cell vaccine for wild boar, but these are not the focus of this review.

VACCINATION OF CATTLE

Historical Studies of BCG

Studies of BCG vaccine in cattle were first reported by Calmette and Guérin (17) and showed that relatively high doses of BCG (20 mg) could induce protection in cattle against experimental challenge with *M. bovis*. In the studies of Calmette and Guérin (18), intravenous challenge of control calves with virulent *M. bovis* resulted in severe generalised TB by 30–60 days. In contrast, the BCG vaccinates which were challenged remained healthy, but virulent *M. bovis* could be isolated from their bronchial lymph nodes when the animals were killed at 3–4 months post-challenge. A number of other researchers reported similar results in experimental challenge studies where BCG vaccination did not induce absolute immunity, but moderated the severity of the infection [reviewed in (19)]. A comparison of routes of vaccination with BCG showed that intravenous, intradermal and oral routes conferred some resistance to feeding milk containing large doses of virulent *M. bovis*, although not greater than that conferred with subcutaneous vaccination (20). Field studies of BCG vaccination of cattle using either a subcutaneous or intravenous route of vaccination showed variable results which may in part have been influenced by the duration and the potency of the exposure. Promising results were shown by Rankin (21) with 86% (37 of 43) non-vaccinates with tuberculous lesions compared to 33% (22 of 66) for the BCG vaccinates by 8–10 months post exposure. Watson (22) undertook a study over a longer duration where new-born calves were vaccinated with BCG subcutaneously (50–100 mg dose), fed pasteurised milk for 1–2 months, and then exposed to *M. bovis* through ingestion of raw milk from infected cows or co-habitation with infected cattle.

The study demonstrated that there was good resistance in calves compared to controls by 1 year post-exposure, but resistance declined steadily up to reproductive age where there was little evidence of protection. A number of uncontrolled trials were undertaken to determine whether BCG vaccination could clear TB infection in heavily infected herds. Some studies reported that BCG vaccination eliminated disease over 7 years (23, 24), while others found this approach reduced the skin test reactivity and resulted in newly introduced unvaccinated animals (n = 100) remaining skin test negative over a 5 year post-vaccination period. This approach was judged to be impractical and slow (25).

These early studies indicated that BCG could induce some protection against TB, although protection was not absolute, appeared to wane after 1–2 years and vaccination could induce positive reactivity in the tuberculin test. It was concluded that TB could be eradicated faster and more efficiently using "test and slaughter" control programmes than relying only on vaccination with BCG. However, it was considered that BCG vaccination could possibly have a role in disease control in countries where "test and slaughter" programmes could not be implemented due to economic or social reasons and a number of trials were conducted in Malawi in the 1970s for this purpose. Ellwood and Waddington (26) showed that the development of tuberculous lesions and progressive infection was less in BCG vaccinates following experimental *M. bovis* challenge compared to controls, providing encouragement to proceed with a field study. In the field trial, 3–12 month old calves were injected with 10^7 colony forming units (CFU) of BCG (Glaxo strain) and revaccinated 6 months later, while alternate calves in each herd were sham inoculated (27). When the animals were slaughtered and necropsied 5 years after the commencement of study, no significant differences could be found in the number of animals with tuberculous lesions, 36 of 204 (17.7%) in the vaccinates and 44 of 210 (21.0%) in the controls. The numbers of cattle which were bacteriologically positive and those with lesions at more than one site were also similar for the two groups.

Possible reasons for the failure to protect in the field trials could include administration of high doses of BCG (1–100 mg parenterally), very high level of *M. bovis* exposure, exposure of young calves to *M. bovis* through consumption of milk from infected cows prior to vaccination, lack of long-term protection, and prior sensitisation to environmental mycobacteria or helminths. In relation to the dose of BCG, Griffin et al. (28) demonstrated in deer that parenteral vaccination of a high dose of BCG (10^8 CFU) was less effective than doses of 10^4-10^7 CFU BCG, whereas higher doses of BCG in badgers appearred more efficacious (29). This suggests the optimal dose of BCG to use in any given species will probably need to be determined empirically. Informative meta-analysis of field trials in cattle have not been undertaken due to varying doses, vaccination routes and strains of BCG used, together with different methods to measure protection and varying levels of exposure to *M. bovis*. Furthermore, robustly controlled designs and statistical analyses of results were rarely undertaken and in most studies, the vaccinated and non-vaccinated animals were kept on separate farms.

Recent Studies to Assess Factors Affecting BCG Vaccine Efficacy in Cattle

Over the past 25 years, a large number of vaccination/challenge trials have been undertaken in cattle using harmonised models, allowing comparisons between varying studies with BCG tested alone or in comparative studies with other vaccines. Challenge models have focused on using a relatively low challenge dose of *M. bovis* (10^3-10^4 CFU) administered via endobronchial/intratracheal inoculation or by aerosol (30, 31). This has resulted in the development of tuberculous lesions mimicking those from the natural disease in the lower respiratory tract. Similar BCG strains have been used (initially Pasteur, then BCG Danish 1331) and protection assessed by quantitative gross, histopathological, and microbiological findings.

It is important to determine factors which influence the efficacy of BCG to optimise the use of the vaccine and **Table 1** summarises many of these factors. Results from a number of studies have shown that doses of 10^4-10^6 CFU of BCG administered parenterally induced equivalent protection (30, 32), while higher doses (10^8 CFU) were required to induce protection when BCG was administered orally (33, 34). When BCG vaccine was administered at optimal doses, protection induced by the subcutaneous or oral route was very similar, although an advantage from oral administration of BCG was slightly lower tuberculin skin test reactivity. Combinations of BCG by parenteral and mucosal routes has provided mixed results with no enhancement of protection observed when BCG was administered subcutaneously and orally on the same day (35), but a small enhancement in protection with simultaneous administration of BCG by subcutaneous and endobronchial routes (36). Pasteur and Danish strains of BCG induced similar protection, although the kinetics of the cellular immune response varied with the two strains (37, 38). Calves vaccinated subcutaneously with the Phipps strain of BCG had lower mean rank for the total number of tuberculous lesions following a high challenge dose of *M. bovis* (10^5 CFU) delivered by aerosol compared to controls, although this difference was not significant (46). Neonatal or very young calves were protected at least as well as older calves (39, 40). In one study, natural pre-sensitisation to environmental mycobacteria appeared to have an adverse effect on subsequent immunity induced by BCG vaccine, with less protection induced compared to that for two other attenuated *M. bovis* vaccines (41). While in another study, there was evidence that *M. avium* exposure induced partial protection against *M. bovis* infection, which could possibly mask subsequent immunity induced by BCG (42). Studies in guinea pigs and mice have provided additional information on the effects of pre-sensitisation with environmental mycobacteria where some strains of *M. avium* masked or blocked any protective effect induced by BCG vaccination, while other strains had no effect (47, 48). Studies in Northern Ireland indicated that co-infection of cattle with a liver fluke, *Fasciola hepatica*, and BCG resulted in a suppression of Th1 type immune responses to BCG, potentially affecting immunity induced by BCG vaccination (49). Vaccination of cattle with BCG 3 weeks after an experimental challenge with *M. bovis*, did not produce a beneficial effect,

TABLE 1 | Experimental challenge studies to assess factors affecting the efficacy of BCG vaccine for protection of cattle against TB.

Factor		Age of vaccination	BCG strain	Vaccine dose (CFU)	Vaccine route	*M. bovis* challenge	Measure of disease[†]	Assessment	References
								BCG vs. Control	
Dose of BCG	1.	5–6 months	Pasteur	6×10^4	S/C	I/T	Proportion with lesions	2/15 vs. 10/16*	(30)
		5–6 months	Pasteur	6×10^6	S/C	I/T	Proportion with lesions	4/15 vs. 10/16*	
	2.	5–6 months	Danish	2×10^5	S/C	E/B	Proportion with lung lesions	3/9 vs. 10/10*	(32)
		5–6 months	Danish	2×10^6	S/C	E/B	Proportion with lung lesions	2/9 vs. 10/10*	
	3.	6 months	Danish	2×10^7	Oral	I/T	Median total lesion score	5 (0, 8)[‡]vs. 11 (8, 13.5)*	(33)
		6 months	Danish	2×10^8	Oral	I/T	Median total lesion score	3.5 (0, 10.5) vs. 11 (8, 13.5)*	
	4.	6 months	Danish	1×10^6	Oral	I/T	Median LN lesion score	4 (1, 12) vs. 3.5 (0, 12)	(34)
		6 months	Danish	1×10^7	Oral	I/T	Median LN lesion score	4 (0, 12) vs. 3.5 (0, 12)	
		6 months	Danish	2×10^8	Oral	I/T	Median LN lesion score	1 (0, 9) vs. 3.5 (0, 12)*	
Route(s) of vaccination	1.	6 months	Danish	1×10^6	S/C	I/T	Median total lesion score	0 (0, 10) vs. 11 (8, 13.5)*	(33)
		6 months	Danish	2×10^8	Oral	I/T	Median total lesion score	3.5 (0, 10.5) vs. 11 (8, 13.5)*	
	2.	6 months	Pasteur	$10^6 + 10^9$	S/C + Oral	I/T	Median total lesion score	1 (0, 12) vs. 8 (0, 15)*	(35)
		6 months	Pasteur	10^6	S/C	I/T	Median total lesion score	1 (0, 8) vs. 8 (0, 15)*	
		6 months	Pasteur	10^9	Oral	I/T	Median total lesion score	0.5 (0, 7) vs. 8 (0, 15)*	
	3.	5–7 months	Danish	$5 \times 10^5 + 5 \times 10^5$	S/C + E/B	E/B	Median total lesion score	1 (0, 15) vs. 12 (0, 28)*	(36)
		5–7 months	Danish	1×10^6	S/C	E/B	Median total lesion score	3 (0, 15) vs. 12 (0, 28)	
		5–7 months	Danish	1×10^6	E/B	E/B	Median total lesion score	1 (0, 27) vs. 12 (0, 28)	
Strain of BCG	1.	6 months	Pasteur	10^6	S/C	I/T	Median total lesion score	6 (0, 21) vs. 13.5(4, 17)*	(37)
		6 months	Danish	10^6	S/C	I/T	Median total lesion score	7 (0, 13) vs. 13.5(4, 17)*	

(Continued)

TABLE 1 | Continued

Factor	Age of vaccination		BCG strain	Vaccine dose (CFU)	Vaccine route	*M. bovis* challenge	Measure of disease[†]	Assessment	References
								BCG vs. Control	
	2.	6 months	Pasteur	2×10^6	S/C	I/T	Median total lesion score	2 (0, 18) vs. 16 (10, 20)*	(38)
		6 months	Danish	2×10^6	S/C	I/T	Median total lesion score	8 (4, 11) vs. 16 (10, 20)*	
Neonatal vaccination	1.	8 h	Pasteur	10^6	S/C	I/T	Proportion with lesions	0/10 vs. 10/10*	(39)
		6 weeks	Pasteur	10^6	S/C	I/T	Proportion with lesions	1/9 vs. 10/10*	
	2.	1 day	Danish	10^6	S/C	I/N	Median total lesion score	2 (0, 4) vs. 13 (0, 30)*	(40)
Pre-sensitisation with *M. avium* spp.	1	5–6 months	Pasteur	10^5	S/C	I/T	Proportion with LN lesions	14/18 vs. 7/9	(41)
		5–6 months	Atten.1 *M. bovis*	10^6	S/C	I/T	Proportion with LN lesions	3/9 vs. 7/9*	
		5–6 months	Atten.2 *M. bovis*	10^6	S/C	I/T	Proportion with LN lesions	3/9 vs. 7/9*	
	2	4–6 months	*M. avium*	10^6	S/C	I/N	Median total lesion score	4 (0, 9) vs. 9 (0, 15)	(42)
Duration of protection and revaccination	1.	2–4 weeks	Danish	10^6	S/C	E/B (12 mths)	Median total lesion score	8 (0, 16) vs. 16 (0, 38)*	(43)
						E/B (24 mths)	Median total lesion score	8 (5, 17) vs. 10 (8, 17)	
	2.	2–4 weeks	Danish	3×10^5	S/C	E/B (2.5 yrs)	Median total lesion score	7.5 (0, 17) vs. 10 (3, 17)	(44)
		2–4 weeks and 2 years	Danish	3×10^5	S/C	E/B (2.5 yrs)	Median total lesion score	4 (0, 10) vs. 10 (3, 17)*	
Vaccination pre- or post-challenge	1.	5–6 months	Danish	1×10^6 (pre-challenge)	S/C	E/B	Proportion with LN lesions	4/12 vs. 10/12*	(45)
				1×10^6 (3wks post-challenge)	S/C	E/B	Proportion with LN lesions	11/12 vs. 10/12	

[†] *Measure of protection was standardized where possible and in some cases, recalculated from original data.*
[‡] *Median (range); *P < 0.05.*
CFU, Colony Forming Unit; S/C, Subcutaneous; I/T, Intratracheal; E/B, Endobronchial; I/N, Intranasal.
Atten. 1 M. bovis and Atten. 2 M. bovis: Two attenuated strains of M. bovis.

nor increased tuberculous pathology (45). Protection against experimental challenge was shown to be effective at ≤12 months post-vaccination, but had waned by 24 months post-vaccination (43). Together these studies suggest that immunity wanes between 1 and 2 years post-vaccination when protection is measured in a stringent *M. bovis* challenge model.

Two studies report the effect of revaccination with BCG. In the first study, calves vaccinated within 8 h of birth or at 6 weeks of age showed a high level of protection against *M. bovis*, while those vaccinated within 8 h of birth and revaccinated at 6 weeks of age had reduced protection (39). The revaccinated calves with the lowest level of protection had the strongest antigen-specific IFN-γ responses post-initial vaccination, suggesting that revaccination had induced an inappropriate immune response. In neonatal calves, antigen-specific IFN-γ responses remain at elevated levels for longer than those seen in older calves, possibly due to a more active BCG infection and BCG revaccination of young calves may be contra-indicated. In contrast, calves vaccinated with BCG at 2–4 weeks of age and revaccinated at 2 years of age when immunity had waned, showed a significant level of protection when challenged 6 months later, while those receiving only the initial vaccine dose were not protected when challenged at the same time (44).

In the past decade a number of field BCG vaccination trials or experiments have been undertaken under natural transmission (in contact) conditions and have provided insights into the effectiveness of BCG vaccine under different levels of disease prevalence over varying time periods (**Table 2**). The field experiments in Mexico (50) and Ethiopia (51, 52) involved the exposure of vaccinated and non-vaccinated calves to herds of cows which had reactor rates of 40% in the Mexican experiment and 100% in the two experiments in Ethiopia. In the Mexican experiment, vaccination induced a significant level of protection against TB and the vaccine efficacy was estimated to be 59.4%. The level of exposure in the experiments in Ethiopia was very high with ∼85% of the non-vaccinated calves developing tuberculous lesions. Despite this high level of exposure, the vaccine efficacy in the first experiment was considered to be similar to that in the Mexican experiment and there were significantly fewer vaccinated animals with lesions and culture positive for *M. bovis* as well as significantly more vaccinated animals that would have passed slaughterhouse meat inspection than that for the controls (51). The vaccine efficacy in the second experiment conducted in Ethiopia when measured by comparing lesioned, culture or histology-positive animals in the BCG-vaccinated group with naïve controls was relatively low (around 30%) (52). However, in this last experiment, the severity of pathology and dissemination of *M. bovis* was significantly lower in the vaccinated, infected animals compared to that for the non-vaccinated animals, which could relate to a lower ability to transmit disease (onward transmission). The difference between the two Ethiopian experiments was attributed to a higher prevalence of overt clinical signs of TB in the infected herd in the second experiment.

A large field trial was undertaken in New Zealand to evaluate the efficacy of BCG vaccine administered orally (53). Free-ranging, vaccinated and non-vaccinated cattle were stocked at low densities and were naturally exposed to *M. bovis* for periods of 1–4 years from tuberculin reactor cattle (reactor herd prevalence of 5–10%) and a wildlife reservoir of infection (brushtail possums). BCG vaccine was administered orally to cattle in an attempt to reduce tuberculin skin test reactivity. This trial included 1,286 cattle and at slaughter the prevalence of infection was 4.8% among vaccinates and 11.9% in non-vaccinates. The overall vaccine efficacy was estimated to be 67.4%, but higher for those killed within 2 years post-vaccination (77.4%). Vaccination also appeared to slow the progression of TB, with infected vaccinates more likely to have no visible lesions and less likely to have a high lesion score.

In summary, the field experiments and trials have shown that BCG vaccination can markedly reduce the number of cattle infected with *M. bovis*, which is different to that seen in the experimental challenge trials where vaccination only reduced the severity of the disease. However, an exception was in the field trial when there was a very high exposure to *M. bovis* (52). With the longer exposure periods, there appeared to be a waning of immunity after 2 years (53).

Differentiating Infected From Vaccinated Animals (DIVA) Tests

It is well-established that vaccination with BCG can compromise the interpretation of the tuberculin skin test, which serves as the primary surveillance test for "test and slaughter" bovine TB control strategies. Using the single intradermal comparative cervical test, 80% of BCG-vaccinated calves were shown to react in the tuberculin skin test at 6 months post-vaccination, but decreasing to 10–20% by 9 months post-vaccination (54) and in a another study, the maximum skin test reactivity was observed after 5 weeks, but disappeared completely by 18 months after vaccination (55). Positive responses were also observed in the caudal fold skin test at 6 months after BCG vaccination compared to that for a corresponding control group, but there were no differences between the groups by 12 months after vaccination (44). DIVA tests will be required for countries intending to use BCG vaccination alongside conventional "test and slaughter" control strategies. DIVA tests have now been developed using antigens from the *M. tuberculosis* complex which are not expressed or secreted by BCG and can be used instead of bovine PPD in the whole blood IFN-γ or skin tests. Two of the antigens used in the DIVA tests are the ESAT-6 and CFP10 proteins, which are encoded in the RD1 region of *M. tuberculosis* and *M. bovis*, but not in BCG, which has lost this region of its genome (56–58), and a third antigen, Rv3615c, which is not located in the RD1 region, but its secretion is dependent on the esx-1 secretion system located in the RD1 region (59). A recent evaluation of the whole blood IFN-γ test incorporating ESAT-6, CFP10, and Rv3615c indicated that the sensitivity was similar to that with the comparative tuberculin readout using avian and bovine PPD. When tested in non-infected animals, both the DIVA and tuberculin readouts gave similar specificities of between 97 and 99%. The relative specificity of the DIVA blood test was also high (95%) in BCG-vaccinated cattle and was significantly greater than that observed for the tuberculin readout

TABLE 2 | Recent field trials to assess the efficacy of BCG for protection of cattle against TB.

Trial	Country	BCG strain	Vaccine dose	Vaccine route	Age at vaccination	Source of infection	In-contact (exposure) time	Measurement of disease	Assessment	References
									BCG vs. Control	
1.	Mexico	Tokyo	10^6	S/C	1–2 weeks	Infected herd (40% herd reactor prevalence)	10 months	Proportion positive in three tests: PPD skin test, PPD IFN-γ test, ESAT-6/CFP10 IFN-γ test	6/65 vs. 15/66* (9.2% vs. 22.8%) 59.4% efficacy	(50)
2.	Ethopia	Danish	10^6	S/C	2 weeks	Infected herd (100% herd reactor prevalence)	10–23 months	Proportion with lesions	5/13 vs. 12/14* (39% vs. 86%)	(51)
								Mean total pathology score (95% CI)	4.6 vs. 14.1* (0, 10.5) vs. (2.5, 24.6)	
3.	Ethopia	Danish	10^6	S/C	2 weeks	Infected herd (100% herd reactor prevalence)	1 year	Proportion with lesions	15/23 vs. 22/26 (65% vs. 85%)	(52)
								Mean total pathology score (95% CI)	4.0 (1.8, 6.1) vs. 7.8* 2.5, 13.1)	
4.	New Zealand	Danish	10^8	Oral	6–30 months	Infected herd and wildlife (5–10% herd reactor prevalence)	1–4 years	Proportion with TB lesions and/or *M. bovis* cultured	31/644 vs. 63/531* (4.8% vs. 11.9%) 67.4% efficacy	(53)

*CFU, Colony Forming Units; S/C, Subcutaneous; CI Confidence interval; IFN-γ, Gamma-interferon; PPD, Purified protein derivative *P < 0.05.*

(71%) (60). One scenario to use the DIVA blood test would be to re-test tuberculin-positive cattle; alternatively, it is also possible to use these antigens in a skin test rather than the IFN-γ test. The DIVA skin test in cattle has now been shown to have a high sensitivity for *M. bovis*-infected cattle, to a similar level than that for the comparative cervical skin test in non-vaccinated cattle while not compromised by vaccination with BCG or with vaccines against Johne's disease (61, 62).

VACCINATION OF GOATS

TB infection of goats is caused by *M. bovis* or *M. caprae* and in the natural disease lesions are predominantly found in the lungs and associated lymph nodes, indicating an aerosol route of infection (63). The disease is responsible for economic losses in endemic areas and infected goats may be a source of TB for cattle or humans. Caprine TB is present in a number of European countries, but currently there are no caprine TB control campaigns in the European Union. To determine protective efficacy of vaccines, gross and microscopic lesions have been assessed by qualitative and quantitative analyses, together with mycobacterial culture from lung-associated lymph nodes. The precise determination of the total lung lesion burden related to total lung volume has been achieved using multi-detector computed tomography (64).

BCG Danish vaccine administered subcutaneously at a dose of 5×10^5 CFU was shown to be safe and no shedding of BCG was detected in the faeces of vaccinated kids or in the milk of vaccinated, lactating goats (65). BCG was isolated from a lymph node draining the site of vaccination from one kid at 8 weeks post-vaccination, but not from any goats at 24 weeks post-vaccination. A single dose of BCG vaccine administered subcutaneously to goats was shown to significantly induce protection against an endobronchial challenge with *M. caprae*, with reductions in pulmonary pathology and bacterial load. Vaccination with BCG appeared to prevent haematogenous dissemination of mycobacteria with extra-thoracic TB lesions only found in non-vaccinated goats (66). A comparison of parenterally administered BCG and heat-inactivated *M. bovis* vaccines showed that both vaccines provided similar levels of protection against a *M. caprae* experimental challenge, with a reduction in the volume of thoracic TB lesions and extra-pulmonary lesions compared to non-vaccinates (67). Use of mycobacterial DIVA reagents, ESAT-6 and CFP10, in the IFN-γ test was able to differentiate TB-infected from BCG-vaccinated goats. A field BCG vaccination trial was recently undertaken in a herd of goats infected with *M. caprae* (68). Twenty-three goat kids were vaccinated subcutaneously with 10^5 CFU of BCG Danish, with a further 22 kids serving as non-vaccinated controls. Two months later, the kids were mixed with a herd of goats which had a TB reactor rate of 79%. Sixteen months later, all trial goats were killed and necropsied. Vaccination significantly reduced the number of animals with TB lesions compared to that for non-vaccinates (35 and 77% respectively; representing a vaccine efficacy of 53%) and when extrapulmonary cases were considered, the reduction were even higher (17 and 68%, respectively; vaccine efficacy of 75%). Vaccination has been seen as a valuable long-term control prospect, reducing the TB prevalence prior to starting a test and slaughter eradication programme which would reduce economic costs for producers and the public sector.

VACCINATION OF SHEEP

Sheep have traditional been considered a rare host for the *M. tuberculosis* complex, but can be part of a multi-species system which may maintain TB in a region, at least in mixed farms where sheep cohabit with TB-infected cattle and/or goats (69). In a trial where lambs were vaccinated parenterally with BCG Danish and subsequently challenged endobronchially with *M. caprae*, the vaccinated lambs had a significant reduction in gross lesions compared to the non-vaccinated controls (70). All challenged lambs developed gross lesions in the respiratory system, which were similar to those observed in goats experimentally challenged with *M. caprae* at a similar dose.

VACCINATION OF DEER

TB in farmed and feral deer is predominantly caused by *M. bovis*, and in the USA and Spain, feral deer also serve as a wildlife reservoir of *M. bovis* infection, acting as a source of infection for domestic livestock (6, 10). Deer serve as important domestic livestock species, farmed predominantly for the production of venison, while feral deer are valued for hunting. Tuberculous lesions are commonly described as liquefied or abscess-like in contrast to the caseous nature of the lesions seen in cattle and goats (71, 72). The most frequent site of the tuberculous lesions is in the retropharyngeal lymph nodes, followed by lesions in the lungs and associated lymph nodes as well as in the mesenteric lymph nodes (73). BCG vaccination studies of deer has been undertaken to assess whether vaccination could be an effective method of protecting farmed deer from TB and in feral deer to prevent reinfection back into cattle herds.

Studies of BCG vaccine in red deer have shown that a single dose of BCG administered subcutaneously to 3 month old deer could reduce disease severity, while revaccinating deer at intervals of 8–16 weeks intervals induced protection against infection, but not at an interval of 43 weeks (74). Increasing the time period between booster dose and *M. bovis* challenge from 6 to 26 or 52 weeks had no significant effect on protection. Two doses of 10^4-10^7 CFU of BCG induced protection against TB, but less with a dose of 10^8 CFU and killed BCG in a mineral-in-oil adjuvant induced no protection (28). A study in red deer in Spain compared oral administration of BCG Danish (10^8 CFU) with oral administration of heat-inactivated *M. bovis*, 10^7 bacilli, followed by an experimental challenge with *M. bovis* (75). Only the heat-inactivated vaccine induced a significant reduction in lesion pathology compared to that for the non-vaccinates, however, the results were constrained by very small group sizes (5 animals/group). Neither vaccine induced a bovine PPD IFN-γ response post-vaccination. Parenteral BCG

administered at a dose of 10^6 CFU or oral BCG at 10^8 CFU induced a similar degree of protection in white-tailed deer (76). Parenteral vaccination with either BCG Danish or Pasteur resulted in decreased disease severity, without sterile immunity (77). A booster dose 6 weeks later did not raise the level of protection (78). BCG was shown to persist for 3–9 months in lymphoid tissues of deer vaccinated parenterally or orally (79). Evidence has been provided of transmission of BCG from parenterally vaccinated deer to in-contact, non-vaccinated deer (77, 79). In another study, deer orally vaccinated with 10^9 CFU BCG Danish were housed with non-vaccinated deer for 27 weeks. There was immunological evidence of transmission of BCG to the non-vaccinated animals, but no BCG could be isolated from the tissues of either group of animals when killed 27 weeks after vaccination (80). There was no evidence (immunologically or by culture) of transmission of BCG to the cattle which were exposed to the room previously occupied by the vaccinated deer. Complications can occur with the delivery of oral vaccine baits to feral deer as the provision of supplementary feed to feral deer can lead to large numbers of deer congregating together resulting in the spread of TB (81), also there are concerns about non-target uptake of live vaccine baits, particularly by cattle. Simulation modelling has examined the potential role that vaccination could play in control programmes to minimise cattle herd breakdowns (82). Vaccination of 50–90% of susceptible deer within a 5 km radius of cattle farms was predicted to result in a 95% probability of having no cattle herd breakdowns in 15–18 years.

BCG VACCINATION OF WILDLIFE

The requirements for a vaccine for wildlife differ to those for domestic livestock in that preferentially, the vaccine would be self-administered via an oral route and animals would only receive a single vaccination. Vaccination should prevent the spread of infection to other wildlife or livestock, but complete protection against infection would not be necessary. Recent studies in multiple wildlife species have shown that BCG vaccine can fulfil these requirements and provide protection against TB (**Table 3**).

Vaccination of Brushtail Possums

The brushtail possum is the major wildlife reservoir of *M. bovis* infection in New Zealand as well as declared as a noxious pest. Possums are highly susceptible to *M. bovis* infection and lesions are found predominantly in the lungs and superficial lymph nodes. Culling of possums by trapping and poisoning has been a major contributor to the dramatic reduction in the numbers of infected cattle over the past 20 years (11). Vaccination of possums against TB has the potential to be an effective TB control measure when it is not suitable to cull possums such as near urban areas.

BCG vaccination of possums via a number of different routes including subcutaneous, intranasal and oral have induced a significant level of protection against experimental *M. bovis* challenge by intratracheal and aerosol routes (83, 93). Oral administration of BCG via baits would be the preferred route of administration of BCG vaccine to wild possums, but it was shown that direct administration of BCG intragastrically was less effective compared to administered by the same route and mixed with a drug to reduce gastric acidity or when administered intraduodenally (94, 95). To increase the efficacy of oral administered BCG vaccine, the BCG bacilli were encapsulated in a lipid matrix which protected the bacteria from degradation in the acidic stomach environment, resulting in improved protection against a *M. bovis* challenge as well as increasing shelf life of the vaccine in the field (83, 96). Vaccine-induced immunity was shown to wane between 6 and 12 months post-vaccination following oral vaccination and there were no differences between BCG doses of 10^7 and 10^8 CFU or between Danish and Pasteur strains of BCG (97). A more recent study indicated that protection against an experimental *M. bovis* infection extended out to

TABLE 3 | Summary of BCG vaccine efficacy studies in wildlife.

Species/Country	Route†	Challenge type	Vaccine efficacy‡	Notes/Particular issues	Key references
Bushtail possum/New Zealand	O,M, P	Aerosol,	+	High vaccine cost compared to that for poisons	(83)
		natural exposure	+		(84)
European badger/ UK, Ireland	O,M, P	Endobronchial,	+	Parenteral vaccine licensed (BadgerBCG). For	(29, 85)
		Natural exposure	+	an oral vaccine: demonstration of consistent protection	(86–88)
White-tailed deer/ USA	O,P	Intratonsilar	+	BCG persistence in tissues, bans on supplementary baiting, non-target bait uptake	(76, 77)
Eurasian wild boar/Spain	O	Oral	+	Non-target bait uptake	(89)
		Natural exposure	±	Regulatory issues	(90)
Ferret/New Zealand	O,P	Oral	±	Rarely maintenance host for *M. bovis*	(91, 92)
African buffalo/ South Africa	P	Intratonsilar	–	Practicality of vaccine delivery in the field	(7)

†*Vaccination route, O, oral; M, other mucosal; P, parenteral.*

‡*Vaccine efficacy, + protection, ± partial protection, – no protection.*

28 months post-vaccination (98). BCG bacilli were shown to be stable in the lipid matrix for 7 weeks under room temperature conditions and 3–5 weeks under field conditions in a forest/pasture habitat, when maintained in weather-proof, bait-delivery sachets. Furthermore, uptake of oral bait placebo vaccines was shown to be high with 85–100% of wild possums accessing baits at bait densities of 40–80 sachets/hectare (96). Possums consuming oral bait BCG vaccine, containing 10^8 CFU of BCG, displayed no adverse clinical signs, but shed relatively low concentrations of BCG in their faeces, 10^2-10^4 CFU/g faeces, for up to a week and BCG could be isolated from their mesenteric lymph nodes for up to 8 weeks post-vaccination (99).

Two field trials have been undertaken in possums to determine efficacy of BCG vaccine against natural exposure to *M. bovis* infection. In the first trial, BCG vaccine was administered intranasally and intraconjunctivally (total dose of 10^6 CFU of BCG Pasteur) to possums trapped in the field, with an equivalent number left non-vaccinated. After vaccination, the animals were released back into the field site, which was endemic for TB in wildlife (100). The animals were trapped, examined for clinical TB and released again every 2 months. Two years after the start of the study, possums were recaptured, killed and examined for TB lesions. Vaccination significantly reduced the proportion of possums infected with *M. bovis* (4/149 for vaccinates and 13/151 for non-vaccinates), with a vaccine efficacy of 69% for prevention of TB. The second field study was of a similar design, but with BCG vaccine administered orally in a lipid matrix (total dose 10^8 CFU BCG Danish). Again, there was a significant reduction in the proportion of infected possums in vaccinates (1/51) compared to that for the non-vaccinates (12/71), with a vaccine efficacy of 95% for prevention of TB (84). In contrast to the experimental challenge studies, protection against natural exposure to *M. bovis* vaccination resulted in protection against infection. The major constraint for the use of BCG vaccine in possums in New Zealand is cost of the vaccine compared to that for poisons, particularly when possums are considered as a noxious pest.

Vaccination of Badgers

The European badger is the major wildlife reservoir of *M. bovis* infection in Great Britain due to their relative abundance and ecology, the prevalence of infection and presentation of TB pathology compared to other sylvatic species (101, 102). Options for preventing the transmission of *M. bovis* from infected badgers to cattle are limited to minimising the potential for contact between them (biosecurity), reducing the number and density of infected badgers via selective and non-selective culling, and vaccination [reviewed in (103)]. Badgers are protected by law in the UK and Ireland which limits the public acceptability and practicality of culling and for disease control, and culling of badgers in England and Ireland has sometimes delivered conflicting results that likely reflect subtle differences in the epidemiology of the disease locally (104). Vaccination of badgers against TB has the potential to be an effective TB control measure, especially in combination with other control measures (105) and considerable progress has been made in testing the efficacy of BCG vaccine in badgers.

BCG vaccine has been administered to badgers via a variety of routes, including subcutaneous, intramuscular and mucosal (conjunctival and oral) and vaccination by all these routes has induced significant protection against experimental endobronchial challenge with *M. bovis* [reviewed in (103)]. The use of BCG to vaccinate badgers against TB in the UK by the intramuscular route was licensed by the UK Competent Authority (Veterinary Medicines Directorate) in 2010 as BadgerBCG and is available for use by veterinarians and trained lay vaccinators under prescription from a veterinarian. Licensing of BadgerBCG required evidence of vaccine safety and efficacy and laboratory and field studies showed that vaccination of badgers by injection with BCG was both safe and significantly reduced lesions of TB caused by *M. bovis* (29, 106). Protection was incomplete, in that *M. bovis* infection of vaccinated badgers still produced either visible pathology or *M. bovis* was isolated from organs at necropsy. Results from a 4-year field study of BCG in wild badgers were consistent with the direct protective effect of BCG observed in experimental studies. Individual badgers that initially tested negative to a panel of diagnostic tests, presumed uninfected, were significantly less likely to subsequently test positive to serological and immunological tests for TB following vaccination, compared to non-vaccinated control animals (86, 107). Furthermore, non-vaccinated cubs captured in vaccinated social groups were significantly less likely to test positive to TB when more members of their group had been previously vaccinated. The most plausible explanation for this result is that BCG had caused a herd immunity effect, with the rate of *M. bovis* transmission being more effectively reduced in social groups where a higher proportion of animals had been vaccinated.

A practical limitation to the extensive use of BadgerBCG is the need to trap badgers before the vaccine can be injected and the use of an oral bait delivery system would be advantageous. BCG has been incorporated in a wide variety of baits, including encapsulation in the same lipid matrix used to deliver BCG orally to possums. Administration of BCG orally to captive badgers, either directly to the back of the throat, or indirectly via ingested bait has been shown to protect badgers against experimental challenge with *M. bovis* and there was no difference in the levels of protection induced by Pasteur and Danish sub-strains of BCG (85, 108). To assess the vaccine safety, badgers were orally dosed with 10^9 CFU of BCG, followed 14 days later by a single oral dose of 10^7 CFU BCG (109). No adverse physical effects were observed, nor effects on the social behaviour and feeding habits of the vaccinated animals. BCG was cultured from the faeces of two of nine vaccinated animals (10^2 CFU/g) ∼48 h after the higher dose of BCG was administered and by one of the nine vaccinated animal (80 CFU/g) ∼24 h after receiving the lower dose of BCG. No evidence was found for the transmission of BCG to non-vaccinated, sentinel, badgers housed with the vaccinated animals despite the occasional excretion of BCG in faeces. The target dose of BCG for the oral vaccination of badgers is yet to be defined.

A field trial was recently completed in Ireland that provided the first estimate of oral BCG efficacy under field conditions (87). Lipid-encapsulated BCG was delivered to the back of the

throat of anaesthetised badgers, whilst other badgers received only the lipid as placebo. The study area was divided into three equally representative zones with different proportions (0, 50, and 100%) of the badger population in each zone being vaccinated with either BCG or placebo. Attempts were made to capture badgers every 6 months and between the first two capture periods the vaccine efficacy was estimated to be 36%, while it was 84% for capture periods 3–6. Among the vaccinated badgers that seroconverted, the median time to seroconversion (413 days) was significantly longer when compared with non-vaccinated animals (230 days). In addition, there was a significant reduction in the proportion of animals presenting with *M. bovis* culture confirmed lesions in the fully (100%) vaccinated zone (9%), compared with the non-vaccinated (0%) zone (26%).

Vaccination of Wild Boar

Wild boar serve as the main wildlife reservoir of the *M. tuberculosis* complex (MTC) in the Mediterranean regions of the Iberian Peninsula, Spain and TB prevalence in wild boar has been associated with TB occurrence on cattle farms (110, 111). Wild boar are widespread in Eurasia and can be found in high densities, particularly on hunting estates (112). These animals are highly susceptible to MTC infection and lesions are most frequently found in the mandibular lymph nodes, although generalised disease is often seen, with involvement of the lungs and thoracic lymph nodes (113). Direct contact between wild boar and other species is thought to be very rare in Mediterranean habitats and inter-species transmission of MTC involving wild boar is considered to occur indirectly at locations such as waterholes (114). Although, transmission of TB between wild boar and cattle could be minimised by culling of wild boar and preventing inter-species contact, vaccination could be a more cost-effective and sustainable disease control measure.

Oral vaccination with BCG Danish (10^6 CFU/dose) vaccine has produced significant protection (70–80% lesion score reduction) in laboratory challenge trials (89, 115). The focus has been to vaccinate piglets as they are less likely to be infected and can be targeted by appropriate timing of bait delivery and with the use of a patented bait delivery system that reduces uptake by non-target species and excludes adult boar (116). In a recent safety study, wild boar were dosed with an oral bait containing 10^6 CFU of BCG and groups of vaccinated animals were killed at 1, 3, 5, and 9 months post-vaccination (117). No adverse clinical signs were observed and tissues collected from the animals were culture negative for BCG. A field trial undertaken from 2012 to 2016 tested the uptake rates and efficacy of orally delivered BCG and heat-inactivated *M. bovis* vaccines in high prevalence settings (40–80% wild boar infection prevalence) in Montes de Toledo, Spain (90). The two vaccines were tested at different sites, one managed and one natural (or unmanaged) site for each vaccine, with an additional 15 non-vaccinated control sites. Vaccine baits were deployed using selected piglet feeders and the uptake rates were 50–74% in natural sites and 89–92% in managed sites. A significant reduction in the TB prevalence was only seen from one vaccinated site: heat-inactivated *M. bovis* vaccine in the managed site; with a 34% reduction in the prevalence of animals with lesions. A limitation of the study was that vaccines were deployed at different sites and efficacy was measured by the change in TB lesion prevalence compared to time zero.

Vaccination of Ferrets

In New Zealand, ferrets (*Mustela furo*) can become infected with *M. bovis* via feeding on tuberculous carcasses, particularly possums and potentially can become a source of infection for other wildlife or cattle (118). In most circumstances, ferrets are simply spill-over hosts and as yet, there is no confirmation that ferrets act as true maintenance hosts in New Zealand. Rather, ferrets could be characterised as extended spill-over hosts in which *M. bovis* infection originally acquired from possums could occasionally cycle within a ferret population before disappearing (119). Vaccination has been considered as a possible control measure for ferrets and in the first of two vaccination trials, ferrets orally vaccinated with BCG incorporated into dietary meat were partially protected against oral challenge with virulent *M. bovis* (91). In the second trial, vaccination of ferrets with BCG by the subcutaneous route resulted in reduced severity of disease following experimental infection with *M. bovis* (92).

Vaccination of African Buffalo

M. bovis infection is currently endemic in the Greater Kruger National Park Complex and the Hluhluwe-iMfolozi Park (120, 121), as well as in several private farms and conservancies in South Africa (122). African buffaloes are likely to be major maintenance hosts of TB (123) and play an important role in spill-over infection to other wildlife species, and of particular importance is spread of infection to predators (lions), large browsers (white rhino) and other co-located species such as kudu, baboons, and warthogs (124). In addition, there has been "spill-back" to domestic cattle (125). As "test and cull" is not a viable option for free-ranging buffaloes due to logistical impracticality and the animals' extensive geographical range, vaccination remains the only realistic alternative.

A preliminary vaccine trial was undertaken in semi-free-range buffalo to assess the efficacy of BCG vaccine. Two doses of BCG were administered subcutaneously (10^7 CFU of BCG) and the buffaloes were challenged with virulent *M. bovis* via the intratonsilar route. The study did not reveal significant differences in the number of lesioned animals between the vaccinated and control groups (7). There were various contributing factors which could have played a role in the perceived negative results such as the age of vaccinated animals with the majority being older than 12 months at the start of the study, the route of vaccine application, challenge dose, exposure to non-tuberculous mycobacteria and stress on the animals with the grazing limitations. Future studies should aim to determine if BCG vaccination could reduce TB in vaccinated herds compared to non-vaccinated herds by targeting buffaloes <12 months old and monitoring over a period of 5–10 years in order to determine true disease status. If successful, vaccination could have a positive

cascading effect, reducing *M. bovis* disease rates in other animal species. The available data does not suggest any risk to "off-target" species from BCG delivery, which reduces the ethical barriers to implementation.

SAFETY OF BCG VACCINE IN TARGET AND NON-TARGET SPECIES

BCG vaccine is one of the most widely used human vaccines, with 100 million children receiving the vaccine annually and remains one of the safest vaccines available. Reports of adverse reactions arising from BCG vaccination of children are relatively uncommon and a review of reactions to BCG vaccine in humans and animals has recently been provided by Murphy et al. (126). More severe reactions to BCG vaccine in humans were often the result of vaccination of immune-compromised individuals and factors influencing the development of adverse reactions included the potency and dose of the vaccine strain, route of delivery, age and immune status of the host and skill of the operator administering the vaccine. The most common reactions were local and regional reactions, which were generally self-limiting where suppurative lymphadenitis and abscesses were the most frequent occurring reactions.

BCG vaccine has been tested in a large number of animal species (**Table 4**) and relatively minor adverse clinical signs have been observed in some cases. In cattle, Francis (19) described local lesions arising following subcutaneous administration of large doses of BCG, similar to those seen with inoculation of large doses of dead bacilli, but no progressive lesions were produced and bacilli were gradually eliminated from the body. Repeat passaging of BCG vaccine in animal species is still to be undertaken to ensure no reversion to virulence, but the evidence from its use in humans for nearly a century has emphasised the safety of the vaccine.

Local abscesses or nodules have been observed following subcutaneous injections of BCG in a number of other animal species and these resolved relatively quickly (**Table 4**). No adverse effects have been observed after oral administration of BCG in animals other than cervical lymphadenitis observed in mice (127), similar to a reaction observed occasionally in young vaccinated children (126). Following oral dosing with BCG of possums and badgers, transient shedding of low numbers of BCG in faeces was observed (99, 109). Transmission of BCG from vaccinated animals to in-contact non-vaccinates has only been recorded in white-tailed deer (79). There is a risk that distribution of oral baits containing BCG for wildlife could lead to uptake by non-target animal species such as cattle, resulting in a subsequent positive tuberculin skin test response and therefore special care with regards to bait distribution is essential. The chance of cattle becoming infected with BCG from faecal contamination of pasture or feed from vaccinated wildlife would be very rare as tuberculin skin test reactivity following oral administration of BCG to cattle has only been recorded with high doses of BCG ($\geq 10^7$ CFU) (34). Similar to the situation in humans, BCG vaccine is considered to be a safe vaccine in all animal species tested.

TABLE 4 | Studies of safety of BCG vaccine of different strains in target and non-target animal species.

Animal species	BCG strain	Adverse clinical signs	Key references
Mouse	Pasteur	Cervical lymphadentitis—oral, None—S/C	(127)
Hamster	Tice	Pleural reaction—I/P high dose	(128)
Guinea pig	Tice	None—I/D	(129)
Rabbit	Phipps	Local abscess—I/D	(130)
Dog	Tice	Mild pleural reaction—I/P	(128)
Monkey	Pasteur	Axilliary lymphadenitis- high dose I/D	(131)
		Local draining abscess—medium dose S/C	
Sheep	Danish	None—S/C	(70)
Horse	Pasteur	None—intralesion injection	(132)
Goat	Danish	None—S/C, no shedding in milk or faeces	(65)
Cattle	Pasteur	Local swelling at injection site—S/C	(19)
White-tailed deer	Danish	None—S/C, Oral, BCG persisted in draining LNs (12 months), transmission to in-contacts	(79, 80)
Red deer	Pasteur	None—S/C, persisted in draining LNs (14 wks)	(133)
Possum	Danish	None—Oral, shedding in faeces (1 wk), persisted in mesenteric LNs (8 wks)	(99)
	Pasteur	None—Aerosol, S/C	(93)
Badger	Danish	None—Oral, single and repeat dosing, shedding in faeces (48 h)	(109)
	Danish	I/Mus, S/C, single, and repeat injection, local swelling at injection site	(106)
Wild boar	Danish	None—low dose oral, BCG not re-isolated	(117)
Ferret	Pasteur	None—Oral, S/C	(91, 92)
African buffalo	Pasteur	None—S/C	(7)

Vaccination route: I/D, intradermal; I/M, Intramuscular; I/P, Intrapleural; S/C, Subcutaneous.

CONCLUSIONS

Experimental challenge studies in domestic livestock including cattle, goats, sheep and farmed deer have demonstrated that BCG vaccination can moderate the severity of the disease, while field trials in cattle and goats have indicated that vaccination can also reduce infection. No single vaccine has been shown to be better than BCG in cattle, although combinations of BCG with various subunit TB vaccines have produced encouraging results and could have application in the future [reviewed in (2, 134)]. Vaccination of cattle with BCG would have greatest application in countries where "test and slaughter" strategies are not affordable or socially acceptable and in this situation, BCG could play a role in reducing the spread of bovine TB. It is well-recognised in humans that BCG confers some non-specific protective effects against other pathogens (135), but this has yet to be evaluated in cattle. Improvement in general health of animals *per se* and/or increased productivity post-BCG vaccination could potentially have benefits in developing countries. Strategic use of BCG vaccine for livestock could also be implemented in regions where wildlife serve as reservoirs of infection, particularly where it is not feasible to contain the spread of infection from wildlife. In these situations, DIVA tests, particularly skin tests utilising specific *M. bovis* antigens, could be used in livestock in association with vaccination to allow vaccination to be integrated with "test and slaughter" control measures.

A number of recommendations can be made from the experimental challenge and field experiments in cattle. Calves should be vaccinated with BCG as young as possible, optimally by 2–4 weeks of age, at doses of 10^5-10^6 CFU parenterally or 10^8 CFU orally and no differences have been detected in protection induced by two of the most commonly used BCG strains, Pasteur and Danish. Protection has been shown to wane between 1 and 2 years post-vaccination and revaccination is recommended every 1–2 years to maintain levels of immunity. BCG vaccine has been shown to be safe in cattle and vaccination of cattle pre-infected with *M. bovis* is not likely to exacerbate or cure infection. Vaccination is likely to produce false reactions in traditional TB diagnostic tests in the first 12 months post-vaccination and as protection induced by BCG is not complete, DIVA tests should be used if "test and slaughter" control policies are in place. It would be preferable to use BCG vaccination in association with other TB control measures such as minimising the chance of early exposure to *M. bovis* by feeding young calves with colostrum or milk from non-reactor cattle or with heated milk, segregating reactor and non-reactor cattle into separate herds and keeping vaccinated calves with the non-reactor animals.

The field testing of BCG vaccine in possums and badgers administered via oral or parenteral routes have resulted in the induction of significant reductions in infection of these animals and a parenteral BCG vaccine has now been licensed for use in badgers in the UK. In wild boar, feral deer and ferrets, BCG vaccine has been shown to induce significant levels of protection against experimental challenge with TB. Practical systems for delivery of oral bait TB vaccines to wildlife have now been established, but further research is necessary to improve oral bait formulations with appropriate attractants, systems for optimising bait distribution and avoiding bait uptake by non-target species. BCG vaccine has been shown to be safe in all animal species tested, although BCG has been isolated from lymph nodes draining vaccination sites and from faeces of animals for a short period following oral vaccination. There was evidence that vaccinated white-tailed deer could transmit BCG to non-vaccinated pen-mates, but not to cattle exposed to the room previously occupied by the vaccinated deer.

In summary, there have been major advances in the past 10–20 years in our understanding of the factors influencing BCG vaccine efficacy for domestic livestock and wild animals. To optimise the use of BCG vaccine, it will be important to continue to field test BCG vaccine in the various animal species in different environments, husbandry systems and in the presence of varying levels of disease prevalence as well as evaluating the practical application of DIVA tests. Although BCG vaccine may not provide complete protection against exposure to *M. bovis*, the protection should be sufficient to markedly reduce onward transmission to others animals. This feature could ensure that BCG vaccine could be particularly valuable for reducing infection in wildlife populations and in domestic animals where infection is currently very high and where "test and slaughter" control strategies are not able to be undertaken. There are numerous technical hurdles still to be overcome before an economically viable oral vaccine for use in badgers in the UK might be available. In the meantime it is beholden on stakeholders to make the best use of the existing tools available, this includes the intramuscular BadgerBCG vaccine. Cattle BCG vaccination in countries using "test and slaughter" control strategies also face significant hurdles. For example, a BCG vaccination-compatible DIVA test needs to be validated to allow vaccination to continue alongside traditional "test and slaughter" control programmes; currently BCG vaccination is prohibited under EU and some other countries' legislation and this would need to change; finally, cost-benefit analyses would decide whether deployment would proceed.

AUTHOR CONTRIBUTIONS

BB, HV, MC, and L-MdK-L wrote sections of the manuscript. All authors contributed to the manuscript revision, read and approved the submitted version.

ACKNOWLEDGEMENTS

The initiative to prepare this review arose from discussions at a workshop under the auspices of the Faculty of Medicine, Hebrew University, Israel which recognised that BCG vaccine could play an important role in control of bovine TB, but up to now its use has been limited. The authors wish to acknowledge the funding received from AgResearch New Zealand and the Department for Environment, Food, and Rural Affairs UK.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 21 December 2018
doi: 10.3389/fvets.2018.00327

Wild Animal Tuberculosis: Stakeholder Value Systems and Management of Disease

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When human health is put at risk from the transmission of animal diseases, the options for intervention often require input from stakeholders whose differing values systems contribute to decisions on disease management. Animal tuberculosis (TB), caused principally by *Mycobacterium bovis* is an archetypical zoonotic pathogen in that it can be transmitted from animals to humans and *vice versa*. Although elimination of zoonotic transmission of TB to humans is frequently promoted as the *raison d'être* for TB management in livestock, in many countries the control strategies are more likely based on minimizing the impact of sustained infection on the agricultural industry. Where wild animals are implicated in the epidemiology of the disease, the options for control and eradication can require involvement of additional stakeholder groups. Conflict can arise when different monetary and/or societal values are assigned to the affected animals. This may impose practical and ethical dilemmas for decision makers where one or more species of wild animal is seen by some stakeholders to have a greater value than the affected livestock. Here we assess the role of stakeholder values in influencing TB eradication strategies in a number of countries including Ireland, the UK, the USA, Spain, France, Australia, New Zealand and South Africa. What it reveals is that the level of stakeholder involvement increases with the complexity of the epidemiology, and that similar groups of stakeholders may agree to a set of control and eradication measures in one region only to disagree with applying the same measures in another. The level of consensus depends on the considerations of the reservoir status of the infected host, the societal values assigned to each species, the type of interventions proposed, ethical issues raised by culling of sentient wild animals, and the economic cost benefit effectiveness of dealing with the problem in one or more species over a long time frame. While there is a societal benefit from controlling TB, the means to achieve this requires identification and long-term engagement with all key stakeholders in order to reach agreement on ethical frameworks that prioritize and justify control options, particularly where culling of wild animals is concerned.

Keywords: tuberculosis, *Mycobacterium bovis*, animals, wildlife, stakeholders, value systems

OPEN ACCESS

Edited by:
Andrew William Byrne, Agri-Food and Biosciences Institute (AFBI), United Kingdom

Reviewed by:
Philip A. Robinson, Harper Adams University, United Kingdom
Sarah Louise Crowley, University of Exeter, United Kingdom

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *03 September 2018*
Accepted: *10 December 2018*
Published: *21 December 2018*

Citation:
Gormley E and Corner LAL (2018) Wild Animal Tuberculosis: Stakeholder Value Systems and Management of Disease. Front. Vet. Sci. 5:327. doi: 10.3389/fvets.2018.00327

INTRODUCTION

With increased global interest in the emergence of new infectious diseases, the role of animals in the transmission of infection to humans has become a focus of attention (1). The reasons for the spread of infections are complex and multifactorial and can involve changes in human populations and densities, modifications in animal husbandry practices, and changes to the ecological environment

leading to human intrusion into wildlife habitats that hitherto remained undisturbed (2, 3). It is the increased risk of transmission to humans that is most often the foundation for efforts to understand the epidemiology of animal disease and the implementation of preventative measures to minimize transmission (4, 5). A case in point is tuberculosis (TB) in animals and the danger it has historically posed to humans. Commonly referred to as "bovine tuberculosis" despite the causative organisms, most frequently *Mycobacterium bovis*, being capable of infecting a wide range of mammalian species, the perceived risk is reflective of the historical close association between livestock and humans (6, 7). During the early part of the twentieth century, in the period preceding the pasteurization of milk, transmission of infection *via* contaminated milk was a serious public health problem in the industrialized world, leading to many thousands of cases of human TB with high mortality rates (8, 9). The discipline of epidemiology (as we understand it today) was then largely non-existent. To the extent that attempts to address the disease in all its forms (cattle and human TB) were driven by competing stakeholder interests (e.g., dairy industry, public health agencies, government), more often than not it resulted in stasis and a complete failure to reduce disease incidence (10). Many countries in Europe eventually achieved eradication of TB from cattle through the roll out of government-regulated compulsory national screening programmes in cattle, and have since maintained this status through monitoring of animals for typical TB lesions at the slaughter house (11). For some of those countries that failed to achieve eradication, despite intensive testing, there was an awareness that the epidemiology of the disease was complicated by other possible sources of infection, notably wildlife (12). This militated against any quick-fix solutions to solve the problems. Instead it lead to decades of research to unravel what has turned out in many circumstances to be extremely complex epidemiology.

There have been few systematic studies worldwide to assess the extent of wildlife TB and it is often the case that studies are only initiated when there is spillover of TB into livestock, or where there is a high value placed on the species by particular stakeholders. Wild animal populations infected with TB are currently found in North and South America, Europe, Africa and Australasia (12). However, the finding of TB in wild animals in any particular environment does not constitute proof that they are a significant source of infection for livestock, companion animals or humans (13). Indeed it reveals little in terms of whether the affected species is a self-sustaining maintenance host or a dead-end spillover host. This distinction is critical for the development of strategies to control the disease in livestock as it can impact on the perspectives and level of engagement among a range of stakeholders. Depending on the reservoir status stakeholders may assign different value systems to the wildlife species and this can directly influence the type of management systems put in place. When TB is found in a free-ranging wildlife population the prevention of spread to other animals, especially livestock is often the immediate priority followed by the prevention of geographic spread. The identification of maintenance hosts is therefore of paramount importance in understanding the epidemiology because the disease can persist indefinitely in the absence of specific management and control programs. If it is established that wild animals are important in the epidemiological cycle and act as a source of risk to livestock, the decision making process as to the preferred actions will primarily depend on the considerations of the reservoir status of the infected host and the broader societal values assigned to each species by stakeholders. With spillover hosts, there may be a broad consensus reached among a limited number of stakeholders that an aggressive response to dealing with the reservoir host is the most effective strategy for stamping out the disease. However, if disease becomes established in a maintenance host, this will attract the attention of a broader range of stakeholders and there will be more complex ethical issues raised from culling of sentient wild animals and the economic cost benefit effectiveness of dealing with the problem in one or more species over a long time frame.

How to deal with the disease problems in cattle, arising from infected wildlife, has in the past often proven to be a quandary for stakeholders, in that government and industry supported measures (e.g., wildlife disease surveillance, culling) were not, at least in the beginning, underpinned by strong scientific evidence (14, 15). Rather, they were often pragmatic choices based on basic, simplistic epidemiological principles that aimed to deliver cost-effective beneficial results to the livestock industry in the short to medium term while awaiting the relatively slow pace of research to decipher the epidemiology and translate the results into policy decisions (14, 16). As a result, the primary driver for disease management in livestock has most often been based on economics and the impact of sustained infection on the agricultural industry (17, 18). In countries where wildlife have been considered as a potential source of infection the programmes evolved as the initial poor epidemiological understanding became clear, both from experience and also resulting from focused research both within the targeted species, and from assessing the risk of spread to other species (16, 19). However, as is often the case with scientific investigations into complex problems there can be an absence of certainty, and this has lead to conflict between the demands of different stakeholders (20).

STAKEHOLDER VALUE SYSTEMS AND WILDLIFE

"Wildlife stakeholder" broadly describes any person or group with an interest in wildlife. The levels of interest and the weighted values that each stakeholder assigns to particular wildlife can be highly variable, and defining the moral and ethical viewpoints of stakeholders that influences their level of engagement can be difficult. This is because there is likely to be a complex interplay between the values that each stakeholder places on wildlife and how it is linked to their moral perspectives on animal rights, animal-human health, conservation and biodiversity (21). Value systems for wildlife have been broadly classified into a number of groups according to their (a) economic importance, (b) nutritional value, (c) ecological role, and (d) socio-cultural significance (22). Quantifying the values with a high degree of certainty can be problematic as it mostly relies on data collected from surveys assessing preferences of stakeholders (23).

Stated preference methodologies, such as choice experiments allow for a structured method of data generation that helps to identify the factors influencing alternative choice scenarios (24). This approach is based on the assumption that individuals will select the choice that they expect will give them the highest benefit (utility), when presented with a set of alternatives. Its advantage over simple stated preference methods is that it allows for the valuation of attributes that characterize a particular scenario, rather than just valuing the scenario itself. Within each scenario there can be a scale of positive and negative values. For example, within the large game reserves in Africa, wildlife conservation activities can have a net positive value because of the significant beneficial impact on the local economy, also through the enhancement of local ecosystems from maintenance of biodiversity, and the cultural significance of wildlife for local communities. Negative values can accrue, for example, if there is crop or other habitat destruction because of over-abundance of particular species (e.g., large herbivores). As another example in the context of TB, choice experiment studies carried out in the UK have shown that badger management policies attract very high values: the surveys revealed that the public places high values on government policies that avoid culling of badgers (25).

Where wildlife start to encroach and compete with human interests negative value perceptions can increase among an expanded range of stakeholders. The divergence of values can lead to conflicts between those who place higher values on human activities (e.g., farming) and livelihoods and those who value the protection and welfare of wild animals. How these differences are reconciled can depend on the environmental and animal ethics perspectives of the stakeholders (26). These perspectives range from a contractarian viewpoint where there is a hypothetical social agreement to manage wildlife wisely for human benefit, to an animal rights focused viewpoint where there is no societal obligation to manage or interfere in any way with the well-being of wildlife. For many stakeholders with a general or transient interest in wildlife the ethical perspectives are likely to represent a blend of different viewpoints combining multiple value systems e.g., utilitarian and animals rights based values, such that respect for wildlife is acknowledged while at the same time adopting a value system allowing for the sacrifice of the interests of some animals for the greater benefit of others. The recognition that wild animals are a source of zoonotic diseases, particularly animal TB, can quickly change the number of stakeholders involved and increase the range of ethical perspectives: it can quickly shift the balance from high values associated with the natural rights of wild animals to much lower values as the threat of TB intensifies. The threat from infected wildlife can, on the one hand, be viewed as a serious agricultural problem with potential significance for broader human activities and health. A contrary perspective can assign higher net values to the affected wildlife species because of the belief that the disease impact is mostly restricted to the livestock industry or that the threat is overstated. Where there is a lack of objective data to support a particular perspective, this can lead to disagreements between those stakeholders who primarily value animal welfare and rights, and those who value the perceived greater benefits to society. Added difficulties arise from trying to define measures of benefit, for example, how can society assess and compare the pain and suffering experienced by slaughter of cattle and culling of wildlife? How are ethical views influenced by the presence of disease in one or both species? Do TB control programmes strike the correct balance between protecting the livestock industry and valuing the benefits of wildlife existing in their natural habitats? If TB was restricted to wildlife, how many stakeholders would be concerned for their fate? From studying the evolution and operation of TB control programmes in different parts of the world, we argue that the presence of TB in wild animals can lead to a change in ethical frameworks, and also involve a wider range and higher level of stakeholder engagement in the strategies employed to deal with the problem. The values of the interested parties appear to be based on an *ad hoc* blend of economic considerations, livelihood activities, knowledge, ethical perspectives, social acceptance, ecological concerns, cultural significance, and political will. This results in significant challenges for the selection of control policies where one or more species of wild animal is seen by some stakeholders to have a greater value than the affected livestock. It can also lead to demands for exceptionally high quality scientific evidence to justify particular interventions. Not all species are of equal significance in the epidemiology of disease, not all are considered equal when subjected to disease management, nor are they always equal in the eyes of stakeholders.

To try and get a better understanding of how policy decisions to manage TB in wildlife are influenced by stakeholders, we have looked at a number of established TB control programmes worldwide where there is strong evidence of epidemiological involvement of wildlife in the transmission of infection. We highlight the influence of stakeholder values on the management of the disease where the contexts differed. The approaches to disease control range from relatively uncomplicated management systems in Australia where there was strong consensus between stakeholders because of the negative value pest status of the wild animals to the highly complex epidemiology of disease in South Africa where multiple species of high positive conservation value are affected and a diverse range of stakeholder groups are involved in the debate on how to control and manage the disease.

WILDLIFE TB IN AUSTRALIA

Australia has been uniquely successful in eradication of TB from cattle against the background of a significant wildlife reservoir of infection in an area of one state, the Northern Territory (NT). Eradication was achieved following agreement of key stakeholders to the program, which included addressing the problem of wildlife reservoirs of infection (27). The last known cases of TB in Australia were detected in 2002: two cases in buffalo herds in the NT and a secondary case in a cattle herd in Queensland (28). Studies had revealed that *M. bovis* infection in animals was limited to two maintenance hosts: domestic cattle and feral water buffalo (*Bubalus bubalis*), with infection recorded in only one other wild animal host, feral pigs (*Sus scrofa*) (29, 30). There were only two reports of infection in other domestic grazing animal species: in goats co-grazing with infected cattle (31) and in fallow deer (*Dama dama*) (32). Also, as well as being

a maintenance host for TB, feral water buffalo and feral pigs were classified as invasive pest animal species that were causing a major negative impact on the environment of the coastal wetlands of the NT.

The Australian history of bovine TB control mirrors that of other developed countries, with an evolution from a voluntary program in the early twentieth century to a national program commencing in 1970 (33). The initial focus was on removal of diseased dairy cattle to minimize the threat to the human population. Reduction in prevalence was rapid and by the 1960s only a few pockets of infection remained among the southern states where dairy herds were dominant. However, the threat of trade restrictions for meat and dairy products imposed by trading partners in Europe and US lead to the launch of the national Brucellosis and Tuberculosis Eradication Campaign (BTEC), which ran from 1970–1997. The cattle industry was a key stakeholder in this campaign which included herd test and slaughter, compensation payments, tracing of animal movements, all backed up by a dedicated laboratory service. Aerial mustering and ground shooting was used in the large farms in northern Australia with whole herd culling of infected herds during the final stages. It was notable that domesticated water buffalo herds in this region were managed similar to local cattle herds and were subject to a test and slaughter strategy.

Feral water buffalo were only found in the NT having been introduced there in the mid-1800s. In the 1960s the prevalence of TB in slaughtered bulls was 16%. In 1970 at the commencement of the BTEC program, the disease was endemic in buffalo across most of their range (34) with the prevalence of lesions in abattoir slaughtered animals ranging up to 8.2% (35). The buffalo population peaked in the 1980s at around 350,000 head with the majority being unmusterable feral stock. With agreement between some of the stakeholders, that is, state and federal governments, pastoralists and conservationists, a decision was made to eradicate the wild buffalo herds by culling. The culling operations were effective and buffalo were eradicated from the coastal plains of the NT, except for a few domesticated buffalo herds and, at the request of the indigenous Aboriginal land owners, up to 60,000 animals were allowed to remain in the northeast corner of the state, where no TB was ever recorded in cattle or buffalo.

There was strong social, political, and cattle industry support for eradication of feral buffalo with the principal justification being the risk of transmission to cattle, even though there was only limited interaction between buffalo and cattle and no evidence of significant cross-species transmission (13). There was minimal objection to the eradication program from the small commercial buffalo capturing industry. The scientific evidence of damage to the coast flood plains caused by buffalo, leading to saltwater intrusion into the freshwater flood plains, resulting in the loss of habitat for native animals, and birds, was well documented (36). The coastal plains included the Kakadu National Park, a World Heritage site.

When the focus of the Australian national TB eradication program was extended to the pastoral grazing areas of northern Australia there was trepidation among stakeholders as feral pigs were considered as a possible reservoir of *M. bovis* infection. These suids were widespread and numerous in the region, and though the prevalence of confirmed *M. bovis* infection in some studies was high at 19.2% (30) it was subsequently considered from the distribution of TB lesions that they constituted a spillover host with a minimal risk of onwards transmission from pigs to other animal species. It is likely the feral pigs became infected by scavenging on carcases of tuberculous cattle and water buffalo (13). No direct intervention was taken against the feral pig population and it was later shown that after eradication of TB from cattle and the eradication of buffalo, TB prevalence in feral pigs declined significantly (29). Unlike in New Zealand, infection with *M. bovis* was never reported in the common brushtail possum (*Trichosurus vulpecula*). Elsewhere, the absence of infection among native wildlife allowed the focus of the TB campaign to remain on cattle and buffalo. Following the end of BTEC, all subsequent buffalo herds were derived from populations where infection had never been present. Since 2011, infection with *M. bovis* has been classified as an exotic disease of cattle in Australia (37).

NEW ZEALAND AND TB IN WILDLIFE

The New Zealand history of bovine TB control parallels that of Australia, starting with voluntary testing of dairy cattle herds in 1941 and moving to stringent and compulsory test and slaughter programmes in 1961 (14, 16). When progress stalled, the discovery of the disease in wildlife was recognized as a possible constraint to eradication (38). Epidemiological studies in New Zealand identified 14 species infected with *M. bovis,* but only three, domestic cattle, domestic deer and brushtail possums, were identified as maintenance hosts, though wild ferrets (*Mustela furo*) were considered as possibly a maintenance host in very limited areas (16, 39). Although not considered as maintenance hosts, feral pigs and wild deer, along with the ferret, have proved invaluable as sentinel hosts for surveillance of TB in possum populations (40). The current testing program for cattle and deer is based on the risks associated with transmission of infection from possums (14). The brushtail possum is a small arboreal marsupial, first introduced into New Zealand from Australia in 1837 to establish a commercial fur trade (41). They were officially classified as a pest species in 1948. The possum population reached a peak of around 50–70 million in the 1980s. The original public perception of possums as harmless changed when it was shown that they might pose a great threat to survival of native fauna, including the iconic kiwi (42). Although first shown to be infected in the mid−1960s, the findings in the 1970s revealed that possums were a maintenance reservoir host for *M. bovis,* and strongly implicated in the transmission of infection to cattle. Studies also showed that possums were highly susceptible to infection resulting in a rapidly disseminated and fatal disease (41, 43). Although generally avoiding cattle, terminally ill possums display abnormal behavior patterns which could bring them into contact with inquisitive cattle (44, 45).

The early government-led initiatives to control TB in cattle subsequently evolved into a public-private partnership between the government and the livestock industries with a remit

to conduct wide scale possum control (16). The objective of the national program was to eradicate *M. bovis* from New Zealand and this received general societal and industry stakeholder support (46, 47). The broad geographic distribution of tuberculous possum populations and the large number of other species affected initially made the prospects of eradication unlikely even though there was support for the TB eradication program, especially the focus on possum culling (48). In recent years a choice experiment survey of the NZ public was carried out to assess the non-monetary benefits to native forest biodiversity arising from TB-related possum control (48). This revealed strong public stakeholder support for the benefits of possum control, particularly the values placed on the observable effects of improved forest canopies and the positive impact on native bird, insect and plant species. The main criticism of possum control has subsequently been aimed at the methods used to cull possums, especially the use of sodium fluoroacetate (1080) by aerial application (49, 50).

The early possum control measures helped to significantly lower the incidence of disease in cattle herds, but relied on basic assumptions of the epidemiology, rather than any hard scientific evidence (41, 51). Where large scale possum control measures were successful, the TB levels in the sentinel species also declined, demonstrating that targeting resources at one key maintenance reservoir had a direct beneficial effect on other species (52). Currently the population of possums is estimated to be in the order of 30 million. As a result of the possum culling, also controls on the movement of cattle and deer, and TB testing, the number of infected herds in NZ has dropped from ∼1,700 deer and cattle herds at the peak in 1995 to 41 herds in 2015 (14, 16).

BADGER TB IN IRELAND AND THE UK

In recent times the most controversial wild animal TB control strategies in Ireland and the UK have revolved around the European badger (*Meles meles*) with deep polarization of opinion among many of the stakeholders, particularly in the UK (53). The role of badgers in the epidemiology of TB in cattle in the UK and Ireland has been subject to intensive investigations since *M. bovis* infection was first identified in badgers in England in 1971 and subsequently in Ireland in 1974 (54, 55). Over the preceding 10 years substantial progress had been made in reducing the incidence of TB in cattle in both countries due to mandatory herd screening programmes (9, 56, 57). When progress stalled, and badgers were found to be infected, local badger culling operations resulted in an apparent decline of disease in cattle (57, 58). Over the next two decades evidence accumulated through large scale culling studies that strongly implicated badgers in the TB transmission cycle (59–63). The advent of DNA genotyping of *M. bovis* isolates also revealed that prevalent genotypes were common in both cattle and badgers sharing the same environment, providing evidence of cross-species transmission (64, 65). Tuberculosis in badgers is a chronic slowly progressive disease (66) and infected badgers satisfy the criteria to be a maintenance reservoir host for *M. bovis* in Ireland and the UK (13). They are highly susceptible to infection and the predominant location of lesions suggest that infection among badgers occurs principally *via* the respiratory route with transmission from infected bite wounds being of secondary importance (67, 68). The social structure of badgers facilitates close interactions that lead to an increased risk of transmission. Pseudovertical transmission from dam to cub is likely to be a key factor in maintenance of infection within local populations (66).

In the Republic of Ireland the national TB eradication plan commenced in the late 1950's, and the strategy has succeeded in decreasing TB incidence in cattle and maintaining it at a relatively low level (69). This has been achieved using a program of sustained cattle testing and targeted badger culling (70). Prior to the implementation of a national badger culling strategy in the Republic of Ireland, two separate badger culling studies (East Offaly Project and the Four Area Study) confirmed the role of badgers in the epidemiology of TB in cattle. Both trials showed a significant drop in cattle TB prevalence in areas where badgers were proactively culled in comparison to the control areas (60, 63). A separate study conducted in County Laois between 1989 and 2005 also provided evidence that badger culling had a positive impact on the risk of future TB breakdowns in cattle and a positive protective effect on herds neighboring the index herd (71). Badger culling was incorporated into the national TB eradication strategy in 2004. As a compromise with stakeholders who had reservations about the strategy, there was a limit imposed on which individual setts could be culled in the relation to the index herd and the proportion of the badger population subjected to culling. Since then, the Irish culling program has focused on areas with high incidence of infection in cattle; areas in which studies have shown the highest infection prevalence in badgers (72). Culling is only conducted following an exhaustive epidemiological investigation to rule out other causes of herd breakdowns (e.g., residual infection, contiguous herd spread, purchase of undiagnosed infected animals), and where badgers are considered as a likely source of infection. Analysis of data generated from culling studies has shown a beneficial long-term decrease in cattle TB (71) and also TB in the badgers of the re-emergent population (73). The culling of badgers in Ireland at national level is considered as an interim strategy to minimize transmission to cattle pending the development of a suitable and effective vaccine. Most stakeholders have accepted culling of badgers, albeit with reservations (72). These reservations are mainly framed around the evidence base that implicates badgers in the epidemiology of TB in cattle, that there is an effective control programme in place for infected herds, and whether culling of badgers is an acceptable measure when the benefits to cattle are difficult to quantify against a background of other control measures focused on cattle.

In the UK there are a large number of stakeholders with diametrically opposing views involved in the debates on the TB control strategy. Culling of badgers to control TB in cattle has proved extremely controversial since it commenced in 1973. Concerns over badger welfare arose from the Ministry of Agriculture, Fisheries and Food (MAFF) policy of gassing setts with hydrogen cyanide, leading to a number of commissioned reports over the following decades, with no clear resolution as to how the scientific evidence should inform policy. The

Zuckermann review in 1980 recommended sampling of badgers in the vicinity of affected farms and culling at setts if badgers tested positive (74). In 1986 the Dunnet report questioned the efficacy and the cost sustainability of this "clean ring" strategy (75). The Krebs report concluded that though the evidence was indirect, badgers were a significant source of infection in cattle and recommended an experimental trial to quantify the impact of badger culling on cattle TB (76). The Randomized Badger Culling Trial (RBCT) was carried out between 1998–2006 with the objectives to generate scientific evidence for the role of badgers in cattle TB, and to help formulate appropriate policy measures. However, it ended up highly divisive and the legacy of the trial continues today. Cassidy (77) argues that the design, scale and complexity of the trial, including ongoing disruption by anti-cull protesters made it extremely difficult to generate a strong evidence base that might have otherwise been gathered in more conventional small case controlled studies. The trial area consisted of 10 sets of "triplets" areas, each containing a proactive-culling area, a reactive-culling area with culling only in response to a cattle TB cattle breakdown and a survey-only area where no culling was conducted. The trial results showed that the incidence of bovine TB in cattle dropped by 19% in the proactive-culling area. However, the proactive culling was also associated with a 29% increase in cattle TB in the area outside the culling zone (62, 78). The increase in cattle TB outside of the culling area was attributed to the "perturbation" effect, where the social behavior of badgers was altered by the culling activities, leading to increased interactions and transmission rates to cattle in the area outside of the badgers normal territories (78, 79). Reactive culling was abandoned early in the trial as it was believed to increase, rather than decrease, the incidence of TB in cattle in these areas.

Since its completion, the conclusions of the RBCT have been disputed and the data re-interpreted many times. In its final report, the government appointed Independent Science Group (ISG), which oversaw the trial, concluded that "*badger culling can make no meaningful contribution to cattle TB control in Britain*" (78). This viewpoint was somewhat contradicted by the (also-) government commissioned follow-up King review which found that badger culling "*could make a significant contribution to the control of cattle TB in those areas of England where there is a high and persistent incidence of TB in cattle, provided removal takes places alongside an effective programme of cattle controls*" (80). Cassidy (77) points out that the ISG took a broad perspective to their remit and combined analysis and policy issues to reach their conclusions, whereas the King review restricted the focus to the scientific evidence, without taking account of policy considerations and animal welfare concerns. The situation has not been helped by the perceived inability of successive governments to formulate a long-term policy that balances the pros and cons of the moral arguments used by stakeholders. Changes in the UK government over the years has lead to major policy shifts on badger control measures, further emphasizing the inability of government stakeholders to implement evidence based policies while taking account of the viewpoints of interested parties (53, 77). The Labor government of 2006–2010 accepted the findings of the ISG and resisted pressure from the Nation Farmers Union (NFU) and the British Veterinary Association (BVA) to endorse culling. The Conservative—Liberal Democrats coalition government (2010–2015) changed policy and agreed to introduce culling on a limited scale. While the majority of politicians and many stakeholder groups recognized the role that badgers played in the epidemiology of TB in cattle, there was less agreement on the strength of the RBCT scientific evidence and also the economics and ethics of large scale culling. Pilot culls commenced in Somerset and Gloucestershire in 2013 attracting major opposition from a large cross section of community groups and organizations. When it was reported that the trials failed to meet the pre-determined limits of humaneness and efficacy, this served only to galvanize opposition that demanded a cessation of culling. The effectiveness of the culling operations was also questioned and despite all of the confounders associated with the design (e.g., failure to achieve reduction of targeted population, differences in levels of implementation), it appeared that badger culling was associated with a reduction in cattle TB incidence (81). As cattle TB rates continued to climb in the UK, the Conservative government in 2015, although fully attuned to the unpopularity of culling, expanded the culling areas to placate the demands of the farming industry. Elsewhere, contrasting policies operated in other parts of the UK experiencing problems with cattle TB. The Assembly in Wales has resisted a badger culling policy but instead has increased the range of cattle control measures and focused on vaccination of badgers (82). In Northern Ireland the local Assembly agreed to a badger Test, Vaccinate and Release (TVR) trial to gauge the effectiveness of this approach on cattle TB rates. The strategy involves capturing live badgers in an area with a high level of cattle TB, testing the badgers for TB, vaccinating those that test negative to the disease and removing those that test positive (83). A survey of farmers attitudes to TB control strategies in Northern Ireland revealed a willingness to allow vaccination and culling of badgers on their land with an overall preference for vaccination, and less concern about public opposition (84).

The multi-dimensional aspects and complexity of the evolution of cattle TB policy in the UK raises many questions on the ethics and value systems of stakeholders in the context of culling of badgers. The role of the media is of key importance in framing the viewpoints of many of the principal actors (85). Where there is difficulty in understanding the complexity of the scientific evidence, the press can influence perspectives by over-simplifying the arguments for or against a particular strategy e.g., culling or vaccination, and this can help to fuel the controversies. This can lead to poorly thought out policy decisions, which may reinforce perceptions of mismanagement. Surveys of farmers in new endemic TB areas in the UK have revealed a fatalistic attitude to the problem, where many believed that bad luck played a role in herds contracting TB, but also mediated by perceptions of the political aspects of the disease and the lack of trust in government (86, 87). Similar perceptions were found in Northern Ireland where interviews with focus groups (cattle and beef farmers, private and state veterinarians) revealed differences in perceptions and knowledge of the disease among the different actors (88). It was concluded that a "one-size-fits-all" approach to control policy would be unlikely to succeed without recognizing

the heterogeneities of many aspects of disease transmission and the multiple framings of the disease by different stakeholders.

McCulloch and Reiss have described the history and evolution of government policy toward control of TB in badgers in the UK (53). They argue that the debate can be distilled into two questions related to quantifying the role that badgers play in the epidemiology and whether the current control options are effective, practical (in controlling transmission) and socially acceptable? They conclude that policy should not be based exclusively on scientific evidence, economics or public opinion. Rather, they propose that the ethical issues need to be addressed by independent experts according to moral frameworks that question what is right, and what is justifiable, and taking into account the impact of policy on the morally affected stakeholders. McCulloch and Reiss separately analyse these frameworks from a utilitarian perspective (89). This approach strives to achieve a balance between the competing interests of stakeholders in order to achieve the greatest utility benefit for all. But it raises the question as to who are the greatest beneficiaries and how can one measure and quantify the utility benefit? In this context there is a generally perceived human benefit from farming arising mainly from production of high quality food leading to good public health. But there is also a strong societal benefit and positive value from maintaining undisturbed badger populations in their native habitats (25). Because of TB there are conflicting viewpoints on these utility benefits among stakeholders. McCulloch and Reiss argue that according to utilitarian theory, "*the slaughter of a cow or the culling of a badger with a life of net positive value will result in a loss of utility. All else being equal, the killing of a cow or badger that could be expected to continue with a life of net positive value is, therefore,* prima facie *morally wrong, simply because it reduces total utility*" They suggest that killing of badgers can be morally justifiable if it results in greater overall utility, e.g., the replacement of the (badger) utility by cattle, or an increase in human utility through improved farming economic benefits accruing from culling of badgers. They then pose the question as to how much culling of badgers is acceptable to justify the increased utility value of cattle? The contention from the ethics of utilitarianism is that the correct policy is one which produces overall highest utility. But this relies on an understanding of the consequences of the policy such that the impact of different policies can be objectively compared and measured. Their analysis concluded that non-culling approaches including badger vaccination policy options resulted in higher overall utility, and was superior to the badger culling option. In the absence of agreement among stakeholders, vaccination of badgers offers a utilitarian solution, and is now considered as a strategy that can address many of the negative issues associated with culling (90).

WILDLIFE TB IN SPAIN AND FRANCE

In continental western Europe, Spain is considered to have a complex epidemiology of TB involving multiple mycobacterium species, animal species and several maintenance hosts including cattle, deer and wild boar (91). Domestic goats, sheep and free-ranging domestic pigs are also implicated in the transmission cycle and often share common pasture land with cattle (92–94). Infections with *Mycobacterium caprae* is common in goats and has been known to spill over to cattle (92). Badgers have also been shown to be infected with *M. bovis* though the impact on livestock is unclear and may differ according to the region (95). For example, badger numbers are more abundant in the cooler Atlantic influenced regions in the north of the country where molecular typing of *M. bovis* strains has shown that they are common to badgers and cattle (96). In the southern mediterranean region of Spain wild boar are believed to be maintenance reservoirs of infection (97). These animals are well-adapted to the seasonal variability in food and water sources, and their mobility and scavenging on infected carrion (e.g., deer) likely influences the pathogenesis of disease which is frequently associated with head, pulmonary and disseminated TB lesions (97). Wild boar are considered as a significant risk factor for TB breakdowns in cattle (98), likely resulting from indirect contact (99).

High densities of wild boar are often maintained by artificial feeding to support a vibrant hunting industry, typically in fenced game estates many of which also house deer, cattle, sheep and pigs in free ranging systems (100). During the hot season experienced in southern regions of Spain, wild boar and other wild species congregate at high densities at watering holes increasing contact rates and the probability of both transmission within and across species (101). Surveys of *M. bovis* prevalence in Doñana National Park (DNP) in southern Spain have revealed prevalences of 52% in wild boar, 27% in red deer and 18.0 % in fallow deer (102). In areas where cattle are absent, prevalences have reached 92% in wild boar (102). The congregation of boar at feeding sites is associated with the high risk of tuberculosis in deer (103). The DNP is also one of the last refuges of the critically endangered Iberian lynx (*Lynx pardinus*), which along with foxes (*Vulpes vulpes*) are considered as spill over hosts (104, 105). In comparison, the prevalence of TB in wild boar in the Atlantic influenced habitats of Northern Spain is significantly lower when compared with the mediterranean habitats (96). This is likely due to lower densities in the northern regions, lower levels of artificial management, less congregation at water holes; all of these factors impacting on infection transmission rates.

As in many other European countries TB eradication in Spain was initially driven by the high prevalence of disease in cattle. When research revealed a multi-host epidemiology of disease, this brought additional stakeholders, including government, hunting lobbies, agricultural industry, and conservationists into the discussion on how best to manage the problem. Culling of wild boar has been shown to be an effective and strategic measure to reduce prevalence, and with the added likely benefit of a decrease in transmission to other species (106). However, culling of animals has also caused conflict among stakeholders while policy makers have attempted to balance the competing interests of hunters, producers, and conservationists. The principal reason is because the hunting estates require managed high densities of

animals to maximize commercial returns and hunting groups are resistant to widescale culling (107).

Research has continued in Spain to monitor changes in the occurrence of TB and to unravel the complexities of the epidemiology with a long term view to measure the impact of interventions that may reduce transmission rates among all affected species (108). A questionnaire survey was carried out among key stakeholders (veterinarians, livestock owners and farmers) in south central Spain to gauge their opinions on specific intervention strategies chosen by a panel of experts that included veterinarians engaged in research into wildlife and disease management in the study area (20). Although banning of supplementary feeding of wildlife on cattle farms was ranked by the experts as the most effective control option, this opinion was not shared by hunters and farmers as a practical measure. Overall, hunters and farmers showed the highest levels of agreement for the top-ranked interventions (ban on supplementary feeding, restricting access to waterholes, increased frequency of cattle testing, removal of discarded offal from hunting land) while hunters and veterinarians agreed least. This study highlighted the diverse attitudes of different stakeholders to a range of intervention strategies and probably reflected differences in opinion on the broader epidemiological picture. The opinions of farmers and hunters were more aligned because of their converged interests in the same parcels of land required for their activities, whereas the perspectives of veterinarians were primarily guided by principles of disease management while trying to balance the interests of all stakeholders including policy makers (20).

France was declared officially TB free in 2000, but since then sporadic outbreaks of TB in cattle have continued in a number of regions in the south-west and east of the country (109, 110). TB was also first identified in wild red deer in the northern Normandy region of France in 2001. By 2006, prevalence rates remained high in deer (24%) and wild boar (42%) despite culling of these animals to reduce densities (111). TB infection was subsequently diagnosed in badgers in the areas most affected by TB in cattle (112). Arising from increased concerns over broader wildlife involvement in cattle TB outbreaks (TB in wildlife occuring in areas with cattle TB), the French General Directorate for Food (DGAL) and institutions involved in animal health and wildlife management established a national surveillance system "Sylvatub" in 2011 (113). This serves to co-ordinate the activities to detect and monitor TB in wildlife, and involves a wide range of national and local stakeholders including hunting and wildlife agencies, cattle breeders, pest controllers, trapper associations, veterinary associations and public administrators. The objective is to develop a broad national understanding of the risks associated with TB in wildlife allowing for the design and implementation of control strategies. An evaluation of stakeholder perceptions of the Sylvatub has revealed overall satisfaction with the system, the utility of helping farmers being the primary motivating factor (114). The improved understanding of TB epidemiology was also cited as a motivating factor for participation. Disincentives to participation included practical difficulties, regulatory hurdles, time-consuming activities, economic and material constraints. The results of this evaluation appear to feed into the same stakeholder narrative in other countries experiencing wildlife TB problems, in spite of a low impact on TB rates in cattle.

TB IN WILDLIFE IN USA

The success of TB control in a wildlife species can crucially depend on the support or otherwise of individual or groups of stakeholders. In the USA white-tailed deer (*Odocoileus virginianus*) are the principal wildlife maintenance hosts implicated in transmission of TB to livestock in Michigan and Minnesota (115). Although there have been significant differences between the two states in the prevalence of TB in deer and the size of areas containing infected deer, the responses to the disease have been contrasted by temporal, social, economic, and logistical factors.

The US National Bovine TB Eradication Program was launched in 1917 following years of often fractious debate on the merits of different control options based around meat inspection and the recently developed tuberculin skin test (8, 15). Pasteurization of milk for human consumption had commenced almost a decade earlier in Chicago and New York, and other major cities, to reduce the risks associated with zoonotic TB and other diseases. Stringent application of the test and slaughter control measures lead to rapid success in controlling the disease and by 1940 prevalence was reduced to $<$0.5% in every state (116). Prevalence in livestock was recorded as 0.003% in 1994. Between 2001 and 2011, 92 US cattle herds were diagnosed as TB infected and several constraints to eradication were identified including changed management practices, importation of infected animals and the emergence of the disease in cervid species, particularly wild white-tailed deer in Michigan and Minnesota (116).

The disease was first reported in wild deer in Michigan in 1975 and was considered an isolated event (117). In 1994 it was detected again in a hunter shot deer close to the original case, providing for early but inconclusive evidence of a possible wildlife reservoir. It was the impetus for the state to initiate a TB control programme targeted at wildlife and farmed deer. In 1995 after the first year of systematic wildlife surveillance, 4.9% of deer sampled were culture positive for *M. bovis* in the core outbreak area ($\sim$1,500 km^2) (118). With the disease eradication plan underway, addressing both the deer and the cattle populations, there was resistance mounted by some of the large number of stakeholders, with no universal acceptance for the proposed control measures (119). While there was overall support among deer hunters, livestock producers and agricultural business for the eradication of TB, there were differences in the knowledge and perceptions of the threats of TB, leading to a lack of support for eradication measures (120). As a major stakeholder, the hunting industry did not consider that the extent of the disease problem warranted reduction of deer numbers in the infected areas, and they were opposed to the banning of supplemental feeding and baiting which had helped to increase deer densities. From an epidemiological perspective, this provided opportunities for

contact between infected and susceptible animals either by direct contact or contamination of food (121, 122). Agricultural producers relying on crop production for sale of feed to the hunters also considered the measures as a threat to their business. Among livestock producers, including those with most to lose from the TB outbreaks, only 57% supported further reductions in deer numbers. These differences in values among the key stakeholders and problems with compliance constrained the ongoing control efforts and TB in cattle and wildlife (123, 124) and TB breakdowns in cattle continued, preventing the state from regaining its former TB free status. Between 1994 and 2010 there were however only 50 cattle farms positive for TB, and of those within the TB core area the most likely source of infection for the herd was wildlife. The majority of TB infections in other wildlife including coyotes (*Canis latrans*), racoons (*Procyon lotor*), red fox (*Vulpes vulpes*), bobcat (*Felis rufus*) and black bear (*Ursus americanus*) have been found in the northern portion of the Michigan's Lower Peninsula which contains the core area and probably amounts to them being spillover hosts (125). The full state of Michigan has still not regained its TB free status from the USDA (123).

When TB was detected in white-tailed deer and cattle in Minnesota in 2005 there was a rapid and aggressive response (119). The control of TB was framed by implementation of a strong management programme by the Minnesota Department of Natural Resources. The outbreak was confined to a small area of $<$425 km^2. By 2011 only 12 beef cattle herds and 27 white-tailed deer had been diagnosed with TB. The result of studies to investigate the factors associated with deer-cattle transmission had implicated deer visits and damage to stored cattle feed as a major risk factor (123). The decision was made to eradicate infection from both the cattle and deer populations by culling both species and this inevitably placed an economic burden on both the cattle industry and recreational deer hunters. A new deer management unit was created that allowed for additional hunting opportunities. Private landowners were issued with shooting permits to remove an unlimited number of deer on their lands, with the proviso that samples were submitted for TB-testing and the carcasses used for venison, thus avoiding wastage. A recreational feeding ban for deer and elk was put in place in 2006 in the TB endemic areas and monitored by enforcement officers. The plan was highly successful in reducing the prevalence of TB and by 2011, there were no recorded cases of TB in deer or cattle in Minnesota (119). As a result the state re-gained its TB free status from the USDA (119).

Although the key stakeholder groups in Michigan and Minnesota were similar and likely motivated by the same concerns, the outcomes of the TB eradication programmes in each state were markedly different. There are a number of factors that may have contributed to the divergent outcomes. The control efforts in Minnesota benefited from the issues revealed from the interventions of the Michigan campaign. With the disease emerging much later in Minnesota there was political pressure to quickly stamp out the disease before it became endemic. Thus, control measures were implemented much earlier after discovery of the outbreak in Minnesota, whereas the disease was present for at least 20 years before control measures were applied in Michigan. Although there was some resistance to deer culling from hunters in Minnesota there was also the realization that TB eradication in the short term was beneficial to the industry in the long term. The demands for strong action from the cattle industry also made it easier for politicians to implement aggressive actions.

Carstensen et al. describe a combination factors that may have contributed to the different levels of stakeholder acceptance in both states and the more aggressive response in Minnesota (119). They highlight that the core area of the TB outbreak in Minnesota was 29% of that in Michigan. Also, the terrain topography and the substantially higher proportion of publicly owned land in Minnesota facilitated access for shooting of deer. Use of helicopters for aerial shooting to remove deer was controversial, though strong engagement with all stakeholders through public meetings helped to alleviate concerns. While baiting and feeding of deer were illegal in the core outbreak areas of both Michigan and Minnesota, baiting was illegal in Minnesota more than a decade prior to the finding of TB in cattle or free-ranging deer. The land ownership in Michigan's core area comprised 90% private land, including hunting areas, making it difficult to enforce compliance with the law. The number of farms in the affected area of Minnesota was twice that of Michigan's core area, helping to increase the political clout of the cattle industry in Minnesota. A buy out program was available to cattle producers in Minnesota's TB outbreak area to help reduce the cattle population at risk. A high proportion of eligible farms accepted the buy-out, and $\sim$6,200 cattle were removed from the TB affected area. A similar buy out was not facilitated in Michigan. What these factors illustrate is how differences in the value systems of the same stakeholders in each state affected the outcome of the disease eradication measures. From a value systems perspective the utility value of the deer in Michigan was given a higher overall nominal score because of the powerful hunting lobby, whereas, in Minnesota the concerns of the agricultural lobby trumped the hunting industry allowing the state officials to implement a much more forceful control plan.

WILDLIFE TB IN AFRICA

The number of wild animal species involved in the highly complex epidemiology of TB in South Africa poses particular challenges for identifying and engaging with stakeholders in order to seek broad consensus on control strategies (126). The African continent is home to a vast and diverse range of indigenous wild mammals, many, if not most, of which it can be assumed are susceptible to infection with TB (127). Given the lack of any reliable hard data, it is not known with certainty if the disease was originally introduced by human activities or if it always had a presence in wildlife at some level, with the open and expansive landscape facilitating interactions and new incidents of infection across multiple species (128). The advent of molecular typing of strains isolated from cattle has revealed the presence of three geographically distinct *M. bovis* clonal complexes in Africa, the African Af1 complex dominant in sub-Saharan West-central Africa (Mali, Cameroon, Chad and Nigeria), African Af2 found in East Africa (Uganda, Burundi, Tanzania and Ethiopia) and

European Eu1 complex in South Africa (129–131). The presence of the Eu1 strain is associated with the arrival of the Dutch and British colonial settlers in South Africa with TB infected cattle, and represented a significant event in the emergence and spread of TB among native animals.

TB was first identified in cattle in South Africa in the late nineteenth century, and in indigenous kudu (*Tragelaphus strepsiceros*) in 1928 (127). In the following decades the disease was diagnosed in an increasing number of wildlife species including duiker (*Sylvicapra grimmia*), and springbok (*Antidorcas marsupilias*). More recently research has focused on the Hluhluwe-iMfolozi Park (960 km^2) and Kruger National Park (19,485 km^2) where it is believed that TB was transmitted to the African buffalo (*Syncerus caffer*) from domestic cattle in the 1950s (132). Among the many wildlife species affected the buffalo is considered to be the principal maintenance reservoir of infection, although kudu also appear to maintain the infection (132–134). By 1995, the disease had spread northwards from the southern part of the Kruger and since then has affected many different animal species including lion (*Panthera leo*), cheetah (*Acinonyx jubatus*), kudu, leopard (*Panthera pardus*), chacma baboon (*Papio ursinus*) (135–137), black rhinoceros (*Diceros bicornis*) (138, 139) and white rhinoceros (*Ceratotherium simum*) (140). There was also evidence of spillover to neighboring livestock (141). Molecular strain typing has shown that the infection had spread by clonal expansion of the Eu1 strain type and spread to game farms and reserves in Mpumalanga, Limpopo, KwaZulu–Natal, Free State and North West Provinces, affecting at least 16 different animal species (142).

In South Africa a voluntary test and slaughter scheme for cattle was initiated in 1969, and by 1991 had reduced the disease prevalence to 0.04%. However, primarily due to financial and resource reasons this level of success was not sustained, and the disease levels increased thereafter (132).

There is only limited basic epidemiology known for most African wild mammal species other than buffalo (143). As the disease became established in maintenance hosts it was inevitable that the infection transmitted to predator species, including lions, hyena (*Crocuta crocuta*), leopard and cheetah, and a range of scavengers and omnivores (142). These, as with other predators, are probably spillover hosts where the infection is unlikely to be sustained in the absence of external sources of infection. The pattern of generalized TB in the prey species (including buffalo and antelope species) increases the likelihood of transmission following ingestion of infected organs and tissues.

In South Africa, all aspects of wildlife have provided lucrative business opportunities with increased global interest in ecotourism, trade in wild animals and conservation (132). The number of wildlife has increased considerably in South Africa in recent years, both in national parks and private game reserves. Iconic African wildlife species are exported worldwide to zoos for conservation and can attract very high purchase fees (139). In the absence of any reliable ante-mortem diagnostic tests for TB this poses great challenges to controlling spread of infection when animals are translocated to reserves within Africa or exported worldwide. There are many recorded examples of tuberculosis in rhinoceros housed in zoos going back over 100 years yet in that time there have been relatively few advances in development of sensitive diagnostic tests other than relying on observation and clinical symptoms (139). The finding in the Kruger National Park of an infected free-ranging black rhinoceros (138) and in the white rhinoceros (140), species recognized as critically endangered and near threatened by the International Union for Conservation of Nature, has serious implications for the conservation measures for rhinoceroses, and movement out of the Park for breeding and conservation reasons.

With the expanding range of African animals infected with TB, it is difficult for programme managers to deal with the problem given the enormous costs involved, notwithstanding the paucity of epidemiological information available for single species let alone unraveling the complexities of the infection in multi-species hosts (132, 144). The deficiency in the epidemiology of the multi-host system prevents any single proposed programme from claiming precedence. In South Africa control of animal diseases is regulated by the Department of Agriculture, Forestry and Fisheries (DAFF) though there are many local, national and international stakeholders involved, including ecologists, veterinarians, conservationists, animal rehabilitation centers, ecotourism companies, game capture operations, national and provincial parks, hunting companies, the cattle industry, wildlife ranching etc. Given the diverse range of the interest groups, there is likely to be as many conflicting opinions on how to manage the problems. For example, although TB is endemic in many buffalo populations, it does not appear to be detrimental to their population structure, nor are TB test positive buffalo more likely to be subjected to predation by lions (145). This may lead to opposing viewpoints from those groups who believe the presence of TB has minimal ecological impact and, for example, veterinarians motivated to eradicate disease. Spillover of disease to high value predators does raise concerns from many additional stakeholders. It is unlikely that TB can now be eradicated from the community of affected species by current available methods and policies are likely to be framed around management of the disease to minimize spread. Test and slaughter programmes, if available, may serve to decrease local prevalence but are unlikely to achieve eradication. Resources may be focused on species of highest monetary or conservation value, thus providing short to mid range economic benefit but achieving little in the context of eradication of the disease from free-living animals. Vaccination may provide a potential solution in the future, however it would need to be cost-effective, and any chance of success will also require many additional studies to improve epidemiological knowledge and understand how control measures directed at one or more species affects the dynamics of disease in multi-host systems (146).

VACCINATION OF WILDLIFE AGAINST TB

Where culling of animals is not considered a feasible option (for whatever reason) as a disease management tool, vaccination of wild animals against TB is often promoted as an alternative strategy, primarily because it provides for a non-destructive approach to controlling disease and addresses animal welfare

concerns, as well as conservation concerns arising from deliberate killing of wild animals (147, 148). The purpose of vaccination is to reduce the incidence of infection leading to lower levels of intra-species spread of infection, as well as transmission to other wild species and livestock (149). By reaching and maintaining a threshold level of coverage the vaccine will also confer protection to the non-vaccinated proportion of the population through the generation of herd immunity. Over time, and with an effective vaccine, the disease will eventually disappear from the vaccinated population. The BCG vaccine, used extensively in humans, has been shown to work in a variety of animal species (147, 150, 151), and more recently an alternative heat inactivated *M. bovis* (HIMB) vaccine candidate has shown some promise in a range of species (152–154). These vaccines can be delivered by injection or oral bait. BCG is a live vaccine and a single dose can provide a long duration of protection against natural exposure to infection (155).

In deciding on the appropriate control strategies to employ, the desired outcome needs to be carefully considered in order to avoid further conflict among stakeholders. For example, vaccination of badgers may address conservation concerns arising from culling a protected species, with the added benefit of protecting cattle from badger—cattle transmission. However, the time frames to achieve eradication will be much longer when compared with culling (156). Studies of UK farmers' perceptions of vaccination as a means to control TB have also revealed cautious attitudes to this strategy (157). It has been noted that the media paid more attention to vaccination when the controversies over culling escalated (85), and wildlife groups have heavily promoted the vaccine strategy. While there is good field data to show that the vaccine can protect badgers in their natural environment, the scientific evidence of a direct link between badger vaccination and time scales for a positive impact in reducing TB breakdowns in cattle is lacking. This serves to reduce farmers' confidence in vaccination, which in part reflects their lack of trust in the ability of government to control the disease (86). There is also a viewpoint among farmers of over-population of badgers that is consistent with a preference for culling of badgers above vaccination (158). If farmers believe that there is little that can be done to control the disease, a vaccination strategy is also unlikely to alleviate such concerns. Elsewhere, BCG vaccination may be of use in countries without established control or eradication programmes where testing and slaughter of reactor cattle is not practiced or considered acceptable for economic, social or religious reasons.

DISCUSSION

The eradication of TB from animals has faced many challenges since studies commenced in cattle, when in the early 1890s Koch's old tuberculin was found to be useful as a diagnostic tool for TB (159). Along with pasteurization of milk and slaughter of infected animals, these measures would eventually herald a new age where the impact of zoonotic TB was effectively controlled. From today's perspective it seems extraordinary to consider that stakeholders did not universally welcome these approaches as a potential panacea to reduce the burden of infection in humans. To understand this we must take account of some of the value systems that underpinned opposition to the policy at the time. In the US, which launched its TB eradication program in 1917, when TB was causing greater morbidity and mortality among cattle than all other diseases, there was often complacency and resistance to mass tuberculin testing of animals (8, 15). The TB problem was seen as wholly intractable and any broad scale measures would result in unacceptable economic losses. At that time there was also considerable resistance, particularly in the UK, from the dairy industry to any government imposed interventions that would increase production costs and where the benefits were largely unproven (10). During this period it was primarily veterinarians who supported the campaign of compulsory inspection, animal slaughter, pasteurization, and any other measures that might help to eradicate the disease (10). However, there were also many in the profession whose livelihoods depended on the custom of farmers and were opposed to some of the proposed measures. Given the high burden of disease in cattle there was also the view among interested parties that mass screening and slaughter of infected animals would decimate the dairy industry (10). The historical record highlights the different perspectives of stakeholders in dealing with a serious zoonotic disease, which in the end only succeeded in stalling progress to reduce the incidence of zoonotic TB. The emergence of the discipline of epidemiology in the past fifty years has increased our understanding of many of the risk factors associated with TB in cattle and wildlife, but it has also generated and molded the viewpoints of many different stakeholders. There is now better knowledge of wildlife sources of TB infection that are implicated in the transmission cycle of disease to cattle. It might be logical to conclude therefore, that the improved scientific knowledge base should lead to more rational and manageable control options. However, where these affected wildlife species have a high societal value, it has created a new set of stakeholders with often conflicting perspectives that is redolent of the antagonism among interested parties in the early twentieth century. Zoonotic TB may no longer be the potent driver for disease control that it was in previous decades. Instead, the rationale for TB eradication is now driven mainly by economic, trade, animal rights and conservation concerns (18, 132, 160). Each of these drivers brings elements of different moral frameworks and ethical perspectives, which sometimes clash because of the difficulties and uncertainties associated with control of the disease.

The examples of TB management in the different countries portray a range of single and multi-host wild animal systems implicated in the transmission cycle of TB that involves livestock. In most cases epidemiological investigations have helped reveal the reservoir status (maintenance or spillover) of many of the species involved, and this has informed the type of control measure applied (14, 161). How policy makers decide on the appropriate intervention strategies to address each concern is extremely difficult, but it must, by necessity, take account of the stakeholder perspectives in the local environment where the disease is proving problematic to eradicate (46). In New Zealand,

for example, the economic impact of TB transmission from possums to cattle has been reduced significantly in recent years. Nevertheless, there is broad acceptance for continued culling of possums given their perceived status as an environmental pest species. Although there has been disquiet about the widespread use of sodium fluoroacetate (1080) in the environment (49), studies have shown high societal value placed on the conservation co-benefits as a result of culling (48). Adopting the rationale of McCulloch and Reiss (89), there is measurable net utility benefit to New Zealand biodiversity, ecology and agriculture arising from culling of possums, which validates the utilitarian approach to solve the problem.

In contrast, the culling of badgers in the UK is not short of controversy and reflects the polarized perspectives and viewpoints of the principal stakeholders. These would include the dairy and beef cattle industries and associated beneficiaries on one hand, and conservationists, animal rights groups and environmentalists on the other (53). The broad middle ground of opinion may be influenced by arguments from either side. All would agree that eradication of TB is a desirable goal though they might disagree on where the control programme should be focused. The issue at hand is how TB control is best achieved and what strategies are likely to be most effective (162). Despite being a protected species in the Republic of Ireland and the UK, the culling of badgers in order to reduce densities as a means of minimizing transmission to cattle has been central to the wildlife disease control programmes (72). This is justified by the positive outcomes achieved in New Zealand following culling of possums (14). Nevertheless, in the UK this has not detracted from the determination of opponents of the current policy to resist expansion of culling areas and to advocate for complete cessation of culling (53). It appears that there is a broad range of complex evidential and ethical perspectives at play among the principal actors. Arising from the RBCT, there are continued debates as to whether reactive or pro-active culling is the most effective strategy (163, 164). It is argued by some that the scientific evidence is not sufficiently strong to warrant culling policies (165). Others adopt a moral framework based on animal health and welfare (i.e., the moral harm from culling wild animals is inconsistent with empathy, compassion or benevolence) concluding that it is fundamentally unethical and inhumane to indiscriminately kill a protected wild species that is an integral part of the natural countryside (166). The impact of culling badgers on other animals also comes into play: opportunistic analysis associated with the RBCT has shown that population counts of hedgehogs doubled over a 5-year period from the start of cull, demonstrating potential ecological consequences of badger culling and the direct impact it has on other animal species (167). These viewpoints reflect the different moral and ethical frameworks underpinning the diverse range of opinions. According to Cassidy, the societal values and cultural framing of the badger in the UK as being "good" or "bad" is at the root of the polarized opinions on how to deal with the TB problem (168). In her essay she traces the conflict as far back as the sixteenth century when badgers were listed in the Tudor Vermin Act among animals believed to interfere with human activity, and attracted a bounty per head killed. The notion of badgers being a positive cultural iconic wildlife species was promulgated in early twentieth century literature, particularly through the influence of stories such as "The Wind in the Willows" (169), notwithstanding the social attitudes that lead to ambivalence over cruel practices such as badger baiting, which took place widely over many decades until recently.

The current arguments for and against culling of badgers in the UK broadly align with the opposing framings of the badger and the societal values assigned to the badger by either side of the debate (53, 168). On the one hand they play a defining role in the perceptions of a healthy natural countryside, while on the other they pose a serious economic threat to the cattle industry by virtue of their TB status. The approach of McCulloch and Reiss is of relevance here in that by comparing the consequential outcomes of different control strategies e.g., culling vs. vaccination, it does allow for a measurable impact of different policies (89). They propose that policy decisions affecting sentient animals be subject to a mandatory Animal Welfare Impact Assessment (AWIA) based on the arguments that (a) sentient animals are owed moral considerations, (b) there is public concern about how policy impacts on the welfare of animals, and (c) international treaties pay full regard to animal welfare (170). The desired endpoint is an overall policy that defines the greatest level of benefit (who benefits and by how much?) while accounting for the different moral frameworks that fuel the disputes. It is of interest to note that the level of acrimony between opposing sides appears to be much greater in England compared with Wales, Northern Ireland and the Republic of Ireland. Although there are no comparable sociological studies, it has been suggested that the controversies in England reflect in some part the traditional different attitudes to the countryside between urbanized and rural societies (168). Ireland has historically been a largely agrarian society with few large urban centers (compared to UK), and this may have informed attitudes to the badgers and to their place in the countryside. This makes it relatively less problematic to generate policies with clearly identifiable beneficiaries.

Some stakeholders have questioned the cost-benefit of continued costly surveillance of TB given that milk pasteurization is highly effective at killing *M. bovis*, and the risk of infection from infected meat is negligible (160). While the case may have merit from the viewpoint of agricultural economics, it does represent a narrow perspective on public spending on an animal health issue. Engaging the opinions of other stakeholders, as we have asserted, serves to broaden the arguments for continued surveillance. Many countries have successfully eradicated TB and there is a societal benefit to having disease-free cattle. In other parts of the developing world, pasteurization of milk or meat inspection is not routine and *M. bovis* in unpasteurized milk poses a zoonotic risk to consumers (171, 172). If developed countries are not seen to lead the way in progressing toward eradication this might dis-incentivize others to follow similar pathways.

We have shown here that the level of engagement and ethical perspectives of stakeholders can change when wildlife disease management becomes part of an eradication programme (46). One of the major problems for policy makers is identifying the main beneficiaries of any programme, simply because there are

so many worthy candidates. In recent years, and driven by the need to better understand the disease, there have been many studies reporting new TB diagnostic tests for a variety of high value animal species (173–182). Knowledge of the extent of the disease in these animals is the first step in addressing the problem, which may prove to be very costly. The control of animal TB needs also to be considered in the context of the OIE "One health" strategy to control zoonotic diseases (183). This will require increased cooperation and communication between an expanded range of stakeholders engaged in human and animal health, the industry sector, conservation, ecologists, educators, farmers, and interested public etc. Reaching agreement on a common and standardized value system for animals may be extremely challenging, but it could represent a first step in devising solutions for TB that are realistic and achievable.

AUTHOR CONTRIBUTIONS

EG and LC contributed equally to this review including critical analysis of published data and preparation of the manuscript. The opinions expressed in this paper are solely those of the authors.

ACKNOWLEDGMENTS

We would like to acknowledge funding and support provided by the Department of Agriculture, Food & the Marine Food (DAFM) for all of the badger studies in Ireland. We are very grateful for constructive discussions with Paul Livingstone (NZ), Ana Balseiro (Spain), Sven Parsons (SA), Dan O'Brien (USA) and Lin Marie De Klerk (SA), and for valuable insights into animal TB in their respective countries.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 08 October 2018
doi: 10.3389/fvets.2018.00244

Use of the Human Vaccine, *Mycobacterium bovis* Bacillus Calmette Guérin in Deer

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The only vaccine ever approved for human tuberculosis was developed a century ago from an isolate of *Mycobacterium bovis* derived from a tuberculous cow. Initial safety and efficacy studies of an attenuated version of this isolate were conducted in cattle and other animals. In 1921 the first human, an infant, was orally dosed with this attenuated strain that came to be known as *M. bovis* bacillus Calmette-Guérin (BCG); named for Albert Calmette and Camille Guérin, the two French scientists that developed the strain. Since 1921, billions of people have been vaccinated with BCG making it the oldest, most widely used, and safest vaccine in use today. It is also the tuberculosis vaccine most studied for use in wildlife, including deer. While BCG vaccination of deer may not reliably prevent infection, it consistently decreases lesion severity, minimizing large, necrotic lesions, which often contain large numbers of bacilli. It is believed that decreased lesion severity correlates with decreased disease transmission; however, this hypothesis remains to be proven. Safety studies in white-tailed deer show BCG may persist in lymphoid tissues for up to 12 months; a factor to be considered in deer used for food. Beyond efficacy and safety, methods of vaccine delivery to free-ranging deer are also under investigation, both in the laboratory and in the field. The ideal delivery method is effective, efficient and safe for non-target species, including livestock. Ingestion of BCG by cattle is of special concern as such cattle may present as "false positives" using currently approved diagnostic methods, thus interfering with efforts by animal health agencies to monitor cattle for tuberculosis. An effective BCG vaccine for deer would be of value in regions where free-ranging deer represent a potential source of *M. bovis* for livestock. Such a vaccine would also be beneficial to farmed deer where *M. bovis* represents a serious threat to trade and productivity.

Keywords: BCG, deer, mycobacterium, tuberculosis, vaccine, wildlife

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *30 July 2018*
Accepted: *14 September 2018*
Published: *08 October 2018*

Citation:
Palmer MV and Thacker TC (2018) Use of the Human Vaccine, Mycobacterium bovis Bacillus Calmette Guérin in Deer. Front. Vet. Sci. 5:244. doi: 10.3389/fvets.2018.00244

INTRODUCTION

Mycobacterium bovis is the cause of tuberculosis in most animal species, including man. Clinical signs and pathological manifestations of *M. bovis* in humans can be identical to infection with the more common cause of human tuberculosis, *Mycobacterium tuberculosis*. The range of susceptible hosts to *M. bovis* is broad and includes most species of both livestock and wildlife. For decades, most developed countries have conducted costly campaigns to eradicate tuberculosis from cattle with varying success (1). In cases where a wildlife reservoir of *M. bovis* infection exists, eradication

has been difficult, if not impossible (2) due to transmission of *M. bovis* from livestock to wildlife (spillover) and subsequent transmission from wildlife back to livestock (spillback). In northeast Michigan, USA there is a focus of *M. bovis* infection in free-ranging white-tailed deer (*Odocoileus virginianus*) where infected deer have been implicated as the source of infection in 69 cattle herds from 1995 through 2017. Control efforts, including increased hunting have been effective in decreasing disease prevalence from 4.9% in 1995 to 1.7% in 2004, but prevalence continues to remain at approximately 2% (3, 4).

In addition to white-tailed deer in the US, there is general consensus that the European badger (*Meles meles*) in the United Kingdom and the Republic of Ireland, the brushtail possum (*Trichosurus vulpecula*) in New Zealand, and the European wild boar (*Sus scrofa*) in the Iberian Peninsula represent wildlife reservoirs of *M. bovis* and can be a persistent source of re-infection of cattle (5–12). Attempts to control or eliminate these reservoirs of infection have involved population reductions through hunting, trapping or poisoning, as well as physical exclusion of wildlife from cattle feeding areas through barrier fencing. In all cases, vaccination of wildlife to reduce wildlife-to-cattle transmission has been investigated, with some vaccines progressing to field trials (13, 14). The goal of vaccination is to induce an immune response such that the animal is resistant to infection or if infection occurs, disease severity is lessened and transmission is reduced or eliminated. Thus, a successful wildlife vaccine need not provide complete protection from infection (15, 16).

Vaccines other than BCG have been successfully used in wildlife to control rabies in raccoons (*Procyon lotor*), foxes (*Vulpes vulpes*), skunks (*Mephitis mephitis*) and coyotes (*Canis latrans*) in Europe and North America (17–19); plague in North American black-tailed prairie dogs (*Cynomys ludovicianus*) (20–23); and classical swine fever in wild boar (*Sus scrofa*) in Europe (24). There have been no widespread efforts to vaccinate wildlife to control tuberculosis, although there is currently one approved vaccine for use in European badgers (25) and field trials are progressing (14).

HISTORY OF BCG

The most studied tuberculosis vaccine in deer, as well as other wildlife is the attenuated strain of *M. bovis* known as bacillus Calmette-Guérin (BCG), named for Albert Calmette and Camille Guérin, two French scientists at the Pasteur Institute that developed the strain (26). BCG vaccines are the oldest vaccines still in use today; moreover, with over four billion people vaccinated in over 180 countries it is history's safest and most widely used vaccine (27) and it remains the only approved tuberculosis vaccine for humans. Protective immunity in adults is highly variable, ranging from 0 to 80% depending on the study (28). In adults, BCG vaccination does not reliably prevent infection, development of latent tuberculosis, or reactivation of latent disease (29). However, in infants BCG has proven beneficial and highly cost-effective in protecting children from tuberculous meningitis (30, 31).

In 1901, French veterinarian and microbiologist, Edmond Nocard transferred to Calmette and Guérin a virulent isolate of *M. bovis* he had recovered from a cow with tuberculous mastitis (32). From this isolate, BCG was developed through continuous subculture on a media composed of ox bile, glycerin and potatoes. In 1919, after 13 years and 231 subculture passages, virulence in various animal models was lost (i.e., rabbits, guinea pigs, cows, horses, hamsters, mice, dogs, chickens, non-human primates) (33–35). The attenuation of BCG was shown to be irreversible upon further cultivation on bile-potato medium (36) and passage through various animal species (33). The first human was vaccinated in 1921 when an infant was orally dosed with live BCG. The infant's mother had died of tuberculosis and the infant's caregiver, the grandmother, had clinical tuberculosis. In spite of what must have been significant exposure to virulent *M. tuberculosis*, the child developed normally with no signs of tuberculosis (33). In the following 3 months after this first vaccination, 317 infants were vaccinated and by 1924 more than 660 infants had been orally vaccinated (26). Oral, subcutaneous, intraperitoneal and intravenous routes of administration all proved safe. Although originally given orally, the current recommendation for BCG vaccination is intradermal injection (37). The original BCG was not cloned, but was distributed to many laboratories worldwide, where the vaccine was propagated, such that today there are many genetically variant BCG strains, none of which are identical to each other or to the original vaccine (26, 32, 38). The various substrains differ in immunogenicity and potency; a possible reason for historically large ranges of observed efficacy in human studies around the world (26, 32, 39). Currently, five strains account for >90% of the BCG used worldwide; Pasteur 1173 P2, Danish 1331, Glaxo 1077, Tokyo 172-1, Russian BCG-I and Moreau RDJ (40). The two strains most commonly used in deer studies are strains Danish 1331 and Pasteur 1173 P2. The isolate that would later become BCG Danish was received directly from Calmette in 1931 by Statens Serum Institut. In 1960, batch 1331 was freeze-dried and eventually adopted as the primary Danish 1331 seed-lot in 1966 (32). The strain Pasteur 1173 P2 originated in 1961; produced from a colony closely resembling the original descriptions of BCG by Calmette (32). In white-tailed deer studies, both strains have demonstrated some degree of protection (41).

Calmette and Guérin recognized in animal studies that vaccination prevented disease, but did not always prevent infection (36), a finding consistent with most modern BCG studies in animals (42–45). Although developed as a vaccine for humans, it was first proven efficacious in cattle circa 1911. Calmette and Guérin recommended widespread oral BCG vaccination of neonatal calves, since older calves may have already been infected with virulent *M. bovis* (36). Safety studies in other mammals including horses, sheep, dogs, rabbits, guinea pigs, non-human primates, rats, mice, chickens, and pigeons showed no untoward effects (33).

MODEL OF INFECTION

To study vaccine-induced protection, a reliable model of infection is of paramount importance. The ideal model is repeatable, technically feasible, and produces disease similar to that seen in natural infection. The best and most widely used model of tuberculosis in deer was developed in New Zealand

using red deer (*Cervus elaphus*) and a low dose (200–500 colony forming units, CFU) intratonsilar inoculation (46); where virulent *M. bovis* is deposited into one or both palatine tonsillar crypts. Using this model, many experiments were carried out to identify critical variables in BCG studies, such as dose, route, boosting and detailed immune responses (47–52). The red deer model has been extended for use in white-tailed deer (53). In both deer species, the intratonsilar model results in primary involvement of the medial retropharyngeal lymph node (46, 53), the most commonly affected tissue in naturally infected deer (54–56). The frequent involvement of the medial retropharyngeal lymph node suggests that the primary route of infection in deer is oral; although contribution by aerosol cannot be excluded (57–59). Further supporting a primary oral route of infection is the finding that experimental infection of white-tailed deer via an aerosol did not result in lesion distribution similar to natural infection, but rather resulted in disease focused on the lungs and pulmonary lymph nodes (60).

VACCINE EFFICACY

Vaccine doses of 10^4-10^7 CFU of BCG provided significant levels of protection against infection and disease (lesion development) in red deer (51), while 10^7 CFU (parenteral) and 10^8 CFU (oral) demonstrated similar efficacy in white-tailed deer (41, 61, 62).

There are no known antemortem immune responses that correlate to BCG-induced protection. Measurements of immune responses to vaccination such as intradermal skin testing or cytokine production do not predict protection in any species. Rather, BCG efficacy is measured through postmortem quantitative or semi quantitative assessments of disease severity, as well as measuring the level of tissue colonization (63, 64). Disease severity assessments include subjective scoring of gross lesions based on size, number, presence of liquefactive or caseous necrosis or fibrous encapsulation, and the number of tissues with lesions and from which virulent *M. bovis* can be isolated. Protection has also been evaluated by considering the extent and distribution of lesions, that is, animals with lesions limited to a single body region are considered more protected than those with lesions in multiple anatomic locations such as cranial lymph nodes, thoracic lymph nodes and abdominal organs (41, 43, 61, 62, 65).

In white-tailed deer and red deer, oral (43, 51, 62) or subcutaneous (41, 43, 51, 61) BCG vaccination results in fewer lesions, as well as fewer tissues from which virulent *M. bovis* may be isolated. Using subjective gross lesion scoring, BCG vaccination of deer decreases lesion severity and limits disease dissemination. Microscopic examination of tissues reveals that vaccinated deer have fewer large necrotic lesions that contain large numbers of acid-fast bacilli compared to non-vaccinated animals (41, 43, 61, 62). Both live and inactivated BCG in saline and oil adjuvant, as well as a recombinant BCG expressing the inflammatory cytokine IL-2 have been evaluated in red deer (66, 67). Detailed studies show significant immune responses to some of these preparations; however, necropsy and pathology results are not always available from these studies making vaccine efficacy determination difficult. Studies in red deer have also shown that a homologous prime boost regime (i.e., two doses 4–8-weeks apart), further reduces infection and disease (48, 68, 69). A single study in white-tailed deer demonstrated no significant difference between a single vaccination and a homologous prime-boost approach (61). Reduction of disease transmission through BCG vaccination remains to be demonstrated in deer.

In other wildlife species, the time to seroconversion, and transmission from adults to offspring have been used to demonstrate BCG-induced protection in European badgers (13, 14). The median time to seroconversion was significantly longer for vaccinated badgers (413 days), compared to non-vaccinates (230 days) (14). In addition to a direct protective effect of badger vaccination, there was a positive indirect effect on unvaccinated badger cubs. When at least one third of a badger social group was BCG vaccinated, the probability of an unvaccinated badger cub testing positive for *M. bovis* infection was reduced by 79% (13). The use of such metrics in deer would be difficult due to differing social structures, fecundity and biology.

VACCINE DELIVERY

The most efficacious vaccine is of little use if it cannot be delivered to the target population. An effective means of delivery requires knowledge of host feeding behavior, climatic effects on bait matrix composition, environmental survivability of the vaccine, and bait attractiveness and palatability to the target host. In most cases the only effective means to vaccinate wildlife is through an oral bait. Oral vaccines have been used experimentally to protect white-tailed deer from the prion-based, chronic wasting disease (70, 71), as well as brucellosis (72).

A variety of oral baits have been evaluated in wildlife. Dried shell corn has been used to deliver an acaricide to free-ranging white-tailed deer (73, 74) while Hakim, et al showed that free-ranging white-tailed deer found a liquid bait composed of apple juice, water and glycerin palatable; thus a plausible means of delivering pharmaceutical agents (75). A molasses-based bait for potential BCG delivery was evaluated for palatability, attractiveness and stability under various environmental conditions (76). Although environmentally stable and attractive for captive deer, field testing demonstrated a lack of palatability to free-ranging deer. A lipid formulation of BCG has been used as an oral vaccine for brushtail possums (77, 78), and European badgers (45, 79). The same BCG lipid-formulated bait has been used in white-tailed deer, and although vaccination was achievable (43, 80), deer found the lipid formulation unpalatable. In Spain, baits prepared from feed mixed with paraffin, sucrose and cinnamon-truffle powder worked well to deliver BCG to wild boar (81, 82), but have not been evaluated in deer.

A potential hazard of oral bait vaccines, is the difficulty of preventing non-target species from consuming the vaccine bait. Cattle are a non-target species of special interest as it is possible that BCG ingestion could result in sensitization to the tuberculin used in intradermal skin testing resulting in false positive results; thus, confounding accurate identification of infected cattle (83). Alternative diagnostic tests, able to

differentiate infected from vaccinated (DIVA) cattle would be needed to avoid this confounding problem (84–86). In addition to exposure of non-target species to vaccine, dosage is difficult to control using oral baits. The effect of higher than recommended doses of vaccine should be evaluated in the target population. In red deer, no untoward effects have been seen using subcutaneous doses of BCG up to 1×10^8 CFU (68); 10–100 times the regular dose, or in white-tailed deer using oral doses of 1×10^9 (80, 87) to 1×10^{10} CFU; 10–100 times the regular dose.

Studies in red deer did not demonstrate shedding of BCG from vaccinates to non-vaccinates (66); however, evidence shows that BCG-vaccinated white-tailed deer shed vaccine and cohorts can become "secondarily vaccinated" (88, 89). It remains to be evaluated whether deer vaccinated secondarily through shed BCG possess any protection against infection with virulent *M. bovis*. If secondary vaccination were to provide protection, this self-disseminating feature could serve to increase vaccine coverage without additional labor or cost. However, the shedding of BCG by deer increases the possibility that non-target species such as cattle could be exposed to BCG. Thus far, indirect contact of calves with BCG-vaccinated white-tailed deer has not resulted in deer-to-cattle transfer of BCG (88, 89).

By comparison, orally vaccinated possums and badgers were shown to shed BCG in feces for up to 7 and 17 days, respectively, after vaccination (44, 90), while excretion could not be detected in orally vaccinated wild boar (82).

SAFETY

Vaccine safety may be viewed from both the perspective of either the vaccinated animal or humans that may come into contact with vaccinated animals. No untoward effects have been reported in BCG-vaccinated deer, possums or badgers (66, 91, 92). In white-tailed deer vaccinated subcutaneously with BCG, but not challenged with virulent *M. bovis*, microscopic, but not gross lesions due to BCG were reported in various lymph nodes (superficial cervical, tracheobronchial, hepatic) as late as 250 days after vaccination (41).

Although BCG has proven safe in humans with uncompromised immune systems, use of BCG in immunocompromised individuals can result in disseminated disease, with infection in various organs and body systems (93, 94). Because BCG may persist in tissues of vaccinated deer, hunters could potentially be exposed to BCG while field dressing vaccinated deer and unlike many other wildlife hosts of *M. bovis*, deer may be consumed as food by humans. In BCG-vaccinated white-tailed deer, vaccine was recovered from lymphoid tissues up to 12 months after oral dosing of 10^9 CFU. Lowering the dose to 10^8 CFU decreased persistence to 9 months. Persistent and viable BCG were limited to lymphoid tissues such as cranial lymph nodes, tracheobronchial, hepatic and mesenteric lymph nodes. Importantly, samples of muscles commonly consumed by hunters (epaxial, sublumbar, supraspinatus, triceps, semimembranosus, semitendinosus and biceps femoris) did not yield viable BCG at any time point (80, 87). In BCG-vaccinated red deer, viable vaccine could be recovered from various lymph nodes and the site of vaccination 14 weeks after vaccination, although the numbers of recoverable CFU were extremely low, 32–57 CFU/node and 150–190 CFU/vaccination site, representing 0.007–0.009% and 0.002–0.003%, respectively, of the original inoculum dose (2×10^6 CFU) (67). It has been shown that thoroughly heating meat products to 60°C (140^0 F) for 6 min kills virulent *M. bovis* (95) and *M. avium* (96). It is assumed the same would be true for *M. bovis* BCG. As humans generally avoid consumption of lymphoid organs and usually cook meat before consumption (97), the potential exposure of humans to BCG from vaccinated deer is very low.

By comparison, BCG has been found in the tissues of orally vaccinated badgers 30 weeks after vaccination (44) and in possums 8 weeks after oral vaccination (90). In contrast, BCG could not be found in the tissues of orally vaccinated wild boar (82) even when examined 30 days after vaccination (98), an important finding as wild boar, similar to deer, are often used for food.

NON-TUBERCULOUS MYCOBACTERIA

Many saprophytic, non-pathogenic species of mycobacteria exist in soil and water. These mycobacteria may be collectively described as non-tuberculous mycobacteria (NTM). Numerous NTM have been isolated from deer (41, 61, 62, 80, 99–101), some of which were found within lesions consistent with tuberculosis. Although some studies have suggested that preexisting sensitivities to *M.avium*, or other NTM, has no effect or confers some degree of protection against virulent challenge (102–106), others show interference with BCG efficacy by NTM exposure in humans, laboratory animals and cattle (102, 104, 107, 108). One proposed mechanism for this reduced efficacy is that pre-existing immune sensitivity to NTM restricts BCG multiplication following vaccination, resulting in dampening of critical cytokine responses, such as that of interferon-gamma (108). For this reason, it is recommended that humans and calves be vaccinated as neonates prior to NTM exposure. It is, as yet unclear how exposure to NTM affects BCG efficacy in deer. Vaccination of neonates, although possible in farmed deer, would prove very difficult in free ranging deer.

FUTURE DIRECTIONS

Self-disseminating Virus-Based Vaccines

One limitation of traditional oral or parenteral vaccination is the need to administer vaccine to every animal individually. Furthermore, with many inactivated vaccines, adequate protection requires subsequent booster vaccinations. In contrast, self-disseminating vaccines are designed to exploit replicating virus-based vectors to spread within the target animal population without the need for individual animal inoculation (109). Vaccination of a limited number of animals introduces the vaccine into the target population and the vaccine is spread naturally as it is shed by vaccinates. Ideal self-disseminating vaccines are viruses with high immunogenicity and high horizontal transmission levels, but with a robust species barrier to minimize infection of non-target species.

Examples of self-disseminating virus-based vaccines include a cytomegalovirus-based vaccine targeting deer mice (*Peromyscus maniculatus*) to interrupt transmission of Sin Nombre hantavirus, and a myxoma virus-based vaccine targeting European hares (*Oryctolagus cuniculus*) to prevent myxomatosis and rabbit hemorrhagic disease [reviewed in Murphy et al. (109)]. A similar self-disseminating viral vectored vaccine targeting white-tailed deer to prevent deer-to-deer and deer-to-cattle transmission of *M. bovis* may one day be possible.

Plant-Based Vaccines

Another alternative to traditional vaccination is the use of plant-based vaccines (110). Selected immunogenic antigens of the pathogen are introduced into a plant, creating a recombinant edible vaccine. Ingestion of the plant material induces a protective immune response against that particular pathogen. Plant-based vaccines are cost-effective and amenable to large scale production (110); moreover, using plants that are part of the normal diet of the target population minimizes issues of palatability and acceptance. Edible vaccines have been produced in tobacco, cereal grains, fruits (banana, tomato), leaves (lettuce, alfalfa), tubers (potato, carrot), and legumes (cow pea, soybean) (111). When produced in plants, antigenic proteins of the vaccine are bioencapsulated in plant cells, to be released when plant cells are digested by microbes of the gut (112). This may be particularly advantageous with diseases such as tuberculosis where mucosal immune responses are critical. Transgenic carrots, tobacco, lettuce and arabidopsis expressing *M. tuberculosis* proteins have been tested in mice and piglets and shown to induce both humoral and cell-mediated immunity (112–115).

Inactivated Vaccines

Attenuated live vaccines, like BCG have some drawbacks. The possibility exists that vaccine shed by vaccinates, may contaminate not only the environment, but also potentially expose various non-target species. Use of genetically altered subunit vaccines may be an alternative; however, there could be public resistance to the use of genetically altered microbes. Heat-inactivated *M. bovis* (oral and parenteral) has been shown to reduce disease severity in wild boar (82, 116) similar to protection provided through vaccination with BCG (117), without risk of environmental contamination or spread to non-target species. Similarly, heat-inactivated *M. bovis* has been shown to decrease disease severity in experimentally infected red deer (118). Another noted advantage to heat-inactivated *M. bovis* is that vaccinated calves did not have false positive responses in either antibody-based assays or interferon gamma release assays measuring cell-mediated immune responses (118) reducing concern that vaccine exposed cattle would be falsely identified as *M. bovis* infected during routine surveillance.

CONCLUSIONS

Between 1940 and 2004, more than 335 emerging infectious disease events were reported in the scientific literature. The majority (60%) of those events involved zoonoses, most of which (72–80%) had an epidemiologically important wildlife host (119, 120). Controlling or eliminating disease, which has become established in wildlife is extremely difficult, with seemingly few solutions, such as population reduction, separation of wildlife from livestock and disease control through vaccination. Varying degrees of success have been achieved with rabies, plague and classical swine fever. In the case of tuberculosis in deer and other wildlife, the challenge is indeed monumental. In spite of millions of research dollars and countless hours of research effort toward a new human vaccine, the only approved vaccine remains one that is 100 years old and provides questionable protection in some settings. Far less money and effort have been expended exploring a vaccine for animal tuberculosis. Nevertheless, there is reason to be optimistic. Regardless of the species, research to date on BCG vaccination consistently demonstrates a decrease in disease severity, which likely results in decreased disease transmission, and progress is being made in the development of oral baits as vaccine delivery devices. Moreover, advances are being made in the next-generation of human vaccines based on BCG (79), some of which may prove useful for vaccination of deer or other wildlife.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work funded by the USDA Agricultural Research Service.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 26 July 2018
doi: 10.3389/fvets.2018.00173

The Distribution of Bovine Tuberculosis in Cattle Farms Is Linked to Cattle Trade and Badger-Mediated Contact Networks in South-Western France, 2007–2015

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Bovine tuberculosis (bTB), mainly caused by *Mycobacterium bovis*, can affect domestic and wild animals as well as humans. Identifying the major transmission mechanisms in an area is necessary *for* disease control and management. In this study, we aimed *to evaluate* the involvement of different types of contact in *M. bovis* transmission between cattle farms of south-western France between 2007 and 2015. We analyzed an empirical contact network of cattle farms as nodes, with known infection status and molecular types (16 circulated during the study period of which 14 affected only cattle and two both badgers and cattle). Edges were based on cattle trade data (T-edges) and on spatial neighborhood relationships between farms, either direct (P-edges) or badger-mediated, when two farms neighbored the same badger home range (B-edges), or two distinct but neighboring badger home ranges (D-edges). Edge types were aggregated so that the contact network contained only unique edges labeled by one or several edge types. The association between the contact network structure and bTB infection status was assessed using a non-parametric test, each molecular type being considered a marker of an independent epidemic. Using a logistic regression model, we estimated the contribution of each edge type to the probability for an edge originating from an infected farm to end at another infected farm. A total number of 1946 cattle farms were included in the study and were linked by 54,243 edges. Within this contact network, infected farms (whatever the molecular type) always belonged to the same component, suggesting the contact network may have supported bTB spread among those farms. A significant association between the pattern of bTB-infected farms and the structure of the contact network was observed when all the molecular types were simultaneously considered. The logistic regression model showed a significant association between *M. bovis* infection in direct neighbors of infected farms and the connection by T-, B- and D-edges, with odds-ratios of 7.4, 1.9, and 10.4, respectively. These results indicate a multifactorial *M. bovis* transmission between cattle farms of the studied area, with varying implication levels of the trade, pasture and badger networks according to the molecular type.

Keywords: bovine tuberculosis, network analysis, cattle herds, badger-cattle interface, cattle trade, pastures

OPEN ACCESS

Edited by:
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Reviewed by:
Joseph Crispell, University College Dublin, Ireland
Helen R. Fielding, University of Exeter, United Kingdom

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *05 May 2018*
Accepted: *04 July 2018*
Published: *26 July 2018*

Citation:
Bouchez-Zacria M, Courcoul A and Durand B (2018) The Distribution of Bovine Tuberculosis in Cattle Farms Is Linked to Cattle Trade and Badger-Mediated Contact Networks in South-Western France, 2007–2015. Front. Vet. Sci. 5:173. doi: 10.3389/fvets.2018.00173

INTRODUCTION

Since its discovery by Theobald Smith in the late 1800's (1) *Mycobacterium bovis*, the main agent of bovine tuberculosis (bTB) has been found in a wide variety of domestic and wild animal hosts, as well as in humans (2, 3). In Europe, the main host of *M. bovis* is cattle (4–6), but sheep (7), pigs (8) and goats (9) can be affected too. Wildlife species found infected on this continent include red deer (*Cervus elaphus*) (10, 11), roe deer (*Capreolus capreolus*) (12), red fox (*Vulpes vulpes*) (13–16), wild boar (*Sus scrofa*) (17, 18) and badger (*Meles meles*) (19–21).

Different routes may allow *M. bovis* transmission between wild and domestic hosts. The largest part of *M. bovis* shedding seems to occur through aerosols (respiratory tract secretions) and to a lesser extent through saliva, urine, feces (20, 22, 23), milk in cattle (24) and even wound exudates in badgers (20). Therefore close contacts (e.g., nose to nose) between infected individuals and susceptible ones can allow the transmission of *M. bovis*. However, several studies have shown that *M. bovis* may survive outside a host in a favorable environment for several months (24–26), allowing transmission through indirect contacts. *M. bovis* transmission between cattle can also involve different susceptible species either wild (27) or domestic [although the implication of other domestic species than cattle remains unclear regarding cattle transmission (24)]. At the herd level, several risk factors of bTB have been identified such as larger herd sizes, neighborhood with other herds, cattle movements, farm management practices such as grazing, dispersion of slurry on pastures or the share of water points (24, 28–31). Environmental risk factors have also been studied, with certain environmental conditions favoring the survival and persistence of *M. bovis* (such as shade, moisture or even some soil types) that foster *M. bovis* transmission (24–26). A third category of risk factors involves wildlife interactions, especially with badgers, wild boars and deer. For the latter two species, the sharing of feed or water on pastures appears to be a risk factor of *M. bovis* indirect transmission (23, 32, 33). The transmission between badgers and cattle seems a bit more complex, with uncertain direct contacts on pastures (34–36) and/or inside farm buildings (37). This interspecies transmission could occur on pastures through the shedding of the mycobacteria in urine and feces of infected badgers (24), and in respiratory tract secretions and feces of infected cattle (6, 29).

BTB molecular types are stable (38, 39) and can be used to trace independent epidemics (4). In France, while the officially bTB-free status was obtained in 2000, *M. bovis* infection has persisted in several regions. In 2014, 46% of incident outbreaks were detected in south-western France, with a national number of 105 cattle herds newly detected infected (40). Molecular typing methods spoligotyping (39) combined to MLVA (Multiple Loci Variable Number of Tandem Repeats, VNTR Analysis) based on MIRU-VNTR [Mycobacterial Interspersed Repetitive Unit–VNTR; (4, 38)] have allowed identifying 16 molecular types in this area between 2007 and 2015 from cattle isolates, two of which were shared between cattle and wildlife (4). Because spoligotype and MIRU-VNTR are considered stable markers (at least at a time horizon of several years), these 16 molecular types allow identifying 16 independent epidemics spreading in the same area during the same time period.

An effective way of representing the structure of contacts between hosts of an infectious disease consists in building networks (41), with epidemiological units as nodes, to which an infection status is associated. Edges linking nodes represent the contacts between epidemiological units that may allow the transmission of the disease agent. Regarding *M. bovis* transmission between cattle in France and in light of the above, nodes can represent cattle farms and edges may represent direct or indirect contacts between them. Two types of direct contacts may be featured by edges between farms: (i) contacts due to the trade of live cattle (42, 43) and (ii) contacts due to pasture neighborhood between cattle belonging to different farms but with nose to nose contacts over the fence (31, 44, 45). Besides, indirect contacts between cattle farms due the presence of wildlife may also be represented by edges. Concerning the badger, a known susceptible species to *M. bovis* infection (21, 40), the spatial organization of social groups with stable home ranges around setts (46, 47) allows us to represent indirect contacts with cattle based on the spatial intersection between farm pastures and home ranges (48).

The aim of our study was to analyze *M. bovis* transmission between cattle farms in a south-western area of France using contact networks and molecular types as infection status information. We built different networks featuring possible direct and indirect contacts between cattle farms and analyzed the association between their structure and the observed pattern of infected farms.

MATERIALS AND METHODS

Cattle Data

The study population was made up of the 1946 farms having reported cattle between January 2007 and March 2016 (end of the 2015 herd skin-testing period) and owning at least one pasture included in a 2,735 km² study area, an area straddling the border of *Pyrénées-Atlantiques* and *Landes* French departments (**Figure 1**). Pastures were defined as land parcels used by cattle for grazing according to the *"Relevé Parcellaire Graphique"* (RPG) of 2013 provided by the French Ministry of Agriculture. Two pastures were considered neighbors if the minimal distance between their borders was less than 3 m. Farm sizes (number of bovine females over two years old) and types (dairy, beef, fattening, mixed, small and other herds) were obtained from the French cattle tracing system (*"Base de Données Nationale d'Identification"* denoted below BDNI) (**Table 1**).

BTB surveillance data were provided by the French Ministry of Agriculture. Herd skin-testing was performed each year in the study area in communes (the smallest French administrative subdivision) where infected farms had been detected the previous year, as well as in the neighboring ones, using either single intradermal comparative tuberculin tests (SICTT) (in all dairy farms or in farms located in the communes with confirmed infected farms) or single intradermal tuberculin tests (SITT) (in all the other situations), both performed in

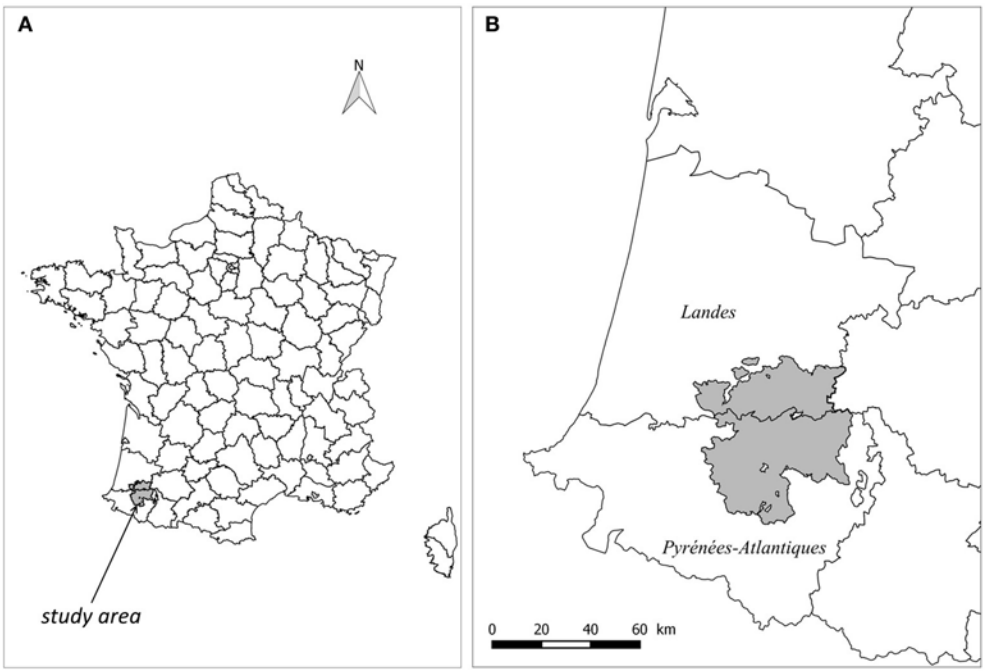

FIGURE 1 | Location of the study area **(A)**, at the border between *Pyrénées-Atlantiques* (south) and *Landes* (north) French departments **(B)**.

TABLE 1 | Description of cattle farms included in the study population.

Farm type	Number of farms	Number of pastures (*)		Herd size (**)		Percentage of farms detected infected (***)
		mean	SD	mean	SD	
Beef	922	9.5	6.5	54.6	34.2	4.2 ($n = 39$)
Dairy	294	8.6	6.0	74.4	43.1	3.7 ($n = 11$)
Fattening	57	7.5	5.8	32.1	28.4	5.2 ($n = 3$)
Mixed	30	12.3	6.5	93.6	32.8	3.3 ($n = 1$)
Other	259	6.3	4.8	21.1	22.9	3.9 ($n = 10$)
Small	384	4.6	3.9	6.7	4.2	1.3 ($n = 5$)
All	1946	7.9	6.1	43.6	39.0	3.5 ($n = 69$)

*, pastures included in the study area; **, number of females of more than 2 years old; ***, at least once over the study period*

the cervical region. In the other communes of the study area, herd testing was biennial in *Landes* department, and triennial in *Pyrénées-Atlantiques* department. *M. bovis* infection was confirmed by polymerase chain reaction (PCR) and/or bacterial culture (either following a positive skin test or the detection of a suspect lesion during routine meat inspection at a slaughterhouse) (40) in 69 cattle farms of the study area during the study period; all the cattle of these farms were subsequently slaughtered and molecular typing was performed on each bovid found infected (with a mean of four cattle per farm detected infected during the study period). Molecular typing results were provided by the National Reference Laboratory (NRL) (Anses, Maisons-Alfort). The combination of spoligotyping and MLVA based on MIRU-VNTR allowed identifying 16 distinct molecular types (**Table 2**). A unique molecular type was identified in all of the 69 detected infected farms, except two where several molecular types were identified.

A farm was classified infected by a given molecular type if this type had been detected at least once in the farm during the study period. Because of the geographic differences in the frequency of skin testing, having detected *M. bovis* earlier in a given farm than in another one does not imply that the former had been infected earlier than the latter. For this reason, the detection dates could not be taken into account.

Badger Data

Two thousand four hundred and 25 badger setts were identified and geolocalised by hunters in the study area, between 2013 and 2015. Around those setts, considered as main setts (i.e., hosting a social group), we defined badger home ranges using a two-step procedure: (i) a Dirichlet tessellation was first built

TABLE 2 | Number of cattle farms detected infected per molecular type during the study period and within the study area.

Molecular types	Number of farms	First and last year of detection
SB0120b	1	2007
SB0120c	2	2009–2011
SB0121a	1	2012–2013[d]
SB0121b	1	2011
SB0121c[b]	1	2012
SB02065[b]	1	2012
SB0295[b]	1	2012
SB0821[a,c]	44	2007–2015
SB0823[c]	1	2010
SB0825[b]	1	2012
SB0827[b]	1	2012
SB0832[a]	13	2012–2015
SB0851	1	2011
SB0853	1	2009
SB0867[b]	1	2012
SB0928	4	2007–2012

[a] *molecular types found both in cattle and badgers.*
[b] *molecular types found in the farm where six molecular types were identified.*
[c] *molecular types found in the farm where two molecular types were identified.*
[d] *the same farm as in 2012 (recontamination).*

around all setts [in which the perpendicular bisectors of each segment between two adjacent setts delineate the home range around one given sett, thus assuming that boundaries were located halfway between neighboring main setts (47)] and (ii) to avoid unrealistically home range large sizes, a home range was defined as the intersection of a tile with a 1,000 m-radius buffer area drawn around the setts (48). Two setts were considered neighbors if the corresponding home ranges were adjacent. A sett and a farm were considered neighbors if one of the farm pastures intersected with the badger home range.

BTB surveillance data were provided by the French Ministry of Agriculture. In the study area, bTB surveillance in badgers was performed according to the "Sylvatub" surveillance network, which started in 2012 in the study area (49). Surveillance protocol included badger trapping (i) within a 1.5 km-radius around confirmed infected farms, (ii) within a 2 km radius around setts with confirmed infected badgers and (iii) in communes at less than 5 km of communes where confirmed infected farms were located (one badger per sett). Trapping was performed using stopped restraints (https://www.plateforme-esa.fr/filedepot_download/35377/100) and snares were checked the morning after the day they were set up within the 2 h following sunrise, in order to limit the stress of trapped badgers. Trapped badgers were culled by head shot except in a minority of cases where they were found already dead (due to trap related injuries that sometimes occurred when snares were placed on sloping terrain, with no possible alternative). Road-killed badgers were also considered. Stopped restraints used for trapping were placed near sett entrances, those setts being considered as the sett of the trapped animals. Where badgers were found dead along roads, hunters reported the most probable sett according to their knowledge of the area (48). All the trapped and road-killed badgers were tested for *M. bovis* infection. Among 401 analyzed badgers (4.5% were road-killed badgers), 11.2% were detected infected (45 animals, one was a road-killed badger), of which 39 harbored the SB0821 molecular type and 6 the SB0832 molecular type, both molecular types having also been found in cattle (**Table 2**). All the badgers trapped could be attributed to 113 distinct setts, of which 33.6% hosted at least one infected badger (32 setts with at least one badger detected infected by SB0821 and 6 by SB0832). Road-killed badgers were attributed to five distinct setts. For four of these setts, the analysis of road-killed badgers did not provide additional information as they had also been subjected to trapping measures. For the fifth sett, the analysis of one road-killed badger allowed the detection of infection (SB0821 molecular type), not revealed by trapping. Setts with at least two badgers tested negative were considered as uninfected ($n = 75$). All the remaining setts, either with only one badger tested negative or without analyzed badger were considered of unknown status.

Contact Network

A contact network was built using farms of the study population as nodes, and four types of edges (**Figure 2**):

- A trade edge (denoted T-edge below) from farms i to farm j represented the sale of one or several cattle by farm i to farm j during the study period, at one or several occasions;
- A pasture neighborhood edge (denoted P-edge below) between farms i and j represented the fact that a pasture owned by i and another one owned by j were neighbors;
- A simple badger-mediated edge (denoted B-edge below) between farms i and j represented the fact that both farms were neighbors of a given sett;
- A second level badger-mediated edge (denoted D-edge below) between farms i and j represented the fact that (i) farm i was neighbor of a sett k_1, (ii) farm j was neighbor of a sett k_2, and (iii) the setts k_1 and k_2 were themselves neighbors.

To avoid duplicated edges, the types of edges (T, P, B and D) were aggregated at the edge level. The full contact network thus contained only unique edges labeled by one or several edge types (**Table 3**). Because the T-edges are directed, each undirected P-, B- and D-edge was transformed into two symmetric directed edges. The full contact network was thus a directed network.

Subnetworks were extracted from the full contact network by restricting the edges to those of specific types (**Table 3**). These subnetworks are termed below T-network, P-network, B-network and D-network. Similarly, we used edge types to split the full contact network in three non-overlapping subnetworks:

- the cattle-specific network incorporated edges labeled T, P or T-P, thus representing only contacts induced by cattle breeding practices;
- the badger-specific network incorporated edges labeled B, D or B-D, thus representing only badger-mediated contacts;
- the mixed network incorporated all the remaining edges, thus representing the co-occurrence of cattle-specific and badger-mediated contacts.

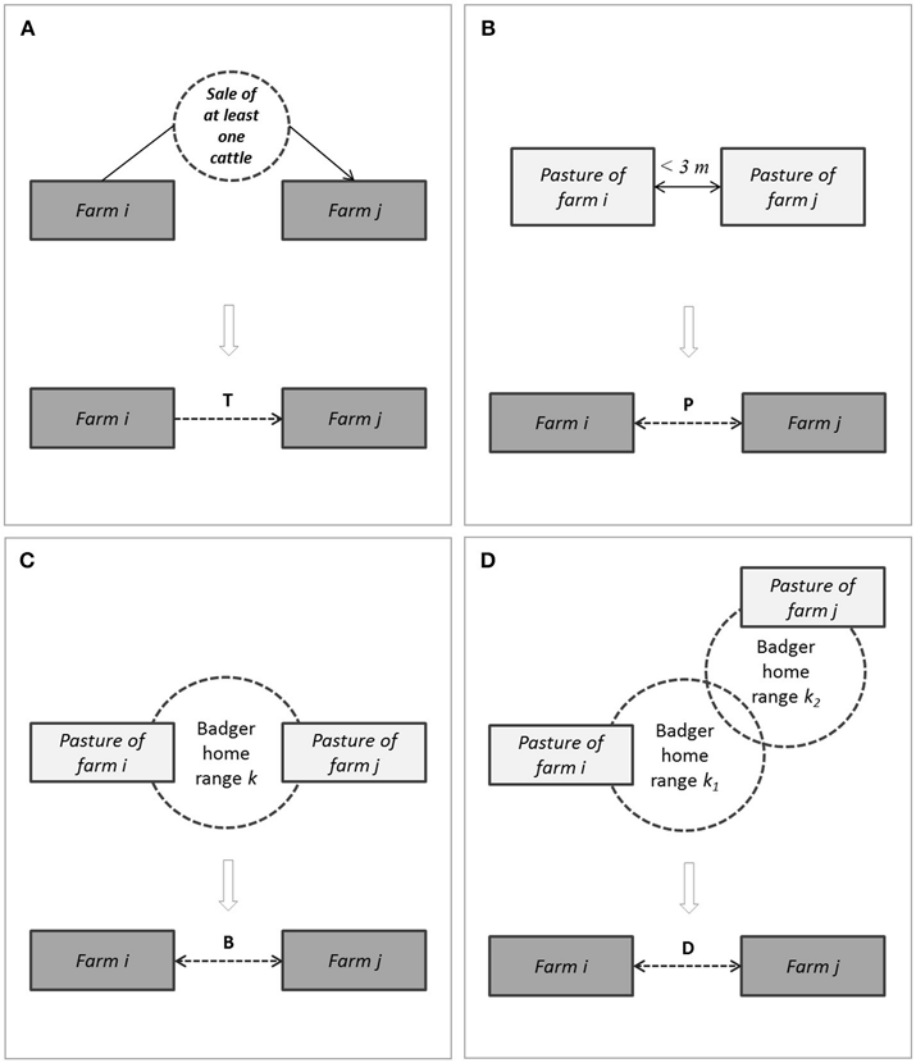

FIGURE 2 | Schematic representation of the four types of edges between cattle farms in the contact network **(A)**, trade edge (noted T); **(B)**, pasture neighborhood edge (noted P); **(C)**, simple badger-mediated edge (noted B); **(D)**, second level badger-mediated edge (noted D).

Statistical Analysis

Each of the 16 molecular types of *M. bovis* identified in the study area was considered as a marker of an independent epidemic. For a given molecular type, the contact network may be considered as supporting *M. bovis* transmission between two farms only if a path exists in the network between these farms. The transmission tree rooted on a detected infected farm should then be entirely located in a single component of the network. The contact network may then be considered as supporting the spread of a given molecular type if most of the farms infected by this molecular type are located in the same component of the contact network. We thus first computed, for each molecular type identified in more than one farm, the number of components in which these infected farms were located (50). For the same subset of molecular types, we also computed, for each infected farm, the length of the shortest path to another farm where the same molecular type was detected.

To evaluate whether the observed pattern of bTB infected farms may have resulted from transmission processes in the contact network, we used the *k*-test proposed by VanderWaal et al. (51). This permutation-based test is based upon the calculation of the *k*-statistic: the mean number of infected cases among the neighbors of an infected node (the approach is easily extended to neighborhoods of order >1). The observed value of this statistic is then compared to the distribution of the same statistic obtained by randomly reallocating the location of cases, thus simulating a possible pattern of cases under the null hypothesis of an absence of association between bTB case location and network structure. The empirical *p*-value of the *k*-test is then the proportion of permutations for which the *k*-statistic is greater than the observed one. We adapted this test to a multi-type epidemic by redefining the *k*-statistic as the mean number of cases among the neighbors of a node, which were infected by the same molecular type as that node.

TABLE 3 | Label of edges in the different networks of contacts between cattle farms in the study area..

Edge label	Full contact network	T-network	P-network	B-network	D-network	Cattle-specific network	Badger-specific network	Mixed network
T	■	■				■		
P	■		■		■			
B	■			■		■		
D	■				■		■	
TP	■	■	■				■	
TB	■	■		■				■
TD	■	■			■			■
PB	■		■	■				■
PD	■		■		■			■
BD	■			■	■		■	
TPB	■	■	■	■				■
TPD	■	■	■		■			■
TBD	■	■		■	■			■
PBD	■		■	■	■			■
TPBD	■	■	■	■	■			■

T, trade edge type; P, pasture neighborhood edge type; B, simple badger-mediated edge type; D, second level badger-mediated edge type; edge labels with several letters correspond to combinations of edge types; gray cells indicate the presence of the label within the network.

The *k*-test was first performed on the full contact network. It was then applied on the cattle-specific, badger-specific and mixed subnetworks; and this, for two groups of molecular types: those observed in cattle only and those observed in cattle and in badgers. Seven tests were thus performed and the Bonferroni correction was applied. Ten thousand permutations were used to compute the empirical *p*-value.

To further analyse the association between edge types and bTB occurrence, we focused on edges originating from infected farms. A binary status was assigned to each of these edges, with a value of 1 when the destination node was infected by the same molecular type as the originating node, and 0 otherwise. The association between this status and the edge type was then assessed using a case-control design: cases were edges having a status of 1, and controls the edges having the status 0. Four binary explicative variables were defined, based on the types labeling the edge: T, P, B, and D. In addition, we took into account the size (number of bovine females over the age of 2 years) of the edge originating and destination farms, herd size being a well-known risk factor for bTB detection in cattle farms (24). We thus modeled the probability for an edge starting from a detected infected farm to end at a farm detected infected by the same molecular type, using a logistic regression model including six independent variables: four binary variables (presence/absence of the T, P, B and D edge type) and two quantitative variables (sizes of the originating and destination farms). We checked the absence of multicollinearity using variance inflation factors (VIF) with a threshold of 10 (52). Odds ratios (OR) and their associated 95% confidence intervals were computed. Finally, attributable risk fractions (AF) were computed for each edge type.

The definition of badger-mediated edges was based upon the neighborhood between pastures and one (B-edges) or two (D-edges) badger home ranges. For some of the corresponding setts, the trapping results allowed defining an infection status: setts were considered as (i) infected when at least one trapped badger had been found infected with an identified molecular type and (ii) uninfected when at least two trapped badgers had been tested negative and no occupant badger had been found infected [for more details, see (48)]. Based on these data, we finally used a Fisher exact test to analyze the association between the status of B- or/and D-edges and the infection status of the corresponding setts.

Dirichlet tessellations were computed using the deldir package (53) and buffers using the sp package (54). Network analyses were carried out using the igraph package (55) and variance inflation factors were computed using the car package (56). Attributable risk fractions were finally computed using the AF package (57). All those cited packages were used in R 3.3.2 (58).

RESULTS

Within the full contact network, the most frequent edge type was the combination of B- and D-edges, followed by single D-, T-, and B-edges. The P-edge type was less frequent alone than in combination with the other types (**Figure 3**).

The largest weak component of the full contact network incorporated 99.8% of the study population. Regarding the four edge-type-specific networks, the proportion of nodes included in the largest component was higher in trade and badger related networks (94.4% for the T-network, 94.7% for the B-network and 93.6% for the D-network) than in the pasture network (50.4%) (**Table 4**) (a more detailed analysis of networks topology is given in Supplementary Tables 2–4 and Supplementary Figures 1, 2).

For each of the 16 molecular types, the farms where the type had been observed were always located in the same component of

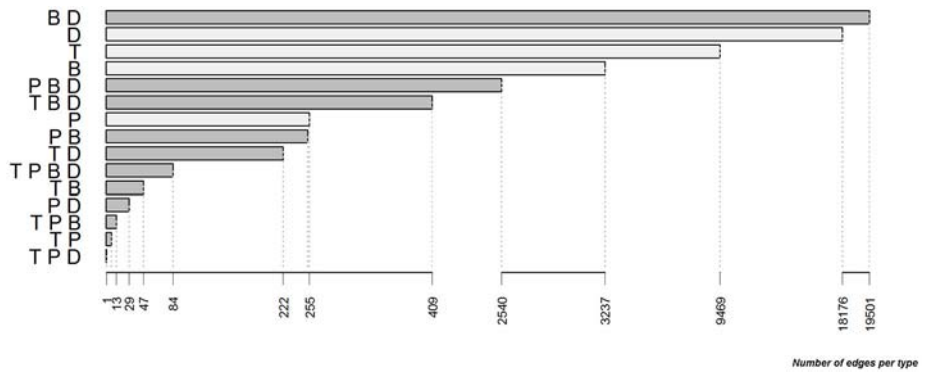

FIGURE 3 | Distribution of the different types and combinations of types for the edges of the full contact network between cattle farms in the study area between 2007 and 2015 (T, trade edge; P, pasture neighborhood edge; B, simple badger-mediated edge; D, second level badger-mediated edge; edges having only one type are in light gray and combinations of several types are in gray).

TABLE 4 | Description of the full contact network and of the four edge-type specific networks.

Indicator	Full contact network	*T*-network	*P*-network	B-network	D-network
Number of nodes (size)	1946	1946	1946	1946	1946
Number of edges	54243	10252	3182	26084	40962
Number of components	5	107	716	93	117
Biggest component size	1942	1837	980	1842	1822
Second biggest component size	1	2	23	6	4
Number of components with one farm	4	103	608	86	112

TABLE 5 | Distribution of detected infected farms in the components of the full contact network and in the four edge-type-specific networks for the molecular types identified in more than one farm.

	Number of components containing detected infected farms				
Molecular types	**Full contact network**	***T*-network**	***P*-network**	**B-network**	**D-network**
SB0120c	1	1	1	1	1
SB0821(*)	1	2	15	1	2
SB0832(*)	1	1	3	1	1
SB0928	1	1	3	1	1

, molecular types found both in badgers and cattle; see **Table 2 for more details.*

the full contact network. This was also the case for the B-network, but not for the T-, P-, and D- networks (**Table 5**).

Four molecular types were observed in at least two detected infected farms (**Table 2**). For 87% of these farms, the path to the closest farm detected infected by the same molecular type was made of a single edge. It included one intermediary cattle farm in 11% of cases (**Figure 4** and Supplementary Table 2). This result suggests a prominence of *M. bovis* transmission between an infected farm and its direct neighbors in the full contact network.

We computed the proportion of shortest paths made of a single edge between farms infected (i) by molecular types found only in cattle and (ii) by molecular types found both in badgers and cattle. The difference between these two proportions was not significant (Fisher exact test: $p = 0.13$).

Using *k*-tests, a significant association was observed between the pattern of bTB detected infected farms and the structure of the full contact network (observed *k*-statistic: 2.3; distribution obtained by randomly reallocating the location of cases: mean = 0.39, $SD = 0.12$; $p < 7.14^{*}10^{-3}$, threshold after Bonferroni correction) (**Figure 5**). No significant association was observed for the cattle-specific network, neither for the molecular types observed in cattle only, nor for those found both in cattle and badgers. Conversely, a significant association was observed between the pattern of farms detected infected by molecular types shared between badgers and cattle and the structure of the badger-specific network ($p < 7.14^{*}10^{-3}$). Finally, the structure of the mixed network was significantly associated with the pattern of bTB-infected farms for both groups of molecular types ($p = 0.006$ and $p < 7.14^{*}10^{-3}$ respectively) (**Table 6**).

The four edge types were included in the logistic regression model as no significant multicollinearity was detected. T-, B-, and D-edge types were significantly associated to the probability of being a case with an OR of 7.13 for the T-edge type (95% CI: [3.39–15.06]), 1.89 for the B-edge type (95% CI: [1.32–2.76]) and 10.44 for the D-edge type (95% IC: [4.38–26.66]). The size of the destination farm of the edge was also significantly associated to the probability of being a case. Regarding edge types, attributable risk fractions were 84% for the D edge type, 32% for the B edge type, and 12% for the T edge type (**Table 7**).

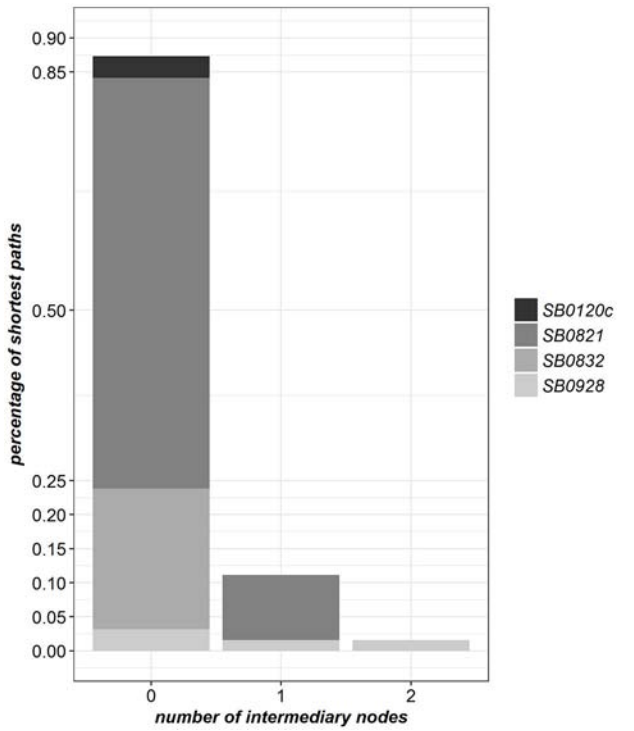

FIGURE 4 | Distribution of the shortest path lengths in the full contact network between pairs of farms detected infected by the same molecular type (only the four molecular types found in at least two farms are considered).

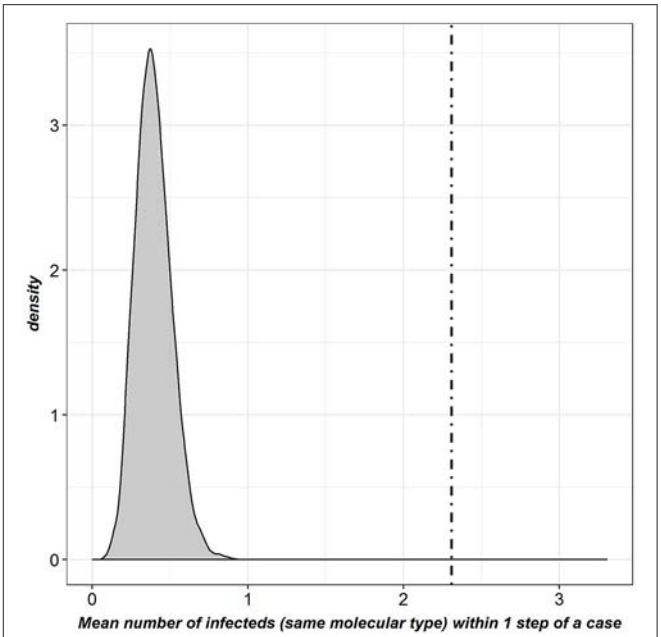

FIGURE 5 | Graphical representation of the *k*-test results for the full contact network [*dot-dashed line: k*-statistic computed in the observed network; *gray density plot:* distribution obtained by randomly reallocating the location of cases; this last distribution was clearly lower than the *k*-statistic observed ($p < 7.14^{*}10^{-3}$, threshold after Bonferroni correction)].

Among edges representing badger-mediated transmission (i.e., B- and D-edges), the infection status of badger setts involved (one sett regarding B-edges and at least one of the two setts regarding D-edges) was known for 264 edges (5%) originating from a farm infected by one of the two molecular types shared between badgers and cattle. Among them, 44 were case edges (i.e., the destination farm had also been found infected by the same molecular type) of which 38 (86%) were supported by positive badger setts; and 220 were control edges of which 102 were supported by positive badger setts (46%). These differences were significant (Fisher exact test: $p < 0.0001$) with an associated OR of 7.3 [95% CI: (2.9–21.9)].

DISCUSSION

The objective of this study was to provide a better understanding of *M. bovis* transmission mechanisms between cattle farms in south-western France using networks which represented the direct and indirect contacts that may allow *M. bovis* transmission among farms of this area between 2007 and 2015.

Four types of edges were represented because of their potential involvement in *M. bovis* transmission between cattle farms and we assumed that they represented the main transmission mechanisms in the study area. Cattle movements due to trade are a known *M. bovis* transmission route in Great Britain (59, 60), but also in France (42). The neighborhood with an infected farm through adjoining pastures (allowing over the fence contacts between herds) has also been identified as a potential risk factor for the *M. bovis* transmission between French cattle farms (31). The intersection of badger home ranges with cattle pastures and between each other's was considered a proxy for badger-mediated transmission, considering the territoriality of badgers (36) and the ability of *M. bovis* to survive in the soil (25, 26). BTB surveillance measures in badgers were not homogeneous among setts of the study area, as they were dependent on bTB detection in the cattle farms in their vicinity. For this reason, although the location of setts was known, we did not model badger setts as nodes in the contact network (we would have been unable to attribute an infection status to each of them). Instead of that, sett location data were used to represent badger-mediated contacts between farms by specific edges, based on neighboring badger home ranges. Two types of badger-mediated contacts were thus modeled by edges. B-edges represented a situation in which two farms neighbored the same badger home range: farm to farm *M. bovis* transmission through such edges thus only assumed cattle to badger and badger to cattle transmission. Conversely, D-edges represented a situation in which two farms neighbored two distinct but neighboring badger home ranges: farm to farm transmission through such edges thus also assumed badger to badger transmission in animals from neighboring setts. Because the epidemiological unit of this study was the farm, P-, B-, and D-edges were built based on the aggregation of pastures of each cattle farm. In the study area, cattle are often moved from one pasture to another one belonging to the same farm,

TABLE 6 | Results of the *k*-tests for the cattle-specific, the badger-specific and for the mixed subnetworks of the full contact network, for the molecular types only found in cattle only and for those found both in badgers and cattle.

			Observed networks	Reallocated networks	
Molecular types found in	**Networks**	***p*-value**	**k-statistic**	**Mean k-statistic**	**SD k-statistic**
Cattle only	Cattle-specific	1	0.00	0.002	0.01
	Badger-specific	0.07	0.11	0.008	0.03
	Mixed	0.006*	0.11	0.0008	0.01
Badger and cattle	Cattle-specific	0.027	0.23	0.09	0.06
	Badger-specific	0*	2.28	0.39	0.13
	Mixed	0*	0.46	0.03	0.03

**significant difference after Bonferroni correction ($p < 7.14^{*}10^{-3}$); SD, standard deviation.*

TABLE 7 | Logistic model of the probability of an edge starting from a detected infected cattle farm to join another detected infected cattle farm and with the same molecular type according to the type of edge.

Variable	**Parameter estimate**	**OR (95% CI)**	***P*-value**	**AF (SD)**
Intercept	−5.02	0.01 [0.00–0.02]	<0.0001	-
T edge type	1.96	7.13 [3.39–15.06]	<0.0001	12% (6.2)
P edge type	0.30	1.35 [0.77–2.27]	0.26	3% (7.4)
B edge type	0.64	1.89 [1.32–2.76]	<0.0001	32% (8.6)
D edge type	2.34	10.44 [4.38–26.66]	<0.0001	84% (6.9)
Size of the destination farm	−0.0045	0.956 [0.910–0.996] *(*)*	0.049	-
Size of the originating farm	0.002	1.02 [0.99–1.06] *(*)*	0.19	-

OR, Odds ratio; CI, Confidence Interval; AF, Attributable Risk Fraction; SD, Standard Deviation.
(*) Odds-ratio corresponding to an increase of ten animals.

e.g., when rotational grazing is used, we thus assumed that this simplification was meaningful.

The frequency of testing cattle was different in the different parts of the study area and this could have biased our results. However, testing was performed each year in communes where infected herds had been detected, and was also performed reactively in farms identified by contact tracing from these herds, based on cattle trade data and on pasture neighborhood. For these reasons, farms directly connected (in the full contact network) to a herd detected infected were considered having been submitted to similar testing regimens, both for B and D edge types (as in most cases the connected farms were located in the same commune), and for the T and P edge types (because of contact tracing). As only edges originating from herds detected infected were considered in the *k*-tests and in the logistic regression model, the corresponding results should not have been biased by geographic variations of the frequency of testing in the study area.

Taking into account the molecular types of isolates allowed considering 16 independent epidemics, of which 12 appeared restricted to a single farm, and 14 to less than 10 farms. All of these 14 molecular types affected only cattle. This predominance of molecular types found in a single cattle farm (75%) was in line with a previous study carried out in France between 1979 and 2000 in which a large majority of molecular types (84%) were found at a low frequency (less than 10 farms). This result has been interpreted as the sign of a poor spread of these strains (61), which could be traces of older epidemics that would have spread prior to 2007, but without significant transmission afterwards. Indeed, in our study, the 14 molecular types found in less than 10 farms were all detected not later than 2012 (**Table 2**).

Farms detected infected by a given molecular type were always located in the same large weak component of the full contact network that contained 99.8% of farms, whereas it was not the case for three of the edge-type-specific networks: the T-, P-, and D- networks. This indicated that, although the T-, P-, and D-edge-type-specific networks could not alone have supported the spread of bTB infection within the study area (contrary to the B-network), the strong connectivity resulting from the union of the four networks into the full contact network provided a structure that might enable the spread of the *M. bovis* infection in the study area. This result is in line with multifactorial mechanisms of bTB spread previously suggested by other studies (24, 29). As an example in Great Britain, dynamic modeling of cattle taking into account farm environment helped understanding *M. bovis* transmission routes (62). Prominent identified routes of *M. bovis* transmission were moving infected cattle between farms and reinfection from an environmental reservoir. The conclusion of this study was that control measures should simultaneously address several transmission routes to be effective.

Using *k*-tests, a significant association was observed between the pattern of bTB-infected farms and the structure of the

full contact network. Moreover, the structures of the badger-specific and mixed networks were significantly associated with the pattern of farms detected infected by molecular types shared between badgers and cattle. This result was expected and confirmed that badger-mediated edges could be viewed as paths for the interspecies *M. bovis* transmission. In addition, the structure of the mixed network was significantly associated with the pattern of bTB-infected farms for molecular types found only in cattle, whereas it was not the case for the cattle-specific network. We could assume that the spread of cattle molecular types would be more efficient when direct contact (trade and/or pasture neighborhood) are associated with indirect badger-mediated contacts. In addition, we should be cautious about the cattle specificity of these molecular types, as these molecular types may be (or have been) present in the badger population without being observed, because of the relatively low sensitivity of bTB surveillance in the badger population.

Considering edges originating from detected infected farms, we used a case-control design and a logistic model to analyse the relationship between the types of an edge and the detection of the same molecular type at the originating and destination farm of the edge (case edges) or at the originating farm only (control edges). Because the detection dates could not be considered in the study to infer dates of infection, the co-occurrence of the same molecular type at both ends of case edges does not model the transmission of *M. bovis* through the edge, although the edges of the full contact network represent possible transmission paths for the bacteria and case edges thus represent possible transmission events. The largest odds-ratio was attributed to the D edge type, followed by the T edge type. This predominance of badger-mediated edges reflects the specific situation of the study area, where molecular types shared between badgers and cattle were predominant (84% of detected infected farms **Table 2**), and the predominant effect of the D edge type suggests a probable spread of *M. bovis* between badgers from neighboring setts, and not only between badgers and cattle. However, B and D edges were defined based on a geographic representation of home ranges, with a maximal distance of 1,000 m to the sett. This distance threshold, the Dirichlet tessellation used to model home ranges, and the fact that some setts may have been unoccupied, are three elements that may have led to an underestimation of home range size, and to an overestimation of the role of the D edge type.

The T edge type was also associated with a putative transmission of *M. bovis* ($AF = 12\%$). This result is in agreement with a previous French study conducted at the national scale, according to which the population attributable risk fraction of bTB infection had been estimated at 12% [5–18%] for cattle trade (42), often allowing long distance bTB spread.

In a previous study conducted in France, pasture neighborhood was found significantly associated with the farm infection status (31). However, in the present study, the P edge type was not significantly associated with *M. bovis* transmission when using the case-control design. This may be first explained by the fact that some of farmers of the study area use rotational grazing, with some pastures left unoccupied for grass re-growth. Furthermore, P-edges were defined based on a direct neighborhood between pastures (<3 m). This short distance does not allow other opportunities of direct contacts between cattle, such as the wandering of livestock, to be represented.

The badger-specific edges (B and D edge types) were defined based on sett locations, one or two setts being associated to each. For some of these setts, an infection status could be determined based on bTB surveillance data. We showed that this infection status was significantly associated with the fact that the sett as well as the originating and the destination farms had all been found infected by isolates of the same molecular type ($OR = 7.3$; 95% CI:[2.9-21.9]). This result supports an actual badger-mediated transmission through these types of edges. Nevertheless, wild boars have also been found infected with *M. bovis* within the study area. Indeed, among 548 analyzed wild boars between 2011 and 2015, 15 (2.7%) were found infected. The corresponding molecular types found in these wild boars were the two molecular types shared between badgers and cattle. Therefore we cannot exclude the role of this wild species that we could not consider in this study because of a lack of field data that would have allowed its spatial organization (captured through radio tracking, for example) to be represented. Not considering wild boars in our analyses could have led to an over-estimate of the role of B and D edge types in *M. bovis* transmission between cattle farms.

Other indirect contacts through herd practices could also have contributed to the predominance of the D edge type. Indeed, this type of edge created links between farms without direct contacts at pasture but being in a kind of vicinity. As examples, the sharing of material or the loan of animals could create links between farms that may overlap the D edges. However, no data were available to investigate this assumption. Its confirmation or refutation would require supplementary investigation.

In conclusion, this study supports the multifactorial nature of *M. bovis* transmission between cattle farms within the *Pyrénées-Atlantiques–Landes* area, France from 2007 to 2015. The largest part of bTB spread seemed to be due to badger-mediated contacts, however cattle trade played a significant role. Consequently, to be truly effective, control measures should not focus on a single type of contact but ought to act on the different mechanisms we raised.

AUTHOR CONTRIBUTIONS

MB-Z, AC, and BD conceived and designed the study. MB-Z prepared the data for the analysis. MB-Z and BD performed the analysis. MB-Z wrote the manuscript. MB-Z, AC, and BD revised the manuscript. All the authors approved the submitted version of the manuscript.

ACKNOWLEDGMENTS

The authors thank the French Ministry of Food, Agriculture and Forest, Directorate General for Food (DGAl) and by the

University of Paris-Sud, which both funded MB-Z's PhD grant. The authors also warmly thank Pierre Jabert (DGAl), Christian Peboscq (*Pyrénées-Atlantiques* Departmental Federation of Hunters-FDC 64), and all the hunters of the *Pyrénées-Atlantiques* and *Landes* for the census of badger setts in the study area.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00173/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 04 December 2018
doi: 10.3389/fvets.2018.00306

Baiting and Feeding Revisited: Modeling Factors Influencing Transmission of Tuberculosis Among Deer and to Cattle

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Although tuberculosis caused by *Mycobacterium bovis* (bTB) is endemic in white-tailed deer (*Odocoileus virginianus*) in northeastern Michigan, USA, baiting and feeding of deer continue despite a regulatory ban. Previous modeling suggests aggregation at bait sites slows the rates at which harvest and/or vaccination decrease bTB prevalence, prolongs time to eradication, and increases the likelihood that once eradicated, bTB will re-establish following an incursion. However, the extent to which specific factors such as food density, attractiveness to deer, and persistence on the landscape influence bTB transmission is unknown. We used an individual-based, spatially-explicit stochastic simulation model of bTB in deer and cattle to investigate effects of feed density, attractiveness, and spatial and temporal persistence on bTB prevalence in deer and the probability of breakdowns in adjacent cattle herds. Because hunter harvest remains key to controlling bTB in deer, and harvest rates are in long term decline, we modeled these feeding-associated factors at harvest rates prevailing both when the model was developed (2003–2007) and in 2018. Food placement at randomized locations vs. fixed sites had little effect on bTB prevalence in deer, whereas increasing the probability that deer move to food piles (attractiveness) had the greatest effect of factors studied on both prevalence and herd breakdowns. Reducing food pile density reduced prevalence, but decreased herd breakdowns only modestly. Consistent availability of food over longer periods of time, as would occur with supplemental winter feeding or persistent recreational feeding, increased both prevalence in deer and cattle herd breakdowns dramatically. Though perhaps implausible to the public, altering how bait and feed for deer are used can reduce cattle herd breakdowns. Baiting and feeding bans have contributed to declining bTB prevalence, but non-compliance and continued legal sales of feed impede eradication. Requiring hunters to move food piles is unlikely to mitigate effects on transmission and is not a useful management tool. Compared to baiting, winter supplemental feeding or extended recreational feeding is likely to magnify bTB transmission by prolonging temporal availability. Because attractiveness of feed is influenced both by type of feed and deer behavior, research to quantify factors influencing deer movement to food should be a priority.

Keywords: baiting, cattle, feeding, management, *Odocoileus virginianus*, simulation model, tuberculosis, white-tailed deer

OPEN ACCESS

Edited by:
Julio Alvarez, VISAVET Health Surveillance Centre (UCM), Spain

Reviewed by:
Javier Hermoso-de-Mendoza, Universidad de Extremadura, Spain
Javier Bezos, Complutense University of Madrid, Spain

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *27 June 2018*
Accepted: *19 November 2018*
Published: *04 December 2018*

Citation:
Cosgrove MK, O'Brien DJ and Ramsey DSL (2018) Baiting and Feeding Revisited: Modeling Factors Influencing Transmission of Tuberculosis Among Deer and to Cattle. Front. Vet. Sci. 5:306. doi: 10.3389/fvets.2018.00306

INTRODUCTION

Infectious disease management in a wildlife reservoir is a contentious issue, especially when changes in human behavior are necessary. While there may be general agreement that control measures are warranted, the specific actions adopted are often controversial. The elimination of human-provided supplemental food for wildlife is of notable debate. Although the use of supplemental food for wildlife is recognized as a mechanism for both inter- and intra-species disease transmission (1–7), disagreements regarding the potential benefits vs. consequences related to this practice are still prevalent. Nonetheless, zoonotic diseases can have serious ecological and economic impacts (5, 8) and the disease management strategies chosen influence the magnitude of the impacts. Advocates for the use of supplement food as bait (an attractant during legal hunting seasons) argue that baiting is necessary to increase and maintain harvest for disease control, yet evidence for this is lacking (9, 10). However, the use of bait increases deer concentrations around these sites, increasing contact rates and so the potential for disease transmission. Supplemental food used in winter to (theoretically) aid survival can have similar consequences (5–7).

In Michigan, baiting and feeding of white-tailed deer (*Odocoileus virginianus*) is recognized as one of the biggest risks for transmission of tuberculosis caused by *Mycobacterium bovis* (bTB) (4, 8, 11, 12). In the wake of extensive logging and the decimation of deer populations by market hunting that occurred in the 1800s, large tracts of land were bought into private ownership and "hunt clubs" were established, both to conserve what deer remained and to provide populations for sport hunting and sustainable harvest. In the 1920's deer numbers in the northern Lower Peninsula of Michigan rose dramatically, in part due to lower harvest pressure on privately owned lands effectively maintained as refuges, and winter starvation became common (13, 14). In an attempt to reduce starvation, supplemental feeding became prevalent, yet starvation continued as the biological carrying capacity of the marginal habitat had been exceeded (13). During this same time, bTB reactor rates in cattle across the state were relatively high, reaching 20–30% in some counties (15, 16). Although Michigan was later successful in lowering bTB reactor rates in cattle and was eventually declared bTB free in 1979, contact between infected cattle and concentrated numbers of deer had been occurring for decades. Large scale supplemental feeding increased in the 1980's as competition increased between private land owners to attract deer to their property. It is hypothesized that spillover of bTB from cattle to deer occurred sometime during the 1950s to 1960s (17, 18), although deer were not recognized as a maintenance host until the 1990's (11). By that time bTB had become self-sustaining in the free-ranging deer herd and persists to the present day (11, 19).

Although bTB is endemic in deer in northeast Lower Michigan, winter feeding and baiting of deer continue despite a regulatory ban, albeit at lower levels than historically practiced. The sale of bait and feed remains legal and widespread, even where its use is banned (8). The current economic value of these sales is thought to be substantial. In 1995, it was estimated that baiting and supplemental feeding generated $15 million for Michigan farmers (20), and predictably, bans are typically opposed by farmers gaining from the sale of crops otherwise unmarketable for human consumption. This presents a persistent struggle to eradicate a contagious disease in the face of exacerbating practices. Previous modeling, using estimated levels of current baiting practices, suggests aggregation at bait sites slows the rates at which harvest and/or vaccination decrease bTB prevalence, prolongs time to eradication, and increases the likelihood that once eradicated, bTB will re-establish following an incursion (21). However, not well known are the extent to which specific factors such as feed site density, attractiveness to deer, and persistence on the landscape influence bTB transmission among deer and between deer and cattle.

Both direct and indirect interactions between livestock and wildlife have been well documented as a source of disease transmission (22–26). Increased bTB reactor rates in livestock resulting from alterations in supplemental food use for deer could be associated as well. We used an existing spatially-explicit model of bTB in deer and cattle (27) to evaluate how altering supplemental food density, attractiveness, and temporal and spatial persistence impact bTB prevalence in deer and the rate of cattle herd breakdowns.

MATERIALS AND METHODS

Study Area

We conducted simulations over a 48 x 51 km land area in the northeastern Lower Peninsula of Michigan consisting mainly of Deer Management Unit (DMU) 452 [12, 27, **Figure 1**]. Deer Management Unit 452 includes parts of Alcona, Alpena, Montmorency, and Oscoda counties, is ∼148,018 ha (1,480 km^2), and comprised of 93% privately-owned land and 7% public land (28, 29). Topography, habitat, and deer management practices are described elsewhere (12, 30, 31). Historically, this area has defined the core outbreak area for bTB in Michigan (8).

The Model

We used an individual-based spatially-explicit stochastic simulation model of bTB in white-tailed deer and cattle for all simulations. The structure, parameters, testing, validation, and assumptions of the model are described at length elsewhere (12, 27, 31). We used two Geographic Information System (GIS) layers to distribute deer and cattle across the landscape and account for movements and spatial concomitance that facilitate bTB transmission among deer and from deer to cattle. We used a deer layer that quantifies winter habitat potential for deer (as a surrogate for biological carrying capacity) to spatially distribute deer throughout the landscape (12, 31–33). We added a second layer that incorporated cattle producer locations, pasture areas, and cattle densities for all farms within and directly adjacent to DMU 452 to approximate cattle distribution (27). The model was originally calibrated to closely approximate steady-state age and sex specific bTB prevalence matched to long-term bTB surveillance data for deer from 2003–2007 (12, 31) and cattle herd breakdown rates 2003–2012 (27). A cattle herd breakdown is defined as a herd having at least one bTB reactor in the herd during whole herd testing.

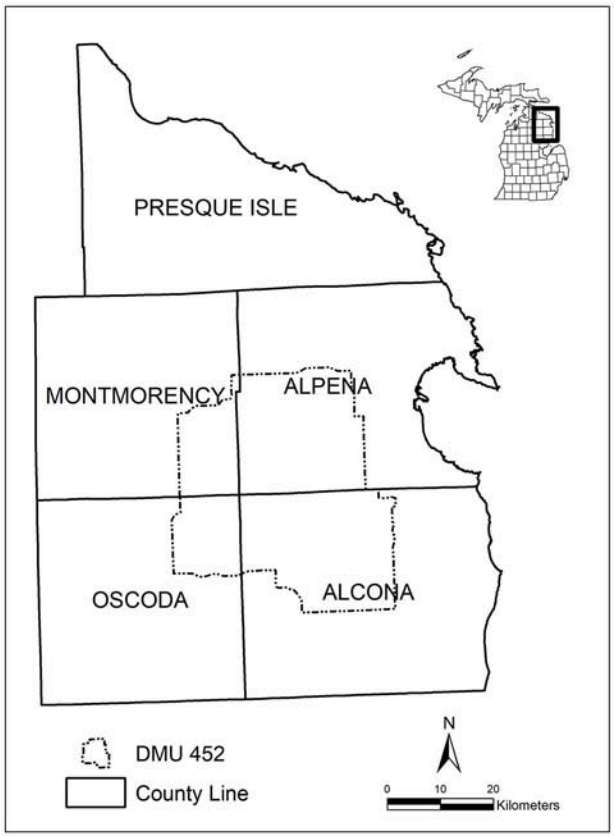

FIGURE 1 | Simulation model study area, Deer Management Unit (DMU) 452, in northeastern Lower Peninsula of Michigan, USA.

Because rates of hunter harvest have declined over the last two decades, primarily due to demographic factors (34), the model was recalibrated in 2018 to adjust bTB transmission rates to accommodate current harvest rates. Briefly, sex- and age-specific harvest rates for DMU 452 were estimated from MDNR deer hunter harvest survey data via the sex-age kill method (35). Using those harvest rates, simulations were run to calibrate the sex-specific transmission rate parameters (betas) so that the predicted sex-specific bTB prevalence closely approximated field prevalence rates recorded from 2012–2016.

The baseline model simulates aggregation of deer around supplemental food sources by estimating the movement of a deer to the food source based on the location of the food source within its home range (12, 31). During each time step (2 months), each deer conducts a search of habitat cells within its home range. If a food pile is encountered, there is assumed to be a 0.2 probability of a change in the deer's current location to the food pile if it occurs at the center of its home range. That probability declines as a half normal function of the distance of the food pile from the home range center and was zero for food piles outside the home range. Food piles were randomly distributed across the landscape. Supplemental food was available from September to December, coinciding with deer hunting seasons in Michigan when bait is used.

In this study, we define supplemental food in two ways: (1) Baiting—the autumnal use of food to attract deer in an attempt to aid harvest; and (2) Feeding—the use of food for deer outside of legal hunting seasons (e.g., to facilitate wildlife viewing, or in an effort to aid winter survival). Our previous modeling suggests that aggregation of deer at food sites has a substantial effect on bTB transmission (12). However, that work only investigated the effects of the presence or absence of bait. The spatial nature of the model affords the ability to assess a variety of parameters associated with baiting and feeding. We altered four different supplemental food parameters to evaluate the effect on bTB prevalence in deer and cattle herd breakdowns over a 30-year period. Other model parameters were kept at default values as described previously (12, 27, 31). We ran each scenario under the original and current harvest rates for 5,000 replicates, discarding simulations for the first 50 and 150 years, respectively, (burn-in period). Due to the stochastic nature of the model, the burn-in period was required to ensure bTB and cattle breakdown rates had stabilized prior to changing the parameters under investigation. For comparison to our treatments, we conducted baseline simulations for both the original and current age- and sex-specific bTB transmission and harvest rates.

Simulated Scenarios

Food Pile Density

Baiting and feeding is illegal in DMU 452 and has been since 1999 with limited use allowed in 2001 (8). However, a non-compliance rate of ∼25% was estimated for hunters in the area (36). By dividing the number of potential baiters (determined from deer hunting license sales; see (12)) by the area of DMU 452, the bait pile density was set at 0.02/ha. We evaluated the effect of reducing the non-compliance rate by 50% (e.g., via more stringent enforcement) which in turn reduced the bait pile density to 0.01/ha.

Food Pile Attractiveness

We define bait attractiveness as the probability that a deer will visit a bait pile if the bait pile is located within the center of the deer's home range. Attractiveness is influenced by both feed type and deer behavior and is thus difficult to quantify with certainty. Consequently, we evaluated three arbitrary variations of this probability: 0.05, 0.1, and 0.5, which were considered to be plausible bounds on the likely attractiveness of bait to deer, assuming the odds of deer being attracted to accessible bait are 50:50 or lower.

Spatial Persistence

Most hunters have preferred hunting locations and often establish permanent tree stands or deer blinds at these locations from which they hunt each year. In turn, if the hunter uses bait, the food piles are located in approximately the same location every year. We simulated the effect of requiring hunters to move food piles to new locations each year by randomizing pile locations, thus potentially affecting contact rates between habituated deer.

Temporal Persistence

Supplemental feeding commonly takes place during winter months when the public attempts to supplement reduced natural food sources for wild deer. This type of feeding can potentially aid winter survival, thus increasing deer densities to a level that exceeds the biological carrying capacity of the habitat. Both supplemental and prolonged recreational feeding (typically for the purposes of viewing) can unnaturally congregate deer for extended periods and in turn increase the probability of disease transmission. We evaluated the effects of prolonged food provision by humans on bTB prevalence and cattle herd breakdowns by expanding the temporal duration of food piles for two different time frames: September–February and September–April. The former simulates feeding through the most severe months of winter in Michigan, and the latter supplementing food through the entire winter.

Analysis

We used R (version 3.0.2, R Foundation for Statistical Computing, Vienna, Austria) for analyzing model output. Summary plots were generated for deer (bTB prevalence) over the simulated 30-year time frame. We compared output from the baseline simulation (no change to current deer management) to output from the simulations evaluating changes in baiting and feeding practices, to detect the direction and magnitude of influence on prevalence and herd breakdowns. Changes in bTB prevalence are expressed as absolute differences in year 30 vs. year zero of each simulation.

RESULTS

The baseline simulation for the original harvest rates resulted in a 0.001 decrease in deer prevalence after 30 years, and no change in prevalence over 30 years under the current harvest rates (**Figures 2**, **3**). Cattle herd break downs were 2.8 per year on average under the original harvest rates and 3.4 per year under the current rates for the baseline simulations after 30 years (**Table 1**).

Food Pile Density

Reducing the baiting non-compliance rate (and thus food pile density) by 50% reduced bTB prevalence in deer by ∼0.005 under both harvest rates, and reduced cattle herd breakdowns by an average of ∼1.5 per year under both harvest rates (**Table 1**).

Food Pile Attractiveness

Under the original harvest rates, reducing the probability that a deer visited a bait pile within its home range to 0.05 and 0.1, reduced prevalence by ∼0.007 and 0.008 respectively, and by ∼0.012 and 0.011 under the current harvest rates (**Figure 2**). Increasing the probability of visitation to a bait pile to 0.5 increased prevalence by 0.06 and 0.069 for the original and current harvest rates respectively. Cattle herd breakdowns were reduced to ∼1 breakdown every 2 years under the original harvest rates and ∼1 breakdown every 5 years under the current harvest rates for the visitation probability of 0.05. For the visitation probability of 0.1, breakdowns were reduced to ∼1 breakdown every 2 years under both harvest rates (**Table 1**). However, increasing the probability of visitation to 0.5, increased herd breakdowns dramatically, ∼22 per year under original harvest and ∼19 per year under current harvest rates.

Spatial Persistence

Changes in harvest rates notwithstanding, randomizing the location of bait piles each year had a negligible effect on bTB prevalence (0.002 decrease) in deer and on cattle herd breakdowns (**Table 1**).

Temporal Persistence

Extending the time supplemental food was available by 2 months (i.e., through February) increased bTB prevalence by ∼0.019 and 0.028 for the original and current harvest rates, respectively (**Figure 3**). When food was available through April, prevalence increased by ∼0.051 and 0.061, respectively. Cattle herd breakdowns were approximately triple for both harvest rates after a 2-month increase in supplemental food (**Table 1**). Breakdowns were 5–7 times higher after a 4-month extension of supplemental food.

DISCUSSION

Over the 10 years since our model was first developed, there has been a decreasing trend in deer harvest, requiring the recalibration of our model. We simulated our scenarios under both old and new harvest rates, to illustrate the effect of this harvest reduction, and to enable comparisons to our previously reported findings. Many of the scenarios simulated under the current harvest rates showed increased bTB impacts as compared to the original harvest rates, emphasizing that hunter harvest remains an important factor controlling bTB in deer. Yet, hunter retention and recruitment are recognized as being in critical decline, and this may have detrimental effects for wildlife disease management (37–40). For density dependent diseases, such as bTB in deer, population control of wildlife reservoirs is crucial. However, after more than 20 years of providing increased harvest opportunity in DMU 452, harvest has likely been saturated and further extending harvest opportunity is unlikely to increase harvest (12). A survey of hunters and livestock producers in northeast Lower Michigan more than a decade ago indicated that only 23% of resident hunters supported a further reduction in deer numbers, whereas and somewhat surprisingly, only 57% of livestock producers in the area were in support of further reductions (41). Future bTB management in Michigan must take into consideration what can realistically be accomplished given the likelihood that reductions in harvest experienced over the past two decades are permanent. Thus far, policymakers have been reluctant to entertain other potential options for bTB control such as culling or vaccination, likely because prevalence in the deer population could be kept low via hunter harvest alone. However, as demographic changes continue to reduce the number of deer hunters (34), harvest may no longer be as efficacious as in the past.

In addition to deer density reduction, prohibiting the use of bait and feed for deer is generally one of the first control strategies

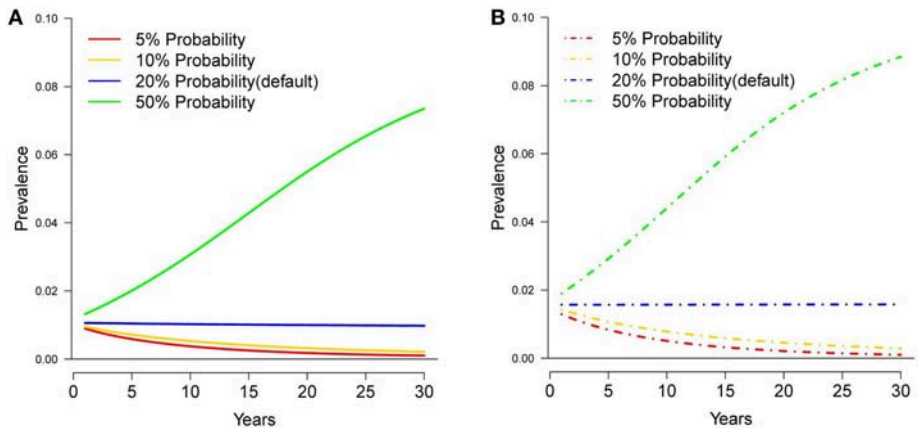

FIGURE 2 | Tuberculosis prevalence in white-tailed deer over 30 years under different probabilities of deer movement to a supplemental food pile, assuming original **(A)** and current **(B)** harvest rates.

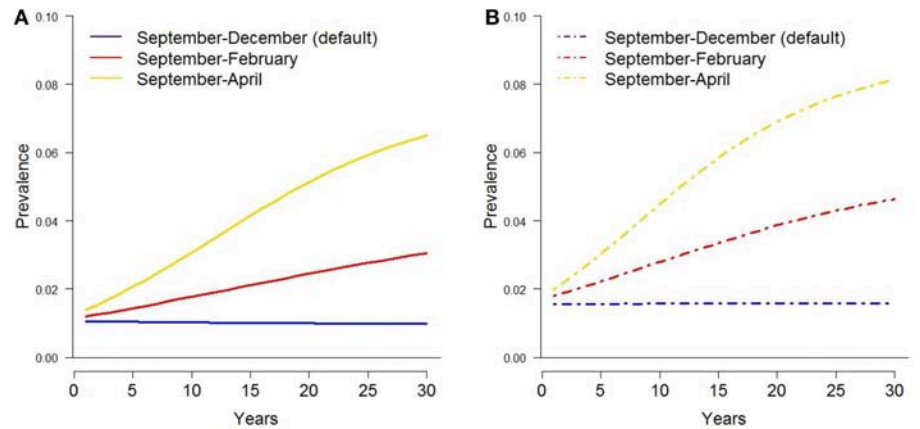

FIGURE 3 | Tuberculosis prevalence in white-tailed deer over 30 years under extended temporal availability of supplemental food piles, assuming original **(A)** and current **(B)** harvest rates.

implemented to limit disease transmission (5). However, a complete ban, in practice, is usually unattainable. Baiting and feeding bans have contributed to declining bTB prevalence in Michigan, but non-compliance, problematic prosecution of violators, and continued legal sales of feed impede eradication. Consequently, attempting to keep the non-compliance rate as low as possible becomes the goal. Even if a 50% reduction of the estimated non-compliance rate in DMU 452 could be achieved, our results show that only modest decreases in bTB prevalence and herd breakdowns would result. While we did not examine the effect of an increased non-compliance rate and an increase in food density, a resurgence in baiting may be occurring in DMU 452. Observations by MDNR field staff have noted an increase in supplemental feed sales (pers. comm. B. Mastenbrook). Moreover, it is not clear that a decreasing number of hunters will necessarily result in fewer bait sites on the landscape. If younger hunters are more likely to employ bait than the older hunters who are gradually leaving the hunting population, it is conceivable that the amount of bait on the landscape may not mirror the decline in hunter numbers.

If a complete prohibition of bait could be achieved, the benefits realized could be diminished by reduced hunter participation (42). In Michigan, there is a cultural significance to deer hunting, and traditions of deer hunting in some regions of the state have long included the use of bait (8, 42). Because reducing deer density is currently the primary strategy for bTB control, the willingness of hunters to harvest deer in endemic areas is crucial for success. If hunters are reluctant to hunt without bait due to the perceived increase in hunter effort, or if hunters choose to hunt elsewhere, baiting bans could potentially decrease harvest further. Following the 2001 hunting season in Michigan, a segment of hunters indicated through surveys that they eliminated or decreased their hunting activities due to the baiting ban (10, 42–44). However, participation and antlerless harvest were similar between DMU 452 and the remainder of the state where baiting remained legal (42). Notably, evidence

TABLE 1 | Average number of cattle herd breakdowns per year under the original and current deer harvest rates after 30 simulated years.

	Harvest rates	
Scenario*	**Original**	**Current**
Baseline	2.8	3.4
Density 0.1	1.5	1.9
Prob 0.05	0.6	0.2
Prob 0.1	0.6	0.6
Prob 0.5	22.4	18.9
Random	2.5	3.0
Sept-Feb	9.3	9.8
Sept-Apr	20.2	17.9

Scenarios: Density 0.1 = Food Pile Density (0.01 food piles/ha); Prob 0.05, 0.1, 0.5 = Food Attractiveness (probabilities of deer moving to a food pile); Random = Spatial Persistence (randomizing food pile locations annually); Sept-Feb, Sept-Apr = Temporal Persistence (extension of time food piles are available).

suggests that in general, baiting does not increase harvest (9, 10, 20, 45).

Even if minimal baiting was allowed in an attempt to maintain hunter satisfaction, a method to mitigate the increased disease transmission resulting from supplemental food has not yet been found (6, 7). We simulated requiring hunters to move bait piles to new locations each year, but the practice had a negligible effect on bTB prevalence and herd breakdowns and is thus unlikely to be a useful management tool. Previous research has shown deer in northeast Lower Michigan to have a high fidelity to baited areas, but not to specific bait locations (46). Effectiveness notwithstanding, requiring hunters to move their bait sites is likely to be impractical. In DMU 452, thousands of hunters are spread over a 1,480 km^2 area, >90% of which is privately-owned land. The effort necessary to enforce such an approach is not feasible and would arguably be more constructively applied to enforcing a strict baiting ban.

The more attractive a bait pile, the more likely a deer is to move to it. In our simulations attractiveness had a considerable impact on bTB prevalence and cattle herd breakdowns. Reducing the likelihood of a deer moving to a bait pile had a desirable impact on prevalence. However, increasing the probability of visitation to a bait pile resulted in 3.5-fold increase in prevalence and an average of 19 cattle herd breakdowns per year after 30 years. Hunters deliberately use bait piles to attract deer. Thus, expecting hunters to use less "attractive" bait defeats the purpose of using bait in the first place. However, attractiveness is also driven by deer behavior. Although supplemental food influences deer behavior (6, 46), quantifying that influence is difficult. Our modeling results are sensitive to variation in this parameter [see (12), Appendix A) and research to better quantify the combined effect of food attractiveness and deer behavior would be valuable.

Compared to baiting, winter supplemental feeding or extended recreational feeding is likely to magnify bTB transmission by prolonging temporal availability. In our simulations, each 2-month extension of food availability increased bTB prevalence 2–3% and herd breakdowns increased dramatically (**Table 1**). The longer food sites are maintained, the greater the cumulative transmission of disease over time (6, 7, 47). These simulations suggest that even 5 years of feeding throughout the winter increased prevalence by more than 50%. Although historic large-scale winter feeding (11) has decreased significantly, a resurgence of this practice could wipe out gains made in bTB control to date after only a few years. Should managers ever allow winter feeding to become widespread again, knowing the likely consequences, the temporal window during which it is allowed must be a critical consideration.

Our temporal persistence scenarios extended the time bait was available on the landscape to simulate winter feeding, a reasonable but imperfect approximation. Bait is used theoretically to aid harvest in autumn. Winter feed sites occur without harvest and generally contain a greater quantity of food than bait sites, potentially attracting more deer to the site. Yet there are usually fewer feed sites in winter than bait sites during the hunting seasons. Historic winter feed site locations were documented previously via aerial surveys when winter feeding was extensive. Future research could incorporate these sites into our model as a new GIS layer to more precisely estimate site densities for comparison against these results.

Our model does not explicitly account for indirect transmission of bTB resulting from environmental contamination; if it did, we hypothesize that both prevalence of bTB in deer and the number of cattle herd breakdowns would likely increase, although the magnitude of those increases is uncertain. Deer infected with *M. bovis* shed infectious bacteria in oronasal secretions (48), and food items contaminated by infected deer are infectious for susceptible deer (49). In a field setting, bait and supplemental feed sites facilitate both the contamination of feedstuffs and surrounding soil with saliva and nasal secretions of deer feeding there (**Figure 4**), and so act as an efficient source of bTB transmission even after both the infected deer, and perhaps the feed itself, are gone. Experimental studies conducted both under laboratory (50) and Michigan outdoor (51, 52) conditions have shown that *M. bovis* can remain viable on feedstuffs for days to several months, depending on the substrate and ambient conditions such as temperature, humidity, and shade. That said, efforts to isolate the bacteria from documented deer feeding sites in Michigan have thus far proven unsuccessful (53). Although yet to be explicitly shown in Michigan field settings, research from other bTB-infected ecosystems has elegantly demonstrated the efficacy of environmental substrates such as watering holes as sources of bTB exposure (54). To parameterize indirect environmental transmission in our model, further research to determine the visitation rates of deer to sites where food was once, but is no longer, present would be necessary, along with rates of soil ingestion. Persistence times for *M. bovis* could be drawn from existing distributions of bacterial survival on foodstuffs (50). Alternatively, a value for indirect transmission could be estimated arbitrarily, and subjected to sensitivity analysis as in previous work (12). In any case, our model has demonstrated the importance of bait piles for bTB transmission in WTD, notwithstanding the additional risk due to environmental contamination.

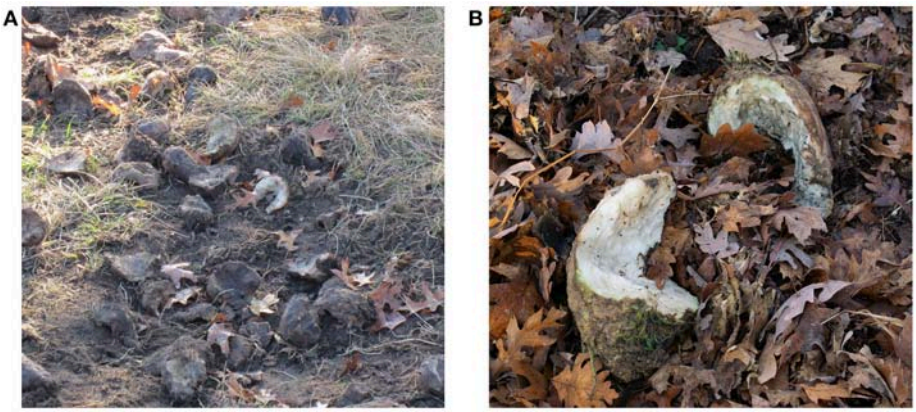

FIGURE 4 | Sugar beets (*Beta vulgaris* subsp. *vulgaris* convar. *vulgaris* var. *altissima*) in an illegal deer baiting site, Alcona County, Michigan. **(A)** Hoof traffic exposes soil which may act as a source of indirect exposure to *M. bovis* when contaminated by infected deer. **(B)** Teeth marks on sugar beets partially eaten by deer. *M. bovis* likely survives for extended periods on these foodstuffs under the cold, wet conditions typical of autumn and winter in northeastern Michigan.

No matter how feed sites are used, their presence, and potentially their past presence, increases bTB prevalence (12). Aggregation, crowding, increased competition, exposure to unrelated individuals, and increased predator-prey interactions are a few of the consequences of feed sites (5–7, 46, 55). These effects in turn increase stress and lower immune response, increasing susceptibility to disease (7, 56). Supplemental feed is often of lower quality than naturally available food and does not provide complete nutrition (57), leading to deficiencies and lowered immune response. Feeding deer to aid winter survival can be successful if begun early, but resulting increased survival and fecundity can increase densities, increasing bTB transmission. Feeding later in winter after nutritional deficits are realized often does not aid survival because body condition is often too poor to be reversed, putting these deer at even greater disease risk (57).

Our results indicate that altering how bait and feed for deer are used can reduce, or increase, cattle herd breakdowns, which is frequently implausible to the public. Discussions regarding the use of bait and feed are often considered of relevance only to bTB transmission among deer. While not effective as the sole management tool (27), how food provided by humans for deer impacts broader issues of bTB eradication from cattle must be considered by both regulators and agricultural producers. While deer bait and feed provide a market for crops that have limited marketability as human food, agricultural stakeholder groups should carefully consider the trade-offs between income generated for crop farmers vs. the economic costs of herd breakdowns to the cattle industry and the larger agricultural economy. Such introspection has largely been lacking in Michigan thus far.

Managing disease in deer must not preclude the use of other means of disease management for livestock. Increased biosecurity on farms in areas where bTB is endemic should remain a priority. Brook et al. (25) argue that a "bottom-up" approach for reducing transmission risk at the wildlife-livestock interface would be more practical and effective. This approach tailors risk mitigation to the individual farm level, taking into consideration spatial overlap and resources, winter feeding areas, animal behavior, fencing, and farm management, in contrast to relying primarily on wildlife culling or harvest, disease testing, baiting and feeding regulations, and cattle depopulation. Clearly, changing farm management can reduce disease transmission between wildlife and livestock substantially (23, 25, 26, 58–60). Our model includes a parameter to account for the proportional reduction in deer-to-cattle contact likely to be afforded by increased biosecurity [see Equation 1 in (27)]. For this study, we chose to hold that parameter at its default value of 1 (unmitigated deer to cattle contact). While heightened biosecurity could help reduce the effects of increased deer baiting and feeding, previous work suggests quite a high level of biosecurity would be necessary in order to have a high probability of reducing herd breakdowns [Figure 10 in (27)]. Although increased biosecurity alone is unlikely to eliminate herd breakdowns in the absence of broader measures to control bTB in deer (27), improving farm management in conjunction with altering supplemental food use for wildlife may facilitate reductions in interspecies bTB transmission.

CONCLUSION

The use of supplemental food for deer continues to be one of the biggest regulatory challenges to bTB eradication in Michigan. As long as widespread baiting and feeding continue, successful eradication of bTB is likely unattainable. As wildlife managers learn to compensate for the decline in hunter numbers and adjust to changes in hunter demographics, the challenges for disease management become more complex. Even if a low level of bTB in deer is acceptable to the public, the ever present and serious risks to livestock remain problematic. Our modeling results demonstrate that a link between supplemental feeding of deer and occurrence of bTB in livestock exists and that feeding has implications not only for deer. Wildlife management necessarily involves managing people and their behaviors as well as wildlife

populations. Disease management programs need to include educating people on how perceived short-term benefits from the use of bait and feed can lead to adverse long-term consequences. Convincing people not only to change their own behavior, but to also encourage others to do so, requires a culture change. Invoking this change is one of the most difficult challenges wildlife disease managers face.

AUTHOR CONTRIBUTIONS

DR developed and recalibrated the model. DO provided the project concept and design. MC conducted the simulations, analyzed output, and drafted the manuscript. All authors contributed to intellectual material, manuscript review, and editing, and have approved the submitted manuscript.

FUNDING

This work was supported by the Federal Aid in Wildlife Restoration Act under Michigan Pittman-Robertson Project W-147-R.

ACKNOWLEDGMENTS

The authors are grateful to A. Locher for allowing use of habitat potential data from which the habitat layers of the deer model are constructed.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 29 November 2018
doi: 10.3389/fvets.2018.00301

Persistent Spillback of Bovine Tuberculosis From White-Tailed Deer to Cattle in Michigan, USA: Status, Strategies, and Needs

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 02 July 2018
Accepted: 12 November 2018
Published: 29 November 2018

Citation:
VerCauteren KC, Lavelle MJ and Campa H III (2018) Persistent Spillback of Bovine Tuberculosis From White-Tailed Deer to Cattle in Michigan, USA: Status, Strategies, and Needs. Front. Vet. Sci. 5:301. doi: 10.3389/fvets.2018.00301

Free-ranging white-tailed deer (*Odocoileus virginianus*) are believed to be a self-sustaining reservoir for bovine tuberculosis (bTB) in northeastern Lower Michigan, USA. Although a comprehensive control program is in place and on-farm mitigation strategies to curtail bTB transmission between cattle and deer have been implemented for over a decade, cattle and deer continue to become infected with the disease. Thus, renewed motivation to eradicate bTB is needed if that is truly the goal. Recurrent detection of bTB in cattle in the region is of mounting concern for state and federal agricultural agencies, producers, and wildlife managers. Current on-farm mitigation efforts include fencing and refined cattle feeding and watering practices. Liberal removal of antlerless deer through hunter harvest and disease control permits (DCPs) issued to cattle producers and agency sharp shooters have also been ongoing. Although these strategies have merit and efforts to reduce prevalence in deer and occurrence of positive farms are elevated, additional actions are needed. Heightened management actions to combat bTB in deer could include deer vaccination programs, strategic habitat manipulations to redistribute deer from farms, and precision removal of deer in proximity to high-risk farms. Foundational research to address development and delivery of vaccine to free-ranging deer is complete. Strategic management and habitat manipulation could reduce and disperse local concentrations of deer while better meeting wildlife, forestry, and agricultural goals. The responses of local deer populations to targeted removal of individuals are generally understood and there is potential to reduce deer activity around agricultural operations while allowing them to persist nearby on natural foods. We summarize the history and progress to date, discuss the realized merit of novel management strategies, and suggest options to rid deer and cattle in Michigan of bTB.

Keywords: bovine tuberculosis, cattle, disease, *Odocoileus virginianus*, transmission, spillback, spillover, white-tailed deer

KEY CONCEPTS

Integrated disease management: employing a variety of proven strategies simultaneously to most efficiently achieve management objectives.

Mitigation measures to protect cattle: specific actions taken to reduce potential for direct and indirect transmission of *M. bovis* from wildlife to cattle.

Management strategies for deer: specific actions designed to reduce potential for maintaining disease within free-ranging deer such as using hunters or professional sharpshooters to reduce deer numbers and eliminating the provisioning of anthropogenic food sources with the intent of attracting and maintaining deer concentrations.

Negative impacts of supplemental feeding and baiting: anthropogenic feeding leads to artificially high and concentrated populations of wildlife which in turn increases disease transmission risk and prevalence.

Setting realistic goals: developing a documented and well-informed formal strategy designed to reach a common and achievable goal.

Public support, political will: varying stakeholder motivations must be considered, reconciled and presented to decision makers so they can empower the pursuit of common goals.

INTRODUCTION

History of Bovine Tuberculosis in Michigan, USA

Bovine tuberculosis (bTB), caused by the *Mycobacterium bovis* (*M. bovis*) bacterium was historically a disease among cattle that spilled over into free-ranging wildlife where it persists (1–3). Bovine tuberculosis is a threat to national and international beef and dairy markets. There are currently more than 13,000 cattle producers maintaining >1.1 million cattle in Michigan. The United States Department of Agriculture (USDA) has 5 levels of zoning regarding bTB status that states, or zones within states, fall into regarding presence of bTB infection in cattle ranging from 1 with no apparent prevalence in cattle and bison (*Bison bison*) to 5 with an unknown or $\geq$ 0.5% herd prevalence. The 5 levels include: (1) Accredited-free zone ("TB free"), (2) Modified accredited advanced zone (MAAZ), (3) Modified accredited zone (MAZ), (4) Accredited preparatory zone, and (5) Non-accredited zone. Zoning enables agencies to tailor surveillance and management strategies relative to regional disease prevalence and potential risk of spread (4). The continual appearance of bTB in livestock facilities in Michigan annually keeps the zoning status of the state at risk while maintaining producer's ability to engage in national and international markets (5).

Movement of cattle from the MAZ must originate from a bTB accredited-free herd or one that has had a negative whole herd test within the previous 12 months and requires a movement certificate, unless the cattle are being moved directly to slaughter. On March 21, 2018 a new TB Zoning Order was signed into effect by the Michigan Department of Agriculture and Rural Development (MDARD) that established the Enhanced Wildlife Biosecurity Area (EWBA; an area slightly larger than Deer Management Unit (DMU) 452 in the center of the MAZ) (6). Development of the EWBA and increased disease mitigation efforts were an intensified effort to avoid another spike in incidence of infected herds like was seen in 2016 when 4 beef herds, 1 feedlot, and 1 dairy herd within the MAZ were found bTB positive (see **Figure 1**) (5, 7). As such, if the incidence of bTB infected cattle herds continues to rise or fluctuate like it has in recent years, there is a chance that the 4-county MAZ status or even statewide status (TB Free) could be in jeopardy (5).

History of bTB in Deer in Michigan

In 1975 and again in 1994 bTB was detected in white-tailed deer in the northeastern lower peninsula (NELP) of Michigan. After which the Michigan Departments of Natural Resources (MDNR) initiated a surveillance program of testing hunter-harvested deer (8–10) (**Figure 2**). A collaborative effort was initiated in 1996 by Michigan Departments of Agriculture (MDA), Community Health (MDCH), MDNR, the USDA, and Michigan State University (MSU) to manage bTB by initiating the Michigan Bovine Tuberculosis Eradication Program (11). In 1997, bTB was identified in the first positive cattle herd in the core disease outbreak area since 1974 (12) (**Figure 2**). In January 1998, the Governor of Michigan directed the MDCH, MDA, and MDNR to develop a plan for eradicating bTB from Michigan deer (13). In summary, the directive included the following components for the 5-county endemic area: (1) implement a deer feeding ban, (2) develop deer harvest quotas consistent with eradication goals, (3) develop methods for eliminating contact between cattle and deer, (4) continue surveillance and determine actual prevalence and evaluate trends, (5) educate stakeholders on managing deer with the goal of eradicating bTB, and (6) enlist a Coordinator to implement the eradication strategy (13). The directive was prepared based on the prioritization of public health and natural resources and insuring the vitality of agricultural industries.

Cattle are acknowledged to be the original source from which bTB or more specifically, *M. bovis* bacterium were disseminated into the spill-over host, deer, which now spill the pathogen back over to cattle (3, 14). The deer in this area of Michigan, then, are acting as a maintenance or reservoir host sustaining the disease on the landscape (see **Figure 2**) (3). Likelihood of maintaining disease would be increased if there was continued spillover from another reservoir host, such as the original source, cattle. Though considerable attention is paid toward protecting cattle and their feed and water sources from potentially infected wildlife species, it must be emphasized that deer are at risk of infection from cattle as well (3). As bTB-positive livestock operations are identified every year, more novel and aggressive approaches will be required to eradicate bTB from the NELP of Michigan, USA.

The infected deer population of the endemic area contributes to continued infections in cattle (1, 3, 10). This area lies within state-designated DMU 452 which is within a 4-county area consisting of Alcona, Alpena, Montmorency, and Oscoda counties. By 1994, the estimated deer densities where bTB occurred were at or beyond biological carrying capacity (19–23/km^2) and there were high densities maintained largely

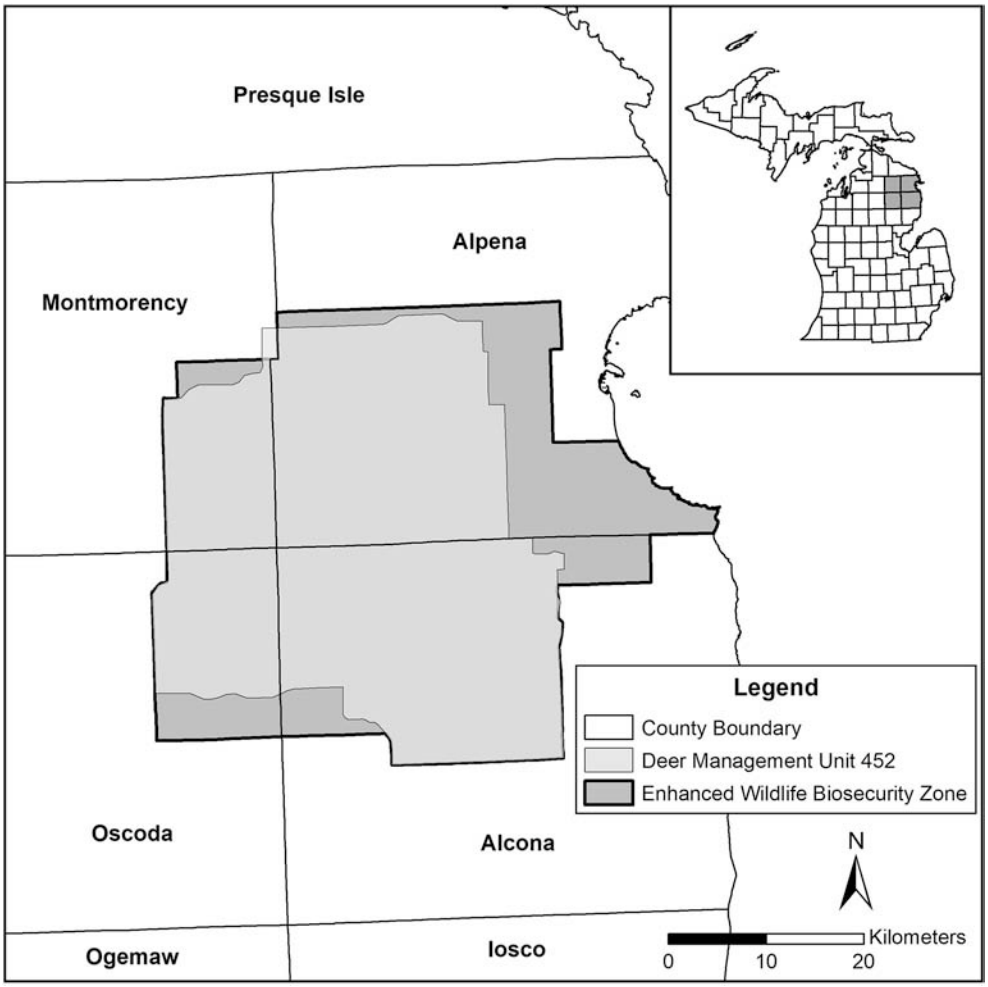

FIGURE 1 | Area of endemic bovine tuberculosis infection in both livestock and wildlife in Michigan, USA, often referred to as the "4-county area" or Deer Management Unit 452 (149,018 ha). The Enhanced Wildlife Biosecurity Zone is an area with increased disease mitigation efforts focused on separating cattle and white-tailed deer (*Odocoileus virginianus*).

through supplemental feeding by hunters and other deer enthusiasts (**Figure 2**) (15). Apparent prevalence rates for bTB in deer in the endemic area as of 2011 ranged from 1.2% (2005) to as high as 4.9% (1995) and has hovered just below 2% over the two decades since (12). Although apparent prevalence rates are an imperfect predictor, they are frequently the best information available to monitor trends in disease (16). From 1994 to 2009 apparent prevalence of bTB in deer correlated with deer population estimates in the endemic area very well (**Figure 2**) (12).

History of Baiting and Feeding Relative to Maintenance of bTB in Deer in Michigan

In general, the bTB endemic area of Michigan consists of several land management types that are relevant to perpetuating the disease and managing the situation: first, several large privately owned parcels of deer habitat are managed exclusively for hunting (17); second, large tracts of public and privately owned forests exist in multiple successional stages thus providing ample deer habitat components in proximity to one another (18); and third, interspersed agricultural lands consisting of dairies, crops, pastures, and beef cattle operations. The makeup of these agricultural lands provides high quality deer habitat in the region.

Supplemental feeding to sustain and concentrate deer and baiting to attract them to specific locations for hunting purposes were common practices in this area and contributed largely to high deer densities and disease transmission (17, 19–21). Prior to restrictions and bans on feeding and baiting, 72% of non-resident and 87% of resident hunters in the NELP of Michigan used bait while hunting (22), illustrating how prevalent these practices had become. Feeding and baiting helped develop a deer population that ultimately exceeded an estimated 20 deer per km^2 (8). As discussed by 8 baiting and feeding are recognized by natural resources professionals as the primary reasons originally enabling deer to become reservoir hosts for bTB in this area.

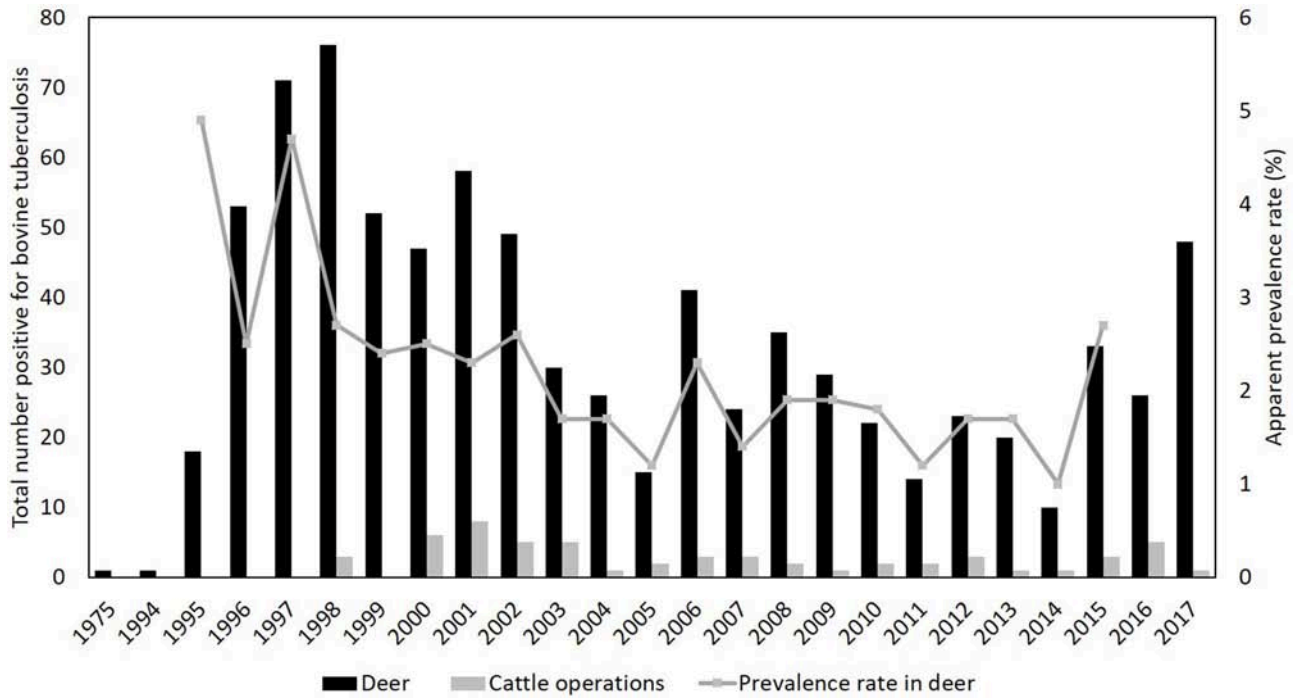

FIGURE 2 | Apparent prevalence rates in white-tailed deer (*Odocoileus virginianus*) as well as numbers of cattle operations and white-tailed deer confirmed positive for infection with *Mycobacterium bovis*.

KEY CONCEPTS IN MOVING FROM MANAGEMENT TOWARD ERADICATION

Approaches to eradicating disease are situational dependent though often include common key components. Components of previous eradication strategies include: (1) implementation of mitigation measures to protect against transmission of *M. bovis* to and from livestock, (2) implementation of management strategies to reduce prevalence in host species including wildlife and livestock; (3) establishment of well-defined goals, plans and policies; and (4) initiation of strategies to build and maintain support of the broad array of stakeholders.

Current Efforts Toward Eradication in Michigan

As in most disease eradication situations, any single strategy alone will rarely eliminate the disease, especially when there are more than a single reservoir host and free-ranging wildlife are involved (23). As such, a combination of strategies need to be implemented in an integrated approach as this will improve efficacy while reducing overall effort and cost (24). In 2008 MDARD initiated the Wildlife Risk Mitigation Project (WRMP) which focused on enrolling and assisting livestock producers in implementing and maintaining an array of measures to reduce risks for transmission of *M. bovis* between deer and cattle on their properties (2, 25–27). Producers were encouraged to participate in the project which entailed education, completing an on-farm assessment of risks, committing to a formal action plan, initiating the action items within the plan, and passing a verification visit to ensure they implemented the plan (25).

A primary risk of transmission between wildlife and cattle stems from shared resources like food, water, and habitat (19, 27–29). Thus, mitigation measures were directed at protecting resources that are concentrated such as stored cattle feed, watering systems and areas routinely occupied by cattle (2, 26–28). It was also recognized that commonly used farm management practices needed to be evaluated and improved upon. Practices such as the collection of waste slurry from cattle that is then applied to crop fields is questionable especially when there's potential for *M. bovis* to be present (30, 31). This practice often occurs during spring green up when nutritionally stressed deer are dispersing from winter concentration areas in search of nutritious food sources like crop residues and lush new growth emerging in crop fields following snow melt (32).

At the initiation of a plan within the WRMP, landowners meet with an agency wildlife biologist on the farm to assess risk factors for disease transmission. Mitigation measures ranging from strategic feeding practices to constructing feed storage facilities are then recommended based on identified risk factors. The WRMP is a science-based program and the efficacy of many of the recommended mitigation strategies have been supported by research findings including the use of fencing (26, 33) and gates (34, 35) to protect stored feed and feeding areas. Risk mitigation strategies prescribed included, but were not limited to: (1) protecting cattle feed by storing it in buildings or within deer-proof fences with gates closed, (2) feeding cattle daily and

away from deer cover, (3) strategically positioning water sources to minimize access and potential contamination by deer, and (4) using disease control permits (DCPs) to reduce antlerless deer numbers on and around farms (5). The majority (545 of 620; 88%) of the farmers in the 4-county MAZ participated in the Program and were subject to annual inspections to insure compliance and maintain their verification (5).

When motivation for deer to access food and water is elevated, such as during late winter, increased vigilance and additional measures to exclude or deter deer may be required (26, 36). The efficacy of mitigation measures is directly related to the motivation of an animal to overcome it and the vigilance of the farmer. Motivation also varies with circumstances relative to season (e.g., severity or length of winter, drought conditions in the summer) and availability of natural foods and water. Producers must be cognizant of these factors, and therefore, when risk is increased, must increase vigilance to maintain an effective level of biosecurity (26, 36, 37). Such mitigation measures and environmental influences are discussed during risk assessments to insure producers understand that wildlife risks are not static and identify factors and scenarios that may increase risk.

Current Efforts: Exclusionary Fences

The use of fencing to exclude deer is an effective means for protecting concentrated resources meant for livestock (2, 26, 33). Numerous fence types exist and fence selection can be based on the predicted level of motivation for deer to breach, the desired longevity, and associated cost (33, 38). In high-biosecurity situations where essentially no deer breaches are acceptable, woven-wire fences $\geq$2.44 m in height are recommended (33, 39). Interestingly, the "weakest link" of a fence is the gate, which obviously must be closed to be effective (26, 34). While this may seem like common sense, in areas where frequent access is needed, livestock producers commonly become lax, leaving gates open, especially during daylight hours. Deer, then, have been documented entering fenced areas of stored feed through open gates in the middle of the day when it was assumed they would not be nearby or active (26).

Current Efforts: Livestock Protection Dogs

Livestock protection dogs (LPDs), traditionally developed and used for reducing the killing of livestock by predators, have also proven effective in keeping deer from directly and indirectly coming in contact with cattle (40). Using specially trained dogs for protecting numerous agricultural resources is becoming more widespread (41, 42). For example, LPDs have proven effective in protecting crops (43), cattle pastures, and feed (40, 44). In the case of transmission of *M. bovis* between cattle and deer in which concerns over indirect transmission through contaminated resources are greatest, LPDs employed to protect stored feed and other resources would be beneficial (40, 44). Although LPDs can effectively repel deer to protect localized areas and livestock, there is a point in which the size of the area or the amount of deer activity exceeds the abilities of a single LPD and either additional LPDs or integrating other measures such as exclusionary fences are needed (33, 44).

Current Efforts: Strategically Locating Feed and Water for Cattle

Currently, 88% of commercial farms in the MAZ are incorporating practices focused on protecting cattle-related resources from wildlife that is potentially harboring bTB (5). Although participation is high, increased emphasis on consistent use and maintenance of mitigation measures is needed (26). Such resources include water, feed, and mineral supplements, all of which are sought by deer and other wildlife and should be a focus of concern regarding the transmission of *M. bovis* (19, 28, 45, 46). Initially, USDA cost-share programs assisted producers in incorporating secure feed storage options including hoop barns and deer-exclusionary fencing to minimize deer access to cattle resources. Refined feeding strategies including limiting provisions to just what a group of cattle will consume that day and constricting the time and duration of availability to just daylight hours can help reduce deer activity in cattle feeding areas (5). Water, though, needs to be available continuously so could be more difficult to protect from contamination by deer (5). Storing and providing cattle resources (feed, supplements, minerals, water, etc.) away from permanent deer habitat and closer to areas of human activity is also recommended.

Current Efforts: Cattle Identification and Tracking

Annual whole-herd testing of cattle for bTB and outfitting cattle with radio-frequency identification (RFID) tags became a requirement for Michigan producers in the endemic area to move live cattle off their farms in 2007. These requirements enable trace-back investigations to locate where and when bTB-infected cattle shared the same space as other cattle, with the goal of identifying other potentially infected animals and premises (29, 37, 47). Although the infection of a herd due to movement of an infected cow into that herd occurs (48), it was presumed to be a lesser risk for cattle producers in Michigan than infected deer (29, 47). Yet recent cases outside of the endemic area and within the accredited-free zone of southern Michigan suggest spread of bTB via infected cattle may actually be increasing (49).

Current Efforts: Reducing Deer Numbers

Population reductions are often considered or used in response to outbreak of disease and involves reducing the density of the host population through strategic lethal removals, usually through culling by professional sharpshooters, or increased recreational hunter harvest (50, 51). Large-scale removals of reservoir species have been implemented and proven effective in some cases (23, 52–55). Though used to a degree in the endemic area of Michigan, these options have proven controversial and have not been wholly accepted by producers, hunters or other publics in Michigan (24, 56).

With the goal of reducing the potential for transmission of *M. bovis* between deer and cattle the MDNR initiated a program in 1998 in which cattle producers could acquire DCPs allowing them to personally address the deer situation on their land by harvesting deer themselves or enlisting the help of sharpshooters with the USDA Wildlife Services (12). Producer use of these permits, though, was low. Only 12% of 6,427 tags were filled in

2008 and deer numbers have since increased as have associated disease prevalence rates (12, 57).

Damage tags or block permits were also available to producers who were experiencing damage to crops by deer, allowing them to harvest deer on their property to alleviate ongoing problems (57, 58). Similar to DCPs, participation was low and lack of local public support was presumed to be the cause (12, 57, 59). Occasionally, negative concerns about these non-traditional deer harvest strategies were voiced by owners of recreational lands adjacent to at-risk farms (12). For example, even when deer density estimates were 8–15/km^2 and crop damage was substantial, only five of 31 alfalfa growers participating in a crop damage project requested permits to control damage on their property and only 42% of issued permits were used. Similarly, red kidney bean growers were issued a total of 88 permits and only 23% were used (60, 61). These data illustrate that even when landowners were faced with substantial amounts of crop damage and provided permits to reduce deer numbers, they were not using them (60, 61).

The MDNR increased the number of available deer tags and the number of hunting seasons, with the focus on removal of antlerless deer, and successfully reduced deer numbers within DMU 452 by 50% from 1995 to 2004 (12, 62). However, deer numbers rebounded rapidly since 2005 to >110,000 and remained steady through 2009 (12). More recently hunting opportunity and harvest potential has been essentially unlimited in DMU 452, though hunters have harvested less than one thousandth of the tags available (i.e., 4,388 deer from 5,575,390 potential tags), demonstrating that demand for opportunity has been saturated (63). Although the MDNR was effective in reducing deer numbers initially, hunters were not overwhelmingly supportive of these actions (22). As a new strategy to address insufficient harvest on farms, it is now a requirement for livestock producers to include and implement a deer reduction component within their EWB Plan, specifically focused on those deer routinely in proximity to farms with cattle (5). As this is a recently enacted requirement, the effects are yet to be seen.

Intensifying Efforts to Reduce Potential Transmission of *M. bovis*

Nearly 20 years ago it was stated that "The measures of apparent bovine TB prevalence have decreased by half since 1997, providing hopeful preliminary evidence that eradication strategies are succeeding" (15), but bTB still persists in Michigan. Ongoing and increased efforts to reduce the persistence of *M. bovis* continue; however, the rate of cattle operations being identified as positive for bTB fluctuates at levels that put the accredited-free status of Michigan in jeopardy (>3 positive herds detected/year) (5). Despite extensive efforts prescribed by the previous WRMP, bTB-positive herds continue to be identified each year, thus new approaches are needed if the goal is still to eliminate the disease. To this end the EWB Project was initiated to involve more thorough on-the-ground assessments of properties housing cattle by an "EpiTeam," similar to what is used following the detection of positive cattle. Each team includes a MDARD veterinarian, a USDA or Alpena Conservation District wildlife biologist, a MSU Extension cattle specialist, and a local producer (5). Each assessment results in an action plan (now entitled "Enhanced Wildlife Biosecurity Plan") that needs to be implemented on the ground by the producer, similar to how the WRMP was implemented from 2008 to present. Producers must implement and maintain all prescribed mitigation measures relating to high-risk areas on their farms by December 31, 2019 or will lose their ability to sell cattle other than directly to slaughter (6).

Intensified Efforts: Targeted Deer Removals

All existing 130 commercial cattle producers in the EWB Area require a deer removal component that enables sharpshooters to remove deer from in and around farms and pastures. For example, in Dressel's (32) study up to 13 deer were removed from a single landowner's property. Action plans in the EWBP are designed to eliminate deer whose home range includes farms and deter others from establishing ranges in proximity to farms (5). The frequency of visitation to highly-desirable resources such as stored feed and agricultural crops may be a learned behavior that could be curtailed by removing mature does or entire family groups of offending deer (64). It has been documented that fawns can learn movement patterns from adult does and that it is typically a few specific deer in a given area that will share space and time with cattle or frequent stored feed areas (27, 36, 37). As such, targeted removal of offending individuals may curb present and future visitation of farms by deer. Research has shown deer frequent farms the most often during: January through mid-April; and Mid-July through August (26, 36), thus these periods are when removal efforts should be focused.

Intensified Efforts: Strategic Habitat Manipulations

Wildlife management consists of three components: (1) the biota or populations, (2) the habitats or ecosystems organisms need to persist, and (3) the people or stakeholders that live in the ecosystems and interact with the wildlife resource (65). To date, bTB research and management practices have been directed primarily at two components, namely the biota (i.e., deer) by reducing numbers through recreational hunting and targeted deer removals and the people (i.e., hunters, producers) by manipulating harvest regulations, deer baiting, and feeding practices, and how producers store and protect feed and water resources. Historically, the third component of wildlife management, the species' habitat, has not been factored into bTB management strategies. Perhaps because, as Felix et al. (66) suggested, "managers may lack sufficient understanding of long-term spatial and temporal links between habitat supply and population response." There has, though, been an extensive amount of research on why and how forest management practices can be used to enhance or reduce quality of deer habitat [e.g., (67, 68)] and potentially influence the distribution of deer across a landscape (18).

The Alpena-Montmorency Conservation District of Michigan has recently initiated a cost-share program that may assist in influencing deer movement patterns, potentially away from stored feed, water sources and livestock concentrations,

by initiating habitat improvements for deer through forest management activities. This new program, if implemented strategically could stimulate deer to redistribute themselves away from agricultural areas to other, naturally occurring vegetation types. The program also includes incentives that encourage data collection and the liberal harvest of antlerless deer (69).

The quality and distribution of a species' habitat is a primary driver influencing the spatial and temporal distribution of species [e.g., (70)], including deer [e.g., (18, 71)] and elk [e.g., (72, 73)]. Recognizing how habitat quality and its distribution can influence the movement patterns of a species, a strategy could be to use this basic ecological principle as a tool to combat bTB in the NELP. An additional step may be to take measures to lessen the quality of habitat for deer on and adjacent to farms, lowering the area's carrying capacity, the desire of deer to be there, and the fitness of deer that persist.

The core of the bTB area, DMU452 is composed primarily of private land (93%) (74) that is dominated by forest cover types. For example, within Alpena County alone, 60% of the area is covered by lowland conifer swamps and northern hardwood forests interspersed among agricultural areas. Much of the forest is relatively later successional stage, especially on private lands. Given that the life requisites of deer in this area include: spring and summer food, thermal cover, and fall and winter food (66), much of the agricultural lands and livestock areas are often under tremendous feeding pressure by deer especially in late-winter through summer (32, 60, 61, 75). A cover type lacking in this area that deer could use extensively for feeding and cover is regenerating deciduous stands (e.g., aspen clearcuts of predominately early age classes) (66). Experimenting with forest management practices as a method to manipulate how deer use the landscape has merit.

Habitat management on public lands is a primary activity used by agencies to meet wildlife management objectives and satisfy a diversity of stakeholders, yet it is poorly understood how frequently or what types of management, if any, occur across private lands. The use of landowner incentive or cost-share programs to manipulate forest cover types to improve habitat conditions away from agricultural lands and livestock operations should be investigated for their efficacy in: (1) providing quality habitat, (2) shifting the distribution of deer away from agricultural areas at high risk for transmission of *M. bovis*, (3) reducing crop damage, and (4) meeting economic objectives of landowners for harvesting forest types. Such a habitat-based bTB management and research approach could be initiated and simultaneously integrated with other bTB mitigation practices. The successful management of this complex problem could be enhanced if the habitat for deer were factored into the management equation.

Potential Future Efforts

Original actions to eradicate bTB in Michigan combined with recently emerging science-based strategies have all been insufficient to date, primarily due to waning stakeholder support. Several new strategies and directions are mentioned above and have begun, here we discuss additional potential measures to consider if the collective desire of agencies, stakeholders, and other publics is to eradicate bTB from Michigan.

Potential Future Efforts: Reducing Deer Numbers

As stated by Riley et al. (76), "An assumption in most conventional deer harvest strategies is that adequate demand for and successful use of antlerless deer permits exists to achieve desired deer harvest." As deer densities decline and number of deer encounters are reduced, hunter perception and support, effort, and desire to continue hunting fade and hunters will often transition to other locations or species (77, 78). When hunter harvest is no longer effective in maintaining deer populations at or below goal, additional measures must be contemplated. In such situations "Hunting eventually may become less a recreation and more a community service or civic duty … Culling may be a more appropriate term for the kind and purpose of hunting under such circumstances" (76). Although recreational hunting is and should remain the primary means for managing white-tailed deer, there are situations in which it may not be safe, feasible, or effective and other means need to be considered (79). Within DMU 452 where deer reductions are needed and current harvest is insufficient, strategies like earn-a-buck or incentivizing hunters by allowing easy donation or profiting from venison may be worth consideration (79–81).

Most (>90%) of the bTB area in Michigan is privately owned (74) which has contributed to challenges in achieving wildlife management goals (9). Although purely speculative, it is uncertain about what the future for large privately-owned "hunt clubs" will be with consistently declining numbers of hunters. Will the owners of these lands want to hunt them in the future or use them simply as family get-aways? How will this affect the local deer population? A decreasing trend in hunters has been well-documented in the US (82, 83) and in Michigan specifically (84, 85). Because of these trends, other approaches might be warranted such as the MDNR purchasing large tracts of hunt clubs or other private lands (farms) to improve access. For example, from January 1998 to November 2018, the MDNR purchased a total of 34,240 ha state-wide with an average of 1,630 ha being purchased annually and the mean amount of land acquired per transaction was 53 ha (K. Wildman, Biologist, MDNR, personal communication, 05 Nov 2018). Non-profit conservation organizations such as the Rocky Mountain Elk Foundation are often partners in purchasing land which the state then manages and oftentimes provides public access for hunting. A local example in northern Michigan was the purchase of the Green Timbers tract in 1982. This property is now attached to the Pigeon River Country State Forest and provides unique walk-in only hunting and other recreational activities (e.g., backpacking, hiking, cross country) for the public. Acquisitions such as this improve the ability of the MDNR to manage the deer population and provide opportunity to its constituents.

Potential Future Efforts: Vaccination Program for Deer

An additional novel tool that could aid eradication of bTB in Michigan is an oral vaccine against bTB for deer. Interest in using a vaccine for bTB in deer is increasing (32, 63, 86). Bacille Calmette-Guerin (BCG) vaccine reduces disease severity

by decreasing gross lesions and sites of infection, suggesting potential for reducing transmission and minimizing endemic infection in wildlife (87, 88). Significant progress has been made in demonstrating the safety, efficacy, and feasibility of implementing a vaccination program against bTB for deer (89–92). Researchers modeled vaccination and demonstrated that vaccinating just 50% of the deer would contribute to an 86% probability of eradicating bovine tuberculosis in DMU 452 in 30 years (63). Interestingly, in the presence of recreational baiting it would be highly unlikely to achieve eradication within the next 30 years at the same vaccination rate (63). A vaccination rate higher than 50% could likely be achieved based on an experiment where placebo vaccine baits were effectively delivered to free-ranging deer (32) which would increase the probability of eradication. Of course, implementing a vaccination program while maintaining the use of additional management strategies; restrictions on baiting, liberal recreational harvest, DCPs, and fencing stored feed and other cattle resources would be the most efficient path to eradication (63, 86).

Potential Future Efforts: Novel Diagnostic Tests for bTB

As current live-test methods involve multiple animal handlings, take 48–72 h to produce results, or require specialized laboratory procedures, improved methods are needed for reliable and timely detection of bTB (93, 94). A "trap–test–cull" project was evaluated using a rapid test and live capture of deer, though it was deemed cost-prohibitive (>$1.5 million US annually) and ineffective in reducing prevalence of bTB (95). Recently developed methods that enable the antemortem detection of unique biomarkers of disease suggest improved diagnostics are becoming available. For example, infection by *M. bovis* results in the presence of specific peptides in the blood which can be detected with common laboratory analyses (96). Additionally, the analyses of breath from cattle to detect bTB-specific volatile organic compounds has proven effective in experimental settings and has potential for applications with deer (94). Also, genotyping particular strains of bTB pathogens enable back tracking to determine the source herd of cattle for the disease (97). New tools like these and the support to develop them are desperately needed.

The People Piece

Public support and involvement is essential if complete eradication is the goal. Are Michigan residents accepting of a low level of bTB sustained in their deer herd? It was apparent in 2006 that Michigan hunters felt bTB was not a problem, ranking it considerably lower than "more extensive problems" including too few mature bucks and too few deer in general (12). Are Michigan livestock producers comfortable with the risk that they may have a reactor cow in this year's whole-herd test and that theirs could be the next positive herd? It is clear that Federal and State agricultural agencies are losing tolerance for reoccurring positive cattle farms. As it should be, input from stakeholder groups and various publics have played a large role in political and management decisions regarding bTB in Michigan since 1994 when the second bTB positive deer in 20 years was found. There is potential that had managers been more empowered or convincing and decision makers more stalwart the bTB situation in NELP may be quite different today. Despite extensive surveys examining strategies used to improve stakeholder appreciation of the situation with bTB in deer (98–101), public and political support has been too little to enable the actions necessary to improve the situation (101). To make better progress going forward, more emphasis must be placed on the human dimensions aspects of the issue by more effectively engaging the diversity of stakeholders associated with this deer-bTB-agricultural industry issue.

Policy Based on Science or Public Demand?

Although state wildlife management agencies are responsible for managing wildlife populations, habitats, and the people who use wildlife resources (65), elected and appointed government officials typically make the underlying decisions driving management actions of agencies (102). In 1996, Michigan voters elected to transfer the responsibility for managing game animals from the MDNR to the 7-member governor appointed Natural Resources Commission (NRC). The NRC was mandated to integrate scientific findings and public input into new policies that the MDNR follows; in turn, the MDNR provides recommendations to the NRC to help them make informed decisions when establishing such policies (20). Policy established by the NRC in 2007 presents the goals of the MDNR as using science-based management practices to maintain a healthy deer population as determined by the carrying capacity of its range and the effects upon native plant communities, crops, and public safety (103). Additionally, they set out to maintain an active educational program to inform the public on practices of deer management for achieving a healthy and vigorous herd (103). Despite these basic, well-intended goals driving policy, public trust (of NELP residents) in the ability of MDNR to set deer hunting rules relative to eradicating bTB was lower than 50% in 2011 (104). This distrust has impacted the ability of MDNR to manage bTB and created backlash by local residents and hunting constituency groups (105).

Tools such as spatial models for forecasting likelihood of disease eradication given various approaches are the types of informative tools needed to aid in establishing goals and creating policy (63). A key strategy for facilitating scientifically based decisions leading to effective management actions lies in providing policy makers with accurate information derived from high quality research while respecting their role of representing those that elected or appointed them (102). Further, educating the general public and earning acceptance and trust are also essential to successful management of healthy wildlife populations and their habitats (15, 22).

Building Widespread Stakeholder Support

Initial efforts by state and federal agencies to eradicate bTB in Michigan were extensive despite minimal public support (106). To be effective and successful, actions initiated by agencies have to be accepted and adopted by citizens including hunters, livestock producers, and wildlife viewers. For example, MDNR initiated strategies to reduce deer numbers through increased

availability of hunting licenses and implemented baiting and feeding restrictions (20, 56). Public support and action was needed to harvest additional antlerless deer and to cease baiting and feeding. Although there was a documented 50% decline in apparent prevalence from 1995 to 2004 due to reductions in deer numbers and restricting baiting and feeding (107), deer numbers and prevalence rates have since rebounded. As demonstrated by the incessant reappearance of bTB in deer and cattle, it is apparent public support and involvement are essential for successful eradication or even tempered control (20, 106, 108). It is also apparent that the lucid presentation of specific disease-related risks to one's personal interests are needed to truly bring about action and change (99, 100). Frequently updated information with an emphasis on successes is essential to maintaining or increasing stakeholder support (98).

In addition to insufficient stakeholder support, there has been decreasing financial support to and from federal and state agencies to enable the eradication of bTB from wildlife and livestock in Michigan. This issue has led to fewer personnel and waning awareness and support from most publics. Thus, current and future efforts toward eradicating bTB require maximizing knowledge gained from past efforts to inform next steps for research and management (62). To this end, modeling efforts have helped predict likely outcomes given the tools and resources available to begin answering questions to help optimize and select combinations of strategies to implement (63). Without incorporating new tools and revising strategies, it was predicted that eradicating bTB from Michigan in the next 30 years was unlikely (63, 95).

It has become clear that ongoing strategies for eradicating or even minimizing the transmission of bTB in Michigan have been insufficient, primarily due to lack of sufficient long-term determination of stakeholders. If the Michigan and US goal is to protect the entire country's cattle herd and trade status, increased support and strategies are needed. Further, it is apparent that increased public acceptance and involvement will be required to defeat the challenges associated with the eradication of bTB (56, 107).

Unfortunately, these challenges are deeply rooted in the culture of the area and will not be overcome easily. There are apparent divides and disconnects amongst the interests and demands of various factions of the public (i.e., hunters, cattle producers, policy makers, general public), with public servants from natural resource and agricultural agencies struggling to regain healthy wildlife and livestock populations for them. It seems that through efforts to achieve healthy deer densities in Michigan following the appearance of bTB, public resentment has actually grown (12, 62). Agencies need improvements in public outreach about all aspects of the bTB issue to reverse this trend and garner support for the intentions behind management actions. Given the current popularity and user involvement in social media (i.e., YouTube, Instagram, Podcasts, etc.), it is a new tool that could be used to aid ongoing and future efforts associated with bTB. Although previous efforts to engage and motivate hunters to actively participate in non-traditional deer management actions (i.e., increased harvest of antlerless deer) failed over the long term, significant changes such as providing extended or alternative seasons and increasing attention on new hunters may improve participation (101). Unfortunately, common trends such as managing for more, larger, and more mature (i.e., older) male deer on the landscape, primarily through imposing antler point restrictions, does not align well with disease management strategies focused on removing more males with an emphasis on older age classes (10).

Optional Approaches Toward Managing bTB in Michigan

Going forward, agencies need to (1) establish long-term, mutually agreed upon objectives, (2) develop well-defined strategies that align with those objectives, and (3) develop and implement practices to evaluate the efficacy of those strategies (109). All options toward managing disease, including no action, need to be considered in establishing objectives (24, 86). First and foremost it needs to be determined what the long-term goal is: status quo, eradicating bTB throughout Michigan, eliminating bTB in deer in Michigan, or eliminating bTB in cattle in Michigan. If the presence of bTB in Michigan truly is acceptable, there is always the option of no additional management action whatsoever, although this may need to be coupled with the buyout of all cattle across the region to eliminate potential for cattle becoming infected. Additionally, compartmentalization could be considered to limit the potential for geographic spread of bTB through the use of significant barriers such as large-scale exclusionary fences for deer (24). It was well-stated by Olmstead and Rhode (110) regarding the interconnectedness of the cattle industry, "Given the benefits from trading in livestock and the contagious nature of the disease, it was more efficient to build a "fence" around the entire country than to create barriers around each and every farm."

If the goal is still to eradicate bTB across Michigan as stated by the Governor in 1998, then the potential exists to make great strides. Actions should include but are not limited to: significantly reducing deer densities with focus on those in the vicinity of cattle operations, eliminating baiting and supplemental feeding, segregating wildlife and cattle/cattle resources, using habitat management to change the spatial distribution of deer, and deploying a vaccine for deer.

If the goal is only to eliminate bTB in cattle, the strategy is relatively straightforward especially if all transmission is occurring only between deer and cattle (111). With cattle being the primary concern, excluding deer from all cattle-related resources with true deer exclusionary fencing (i.e., 2.4-m-h woven wire fence) is needed (24, 26, 39). Where this is not possible, such as a body of water bordered by cover used by deer and cattle pastures, either the deer or cattle must be excluded. Although reliable deer-exclusionary fence is initially expensive and may be considered unsightly, it is effective when maintained and would minimize potential for transmission via indirect and direct contact (24, 26, 33, 38). This level of biosecurity is commonplace in other production animal systems such as within the swine industry (24, 112, 113), especially in areas where the threat of disease transmission is a reality. Permanent deer-proof fences are also commonplace and widely accepted

in areas where the captive cervid industry is active, as well as along expansive stretches of highway systems throughout the US where deer-vehicle collisions had been common. These fences are also used around the world in places such as in Africa because they enable managers to achieve extensive and reliable manipulation and protection of various species (33). Given the serious nature of eradicating bTB, reliable management of deer and cattle are needed in Michigan and thus similar measures could be considered.

CONCLUSION

The ongoing situation with bTB in Michigan has been a persistent and expensive management challenge for livestock producers and state and federal agencies for more than a quarter of a century. As biologists and public servants, we may feel ethically committed to ridding the landscape of this disease that impacts the wildlife resource and a primary agricultural industry. But unless the societal and related political support for this exists, perhaps we need to either stand down or double down. The situation in Michigan is a multi-faceted issue with several imposing barriers, ecologically and socially, that are impeding the possibility for progress toward eradicating the disease. The first and foremost challenge is inadequate public concern over the health of the deer population and cattle herd and subsequent lack of political support and action. This challenge obstructs many crucial steps in wildlife management toward eradication, including the banning of baiting and feeding, reducing host populations, and understanding and accepting the severity of the bTB situation across the landscape.

If there was increased public concern about the occurrence of bTB in wildlife, livestock, and humans there would likely be compounded support and participation in actively pursuing eradication. As demonstrated during the era of market hunting, even before the advent of modern hunting tools and technologies (i.e., high-powered rifles and scopes, night vision, remote cameras, helicopters, drones), Americans demonstrated our ability to severely reduce, and in some cases, decimate deer populations when motivated. Conversely and more recently, due to changes in motivators, we have demonstrated our ability to develop large numbers and concentrations of white-tailed deer. Now we must refocus on maintaining populations of fewer but healthy deer in concert with the limitations of local agricultural goals and available natural vegetation types that can provide deer habitat. In 1949, Aldo Leopold wrote, "A thing is right when it tends to maintain the integrity, stability, and beauty of the biotic community, it is wrong when it tends otherwise" (114). Natural resource professionals can still keep this goal in mind while simultaneously acknowledging and addressing the food production needs of our continually growing and hungry populous.

The toolbox contains much of what is needed to combat bTB in Michigan; including increased hunting license allocations, increased availability of disease permits, financial cost-share programs to increase biosecurity on farms, feeding and baiting bans, the use of educational stakeholder meetings, new novel tools to facilitate diagnosis and surveillance, and even a vaccine for deer or evaluating the use habitat manipulations to redistribute deer. None of these tools will be effective alone, they must be applied aggressively and in unison to complement each other. Progress has been made in understanding and managing livestock-wildlife interactions and the transmission of bTB in the Michigan landscape and recent decisions and new strategies have great potential.

AUTHOR CONTRIBUTIONS

KV, ML, and HC contributed equally in the development of the idea, collection of the data, and preparation of the manuscript. KV fleshed out the original outline. ML drafted the manuscript and continually incorporated and massaged his, KV and HC's thoughts. All authors continually reviewed and edited the manuscript, producing the submitted draft.

ACKNOWLEDGMENTS

We appreciate the assistance and support provided by R. Smith, recently retired MDARD. This research was funded in part by the U.S. Department of Agriculture.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past collaboration with one of the authors HC.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 August 2018
doi: 10.3389/fvets.2018.00190

Bovine Tuberculosis Management in Northwest Minnesota and Implications of the Risk Information Seeking and Processing (RISP) Model for Wildlife Disease Management

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OPEN ACCESS

Edited by:
Daniel J. O'Brien,
Michigan Department of Natural Resources, United States

Reviewed by:
William F. Siemer,
Cornell University, United States
Adam Zwickle,
Michigan State University, United States

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 07 May 2018
Accepted: 25 July 2018
Published: 17 August 2018

Citation:
Cross M, Heeren A, Cornicelli LJ and Fulton DC (2018) Bovine Tuberculosis Management in Northwest Minnesota and Implications of the Risk Information Seeking and Processing (RISP) Model for Wildlife Disease Management. Front. Vet. Sci. 5:190. doi: 10.3389/fvets.2018.00190

Bovine tuberculosis (bTB) is an infectious, zoonotic disease caused by *Mycobacterium bovis* that can spread between domestic and wild animals, as well as to humans. The disease is characterized by the progressive development of lesions that compromise the victim's lungs and lymph system. The disease was first identified in northwest Minnesota in both cattle and white-tailed deer (*Odocoileus virginianus*) in 2005. Due to its risks to human and animal health, bTB has numerous implications related to population management, policy outcomes, stakeholder relations, and economic impacts. When dealing with complicated risks, like bTB, individuals often seek out and process information as a method to learn about, and cope, with the risk. We developed a questionnaire that adapted components of the Risk Information Seeking and Processing (RISP) model and surveyed northwest Minnesota deer hunters. Our objectives were to better understand how stakeholders perceive and act on information regarding disease management in wildlife and to understand the utility of the RISP model for such management contexts. We drew a random proportional sample of licensed deer hunters ($n = 2100$) from the area affected by bTB and conducted a multi-contact mail survey. We found that 43% of the variability in the information-seeking behaviors of respondents was explained by demographics, hunting importance, personal risk perceptions, attitudes, and subjective norms. However, these results are largely attributable to the factors in the RISP model encompassed by components of the Theory of Planned Behavior (i.e., attitudes, subjective norms, perceived behavioral control, and behavioral intentions). This information can help managers contextualize individuals' perceived risks to better frame communication efforts to address stakeholder concerns and develop best practices for disease communication. While the state of Minnesota is currently considered free of bTB, future outbreaks remain possible in Minnesota and elsewhere. Understanding the key factors in the processes through which deer hunters seek out information pertaining

to the disease can help managers collect the data necessary to aid decisions about desired future management outcomes. In addition, testing RISP model performance in applied research improves its future use across a broad spectrum of topics throughout veterinary disease management.

Keywords: bovine tuberculosis, communication, disease management, information-seeking, risk information seeking and processing (RISP) model

INTRODUCTION

Bovine tuberculosis (*Mycobacterium bovis*, hereafter referred to as "bTB") is an infectious zoonotic disease that can spread among domestic and wild mammals and, in rare cases humans. Zoonotic diseases like bTB threaten agricultural economies, pose health risks to humans and wildlife, and disturb the social, political, and economic environments where they occur. In Minnesota, the appearance of bTB among wild white-tailed deer (*Odocoileus virginianus*) in 2005 was not only a health concern but also posed a risk to the state's deer hunt, a major economic industry, cultural event, and the primary method in which the state manages its deer population. Therefore, as bTB engaged numerous stakeholder groups, understanding how hunters viewed the risk, and whether their concerns impacted the decision to hunt, was of particular interest to management agencies.

The human dimensions of disease management in wildlife has increased in importance during recent years (1–5). Following Clarke (1), we used the Risk Information Seeking and Processing model (RISP) (6) as a core framework to discern the key considerations for understanding and better communicating with stakeholders about disease management in wildlife. In this study, we were interested in delineating the processes through which Minnesota deer hunters sought information about risks from bTB.

We addressed the following key questions:

1. How do hunters seek information about bTB?
2. What factors affect hunters' information seeking behaviors?
3. Is the RISP model useful for understanding well-established wildlife disease management issues?
4. What are the implications for natural resource management agencies and professionals?

Such information can help guide managers' decisions regarding the collection and analysis of information related to individuals' perceived risks and improve the development of communication best practices in instances of disease in wildlife.

The initial detection of bTB in northwest Minnesota occurred at a beef cattle operation in 2005 (7). Upon further testing, the disease was found in several other beef cattle operations and detected in wild white-tailed deer (*Odocoileus virginianus*) during fall 2005 hunter-harvested surveillance (7). Epidemiological evidence indicated the disease was introduced into a single beef cattle operation and from there it spilled over to deer (8, 9). Deer presumably served as a spillover host for the transmission of the disease among area livestock operations (8, 10, 11).

In response to the detection of bTB in cattle and deer, the Minnesota Department of Natural Resources (DNR) and the Minnesota Board of Animal Health took joint actions to decrease the likelihood of disease spread, eradicate the disease from cattle (and thus regain Accredited-free status within the United States Department of Agriculture's bTB eradication program), and reduce wild deer prevalence to undetectable levels. These strategies centered on preventative measures to reduce the likelihood of disease transmission. Example strategies included a temporary buy-out of cattle producers, construction of deer exclusion fencing around stored forage, prohibiting recreational deer feeding, and reducing the local deer population using hunting regulations, aerial gunning, ground sharpshooting, and deer shooting permits issued to landowners (8, 10, 12).

Although the disease was successfully eradicated in cattle and reduced to an undetectable level in deer by 2012, deer hunters considered the actions taken to achieve bTB-free status controversial (12). Among the general public, lethal control of deer (sharpshooting) is often contentious (12–14). Further, in instances of zoonotic disease affecting game species, hunters show more concern than the general public about game management (15, 16).

As it was being used in other studies the RISP model is the primary source for the conceptual framework used in our study, and data were collected following the methods outlined in Griffin et al. (6) work on RISP and recent adaptations to that model [see (1)]. The RISP model builds upon an earlier model, the Theory of Planned Behavior, by examining the relationship between information, knowledge, and risk perception (6). Multiple studies in the field of risk and threat perception have shown that information seeking and processing is an important component of how an individual perceives and responds to a risk (17–19).

According to the RISP model (**Figure 1**), an individual's perception of risk is driven by the degree to which they think they are informed about a threat and how he or she seeks out and processes information about the risk (6). Like many other risk perception models, Griffin's RISP model has been used to study health and personal risks. However, environmental risks have been a special emphasis of the RISP model research (18, 20, 21). Clarke (1) presented a modified RISP framework, which integrated values, to examine how individuals perceive zoonotic disease (such as bTB) as a threat to wildlife (**Figure 2**).

The central component that drives the RISP model is "information sufficiency" (6). In the process of developing the perception of a threat, an individual will assess how much information they currently have and evaluate that level based on how much information they think is necessary to

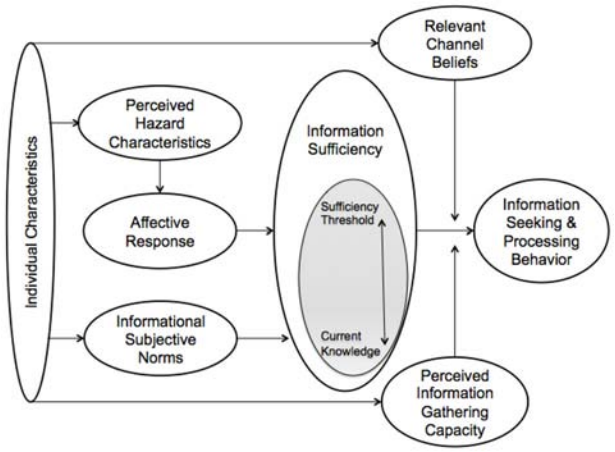

FIGURE 1 | Risk Information Seeking and Processing Model [adapted from (6)].

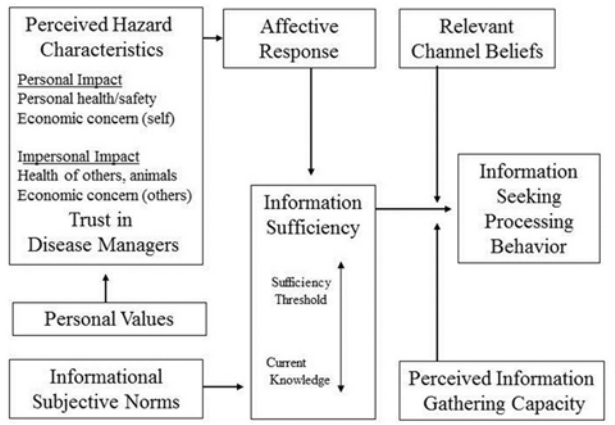

FIGURE 2 | Clarke's RISP framework for zoonotic diseases [adapted from (1)].

understand the threat. If the individual believes they have insufficient information, they will seek out and process additional information about the topic. Demographics, such as age, income, education level, and personal importance of hunting, and informational subjective norms (the social pressure to be informed) may influence individuals' information sufficiency thresholds.

Affective responses may also have an important influence on information sufficiency, and the common affective responses studied by RISP frameworks are worry and anxiety (17). However, fear and anger are also possible responses that could apply, with the degree of perceived personal control influencing with one expresses fear (low control) or anger (high control) to a situation (6, 20). Subjective norms, an individual's assessment of whether his or her peers expect him or her to be informed, can also lead to the information sufficiency stage regarding the threat (6). Even if an individual is not concerned about a risk, they may decide to learn more if they think it will give them more information to talk about with peers.

Another set of components, "perceived information gathering capacity," refers to whether the individual can understand (or comprehend) and access available information. Information that is too complicated or technical may discourage an individual from seeking more information about the risk. Relevant channel beliefs, which we did not collect data on, refer to the "channels," or sources of information, through which an individual learns about a risk (6). In the model, relevant channel beliefs do not interact with other predictor variables and subsequent work on RISP excludes relevant channel beliefs (22). The information sources or amount of information an individual has access to may affect desire to seek information about the source.

These variables are meant to measure an individual's heuristic and systematic processing of information, and in the model, are hypothesized to influence information seeking behavior through the information sufficiency and perceived information gathering capacity variables, which share a direct relationship with information seeking (1, 6, 23). Systematic processing refers to higher order processing, which requires effort on the part of the individual and, more likely than heuristic processing, may lead to attitudinal change (24, 25). Heuristic processing occurs at a comparatively shallow level and uses superficial cues for interpreting information (25). The two forms of information processing are a major field of social-psychological research (26–28). In the context of understanding perceptions about bTB, the distinction is important as hunters who engage in heuristic processing may be more easily discouraged from hunting due to concerns about bTB than those who engage in systematic processing.

In addition to Griffin et al. (6) RISP model, we adopted components of Clarke's (1) Zoonotic Disease Risk Information Seeking and Processing (ZDRISP) framework. Following Clarke (1), we measured hunters' perceptions of the impact of bTB to themselves, other people, and wildlife. The framework includes components that examine how personal impact (e.g., health and financial costs to the individual) and impersonal impact (e.g., health and financial costs to other people, wildlife species, and society) can be included in a traditional RISP model. Clarke (1) also emphasizes the importance of trust in the managing agency on information seeking and processing. Low trust of an agency might discourage, or frustrate people, from learning about the threat. Trust will also likely have an important role in whether an individual supports the agencies policies to manage the threat. Kahlor (19), building off similar communication processing frameworks, argued for a more integrated RISP model that was termed, "A Planned Risk Information Seeking Model" (PRISM). The key aspect of PRISM is the integration of the core RISP model with conceptual components from the Theory of Planned Behavior (24, 29). These components include: (1) positive/negative evaluations of a behavior (attitudes); (2) perceptions of social pressure to engage in a behavior (subjective norms); and (3) perceived ability to engage in a behavior (perceived behavioral control) (19).

Given the controversial nature of wildlife disease management, there is a need to understand how stakeholders perceive, gain, and distribute information. The RISP model hypothesizes that information sufficiency and perceived information gathering capacity directly influence information seeking behavior. Because of the direct relationship of these variables to information seeking in the model (**Figure 1**), we expect them to demonstrate the greatest influence on information seeking behavior relative to other variables. Previous studies conducted using the RISP framework and its variants (1, 19, 20) guided the development of the survey instrument used in this research. We also collaborated closely with the Minnesota DNR regarding their bTB strategy related to deer management. The adoption of this RISP model for evaluating perceived threats from wildlife disease aligns with bTB disease research in Michigan (30), and we were particularly interested in potential insights from the application of the model in the two similar contexts.

METHODS

Sampling

In Minnesota, deer are managed by permit area ($n = 128$) and hunters are asked to identify the areas they intend to hunt that year. Although hunters are not required to hunt only in the area they identify, previous research revealed that most return to the same location annually (31). We drew a proportional random sample from Minnesota's Electronic Licensing System license database of adult individuals who purchased a deer license and indicated they hunted in areas affected by bTB. Our survey followed a modified version of the Tailored Design Method (32). We mailed survey to 2,100 licensed hunters from the bTB affected permit areas ($n = 7$) and used three waves of mailing to maximize response rate. We collected data during late summer and fall of 2012 (University of Minnesota Institutional Review Board Study Number: 0609E92806).

Measurement Variables

Dependent Variable

Following the RISP model, we wanted to understand what variables influenced the likelihood of individuals to actively seek out bTB information. We used five items developed and tested in previous studies of the RISP framework to measure information seeking (**Table 1**). We used a five-point scale ranging from 1 = "*strongly disagree*" to 5 = "*strongly agree*" for each item. Questions that were phrased to report avoidance of information seeking were reverse coded for reliability and subsequent scale formation. We computed the latent dependent variable, "information seeking behavior" as the mean score of the 5 scale items.

Independent Variables

In addition to respondent demographics, we constructed seven latent independent variables as identified in the RISP (6) and ZDRISP (1) models. We measured (1) demographic variables such as age, education level, and importance of hunting, and following Clarke (1), (2) we assessed respondents' general perceptions of bTB impact concerns, or risk judgment (8 items) and personal impact concerns (4 items). Each item was measured on a five-point scale ranging from 1 = "*not at all*" to 5 = *"extremely" concerned*. We also measured the (3) individual affective responses (anger, worry, fear) of study participants to the discovery of bTB and the DNR's subsequent management of the disease on an 11-point scale ranging from 0 = "*none of this feeling*" to 10 = "*a lot of this feeling*." We measured (4) subjective norms using three items on a five-point scale from 1 = "*strongly disagree*" to 5 = "*strongly agree,*" and following Fishbein and Ajzen (29, 33), we used semantic differential scales to assess respondents' evaluation of seeking information about bTB management. Respondents were asked to evaluate (on a 7-point scale) whether their information seeking was worthless or valuable, foolish or wise, and unhelpful or helpful. We used six items to (5) define respondents' beliefs about their personal ability to obtain and understand bTB-related information. Responses related to information seeking capacity were also measured on a five-point style scale ranging from 1= "*strongly disagree*" to 5 = "*strongly agree*."

We also asked survey recipients about (6) their current knowledge and the amount of effort they dedicated to learning about bTB. We asked survey recipients to rate each on a scale ranging from 0 (no information) to 100 (all available information). Following Griffin et al. (20), we did not equate knowledge insufficiency as difference scores between these two measures but rather (7) regressed sufficiency threshold scores on initial knowledge scores to identify "information insufficiency" [as delineated in Cohen et al. (34)].

Data Analysis

Data were analyzed using the IBM Statistical Program for the Social Sciences (SPSS v. 20, 2013). (Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.) We grouped variables into their multi-item scales and tested internal consistency using Cronbach's alpha. Then we used hierarchical multiple regression models to examine the relationship between explanatory variables and the predictor, information seeking behavior as well as the ability of the RISP model to predict information seeking behaviors. We generated 7 separate models to test whether including each additional variable in our modified RISP framework improved the predictive ability of the model.

RESULTS

Response Rates and Respondent Characteristics

Of the 2,100 surveys mailed, 134 were undeliverable. For the remaining 1,966 surveys, 745 were completed and returned, resulting in a response rate of 38%. There was a wide distribution in the responses to survey items for variables included in the models, which suggested that our findings are likely to be representative of the sample population of hunters in all 7 deer permit areas impacted by bTB management. Most of survey respondents were male (93%) and half had completed

TABLE 1 | Measurement of variables.

Item	Mean	SD	alpha
(A) Dependent - Information seeking behavior[1]	0.77		
a) When the topic came up, I was likely to tune it out. (reverse coded)	3.79	0.89	
b) I'd go out of my way to avoid learning more about bovine TB management. (reverse coded)	4.08	0.84	
c) Gathering a lot of information about bovine TB management was a waste of time. (reverse coded)	3.83	0.93	
d) I tried to learn more about TB.	3.48	0.87	
e) I was likely to go out of my way to get more information about bovine TB management.	3.01	0.93	
(B) Independent			
1) Affective response[2]			NA
a) Anger	4.23	3.41	
b) Worry	4.65	3.36	
c) Fear	5.19	3.2	
2) Perceived impacts of bTB[3]			NA
a) Economic impacts to cattle producers	3.07	1.22	
b) Threats to the health of deer	3.81	1.03	
c) Reducing the deer population in the area	3.84	1.15	
d) Economic impacts to businesses that depend on deer hunting	3.23	1.16	
e) Threats to the health of other deer hunters from infected deer	2.74	1.32	
f) Reducing your deer hunting opportunity	3.72	1.2	
g) Threats to your personal health or family members from infected deer	2.68	1.4	
h) Financial costs to you personally	2.08	1.28	
3) Personal impact concerns[3]			0.71
a) Reducing the deer population in the area	0.48	0.65	
b) Reducing your deer hunting opportunity	0.58	0.59	
c) Threats to your personal health or family members from infected deer	0.45	0.68	
d) Financial costs to you personally	0.48	0.66	
4) Subjective norms[1]			0.74
a) People who are important to me thought I should stay on top of information about bovine TB	3.07	0.96	
b) People close to me expected me to get information about bovine TB	2.8	0.99	
c) Most of the people I know wanted to talk about bovine TB	2.99	1.18	
5) Perceived information gathering capacity[1]			0.74
a) I knew what questions to ask of the experts	2.77	0.96	
b) I knew where to go for information	3.39	0.95	
c) I could take the time to gather any information I needed	3.32	0.92	
d) Much of the information was too technical for me to understand (reverse coded)	3.44	0.87	
e) I could separate fact from fiction	3.86	0.86	
f) I could understand the information if I made the effort	2.77	0.7	
6) Attitude toward seeking information[4]			0.90
a) Worthless … valuable	5.18	1.35	
b) Foolish … wise	5.36	1.29	
c) Unhelpful … helpful	5.16	1.25	
7) Trust in DNR[1]			0.93
a) I trust the Minnesota DNR to manage bovine TB	3.14	1.14	
b) DNR officials are concerned about minimizing the impacts of bovine TB on deer hunters	3.43	1.09	
c) The Minnesota DNR does a competent job of minimizing the impacts of bovine TB	3.22	1.06	
d) The DNR is open and honest in the things they do in say when managing bovine TB	2.92	1.12	
e) The DNR makes decisions about managing bovine TB in a way that is fair	2.87	1.08	
f) The DNR listens to deer hunters' concerns when managing bovine TB	2.76	1.13	

[1] *Response options ranged from "1 - Strongly disagree" to "5 - Strongly agree."*
[2] *Scale ranged from 0 to 10, where "0 - None of this feeling" to "10 - A lot of this feeling."*
[3] *Response options ranged from "1 - Not at all concerned" to "5 - Extremely concerned."*
[4] *Response options ranged from "1 - Extremely worthless/foolish/unhelpful" to "7 - Extremely valuable/wise/helpful."*

some education above the high school level (**Table 2**). Many respondents did not respond to the income question ($n = 211$), which is typically a component of personal characteristics in the RISP model. Income, however, did not appear to influence final model results and excluding it from the model provided a larger sample size for analysis. In a simple regression with income predicting information seeking behavior, the two variables were not strongly correlated, with $r_{(519)} = 0.008$, $p < 0.04$. For this reason, we did not use income as an independent variable in the analysis. The final usable response rate for modeling was 30% ($n = 598$) after removing individuals excluded due to incomplete responses to survey items other than income.

Scale Reliability and Model Results

Within the RISP framework, our reliability analyses supported the creation of the latent dependent variable (alpha = 0.77) and the independent latent variables (**Table 1**). Multiple regression demonstrated significant effects of attitudes, subjective norms, information seeking capacity and information insufficiency on the information seeking behaviors of northwest Minnesota deer hunters (**Tables 3**, **4**). The final model in the hierarchical regression suggested the RISP framework explained 43% of the variability in northwest hunters' information seeking behaviors in response to bTB occurrence (**Table 3**).

Model 1 included only individual characteristics and explained a small amount of the variability in information seeking behaviors (6.5%) (**Table 3**). The addition of variables pertaining to personal impact, risk judgment, trust in DNR, and self-efficacy (Model 2) resulted in a ΔR^2 of 5.3% (**Table 3**). Affective response (Model 3) slightly increased the amount of explained variability in information seeking behaviors ($\Delta R^2 = 6.8\%$; $R^2 = 17.1\%$). Subjective norms and attitudes (Model 4) more than doubled the amount of explained variability in the RISP model ($\Delta R^2 = 19.9\%$; $R^2 = 37.0\%$). Current knowledge (Model 5) increased R^2 by 5.0%; whereas the addition of information insufficiency (Model 6) changed the amount of explained variability minimally ($\Delta R^2 = 0.1\%$). Each iteration of our global model was statistically significant.

In model 7, findings suggest only education, worry, subjective norm, information seeking capacity, and information insufficiency are statistically significant ($p < 0.05$) predictors of information seeking behaviors in the RISP model (**Table 3**). Information seeking capacity ($p < 0.001$) and information insufficiency ($p = 0.019$) were significant predictors of information seeking behaviors of northwest Minnesota deer hunters, after controlling for individual characteristics, perceived hazard characteristics, affective response, attitudes and informational subjective norms as the model suggests. As the variables most proximal to information seeking behavior in the RISP framework, the result is expected (**Figure 1**). However, while information insufficiency was significant ($p < 0.05$), the amount of additional variability it explains in the model was small (<1%). Changes in the amount of variability explained in the models are greatest with the addition of subjective norms and attitudes (19.9%) and affective response (6.8%) (**Table 3**).

In addition to the application of the RISP framework, we found that northwest Minnesota hunters reported their family, friends, and social network as their greatest source of information about bTB and bTB management, followed by statewide newspapers and news magazines. Respondents considered state agencies and public meetings as the least-utilized information source, except for the Minnesota Department of Natural Resources which had the third highest average response (**Table 4**).

TABLE 2 | Respondent characteristics.

	N	%
GENDER		
Male	675	93.0
Female	51	7.0
EDUCATION		
Grade school	7	1.0
Some high school	10	1.4
High school diploma or GED	151	20.9
Some vocational or technical school	65	9.0
Vocational or technical school (associate's)	126	17.5
Some college	118	16.4
Four-year college	164	22.7
Some graduate school	29	4.0

DISCUSSION

Our study aimed to better understand the information seeking behaviors of deer hunters regarding disease management. Specifically, we use the RISP model to investigate the relationships of individual variables on information seeking and processing behavior. To achieve this, we used hierarchical multiple regression analysis utilizing the RISP framework. Results revealed several discrepancies from the expectations of the RISP model and from studies using the RISP model in other contexts. These include research on topics ranging from environmental risks to the health communication sciences (19, 20, 30).

Unlike the findings of Kahlor's PRISM research (19), our application of the RISP model variable "information sufficiency" was statistically significant. However, its importance in predicting information seeking is diminished by its lack of predictive power. The addition of information sufficiency in the RISP model yielded almost no increase in variance in information seeking behavior explained (< 1%). Our finding differs from our original expectation—that variables most proximal to information seeking behaviors in the model would explain the greatest proportion of variation.

In our model of information seeking behaviors, we found that attitudes explained the greatest amount of variability in information seeking behaviors of northwest Minnesota hunters. We believe this relates to the time that elapsed between the survey implementation and the initial outbreak of bTB in MN. Bovine TB was originally detected in a wild deer in 2005, and this research was conducted in the summer of 2012. During that

TABLE 3 | RISP framework hierarchical regression models of self-reported information seeking behaviors in northwest Minnesota hunters, data from 2012.

Predictor	SE	Std. β	*t*	*p*	R^2	ΔR^2	F	*p*
Model 1					0.050		10.4	<0.001
Age	0.00	0.05	1.148	0.25				
Education	0.01	0.16	3.993	<0.001				
Hunting importance	0.03	0.16	4.012	<0.001				
Model 2					0.102		9.60	<0.001
						0.053	8.64	<0.001
Age	0.00	0.04	0.931	0.35				
Education	0.01	0.19	4.71	<0.001				
Hunting importance	0.03	0.13	3.18	0.00				
Personal Impact	0.03	0.15	3.57	<0.001				
Risk judgment	0.00	0.12	2.94	0.003				
Trust in DNR	0.03	0.09	2.20	0.029				
Self-efficacy	0.03	0.07	1.83	0.068				
Model 3					0.171		12.1	<0.001
						0.068	16.1	<0.001
Age	0.002	0.04	1.06	0.29				
Education	0.013	0.19	4.87	<0.001				
Hunting importance	0.031	0.10	2.40	0.02				
Personal impact	0.031	0.10	0.211	0.83				
Risk judgment	0.000	0.08	1.97	0.05				
Trust in DNR	0.027	0.10	2.58	0.01				
Self-efficacy	0.025	0.08	1.99	0.47				
Anger	0.009	0.06	1.16	0.25				
Worry	0.014	0.30	4.11	<0.001				
Fear	0.014	−0.04	−0.531	0.60				
Model 4					0.370		28.6	<0.001
						0.199	92.4	<0.001
Age	0.00	−0.02	−0.47	0.64				
Education	0.01	0.14	4.10	<0.001				
Hunting importance	0.03	0.07	2.02	0.04				
Personal impact	0.03	−0.06	−1.58	0.12				
Risk judgment	0.00	0.03	0.79	0.43				
Trust in DNR	0.02	0.03	0.69	0.49				
Self-efficacy	0.02	0.02	0.65	0.52				
Anger	0.01	0.01	0.17	0.87				
Worry	0.01	0.15	2.22	0.03				
Fear	0.01	−0.02	−0.25	0.81				
Subjective norm	0.03	0.26	6.71	<0.001				
Attitude	0.02	0.34	8.51	<0.001				
Model 5					0.420		32.5	<0.001
						0.050	50.7	<0.001
Age	0.00	0.01	0.322	0.75				
Education	0.01	0.09	2.71	0.01				
Hunting importance	0.03	0.06	1.79	0.07				
Personal impact	0.03	−0.04	-952	0.34				
Risk judgment	0.00	0.02	0.507	0.61				

(Continued)

TABLE 3 | Continued

Predictor	SE	Std. β	*t*	*p*	R^2	ΔR^2	F	*p*
Trust in DNR	0.02	0.02	0.557	0.58				
Self-efficacy	0.02	−0.01	−0.411	0.68				
Anger	0.01	−0.01	−0.323	0.75				
Worry	0.01	0.16	2.57	0.01				
Fear	0.01	0.01	0.092	0.93				
Subjective norm	0.03	0.20	5.32	<0.001				
Attitude	0.02	0.29	7.34	<0.001				
Capacity	0.04	0.25	7.12	<0.001				
Model 6					0.421		30.3	<0.001
						0.001	0.848	0.357
Age	0.00	0.01	0.24	0.81				
Education	0.01	0.09	2.70	0.01				
Hunting importance	0.03	0.06	1.76	0.08				
Personal impact	0.03	−0.04	−1.00	0.32				
Risk judgment	0.00	0.01	0.43	0.67				
Trust in DNR	0.02	0.02	0.67	0.50				
Self-efficacy	0.02	−0.01	−0.43	0.67				
Anger	0.01	−0.02	−0.43	0.67				
Worry	0.01	0.16	2.56	0.01				
Fear	0.01	0.01	0.16	0.87				
Attitude	0.03	0.20	5.10	0.00				
Subjective norm	0.02	0.29	7.32	0.00				
Capacity	0.04	0.25	6.83	0.00				
Current knowledge	0.00	0.03	0.921	0.35				
Model 7					0.427		28.9	<0.001
						0.006	6.02	0.014
Age	0.00	0.01	0.43	0.67				
Education	0.01	0.09	2.54	0.01				
Hunting importance	0.03	0.06	1.79	0.07				
Personal impact	0.03	−0.04	−0.97	0.33				
Risk judgment	0.00	0.01	0.25	0.80				
Trust in DNR	0.02	0.03	0.83	0.41				
Self-efficacy	0.02	−0.01	−0.20	0.84				
Anger	0.01	−0.02	−1.55	0.58				
Worry	0.01	0.16	2.50	0.01				
Fear	0.01	0.01	0.12	0.90				
Attitude	0.03	0.20	5.18	0.00				
Subjective norm	0.02	0.26	6.28	0.00				
Capacity	0.04	0.25	6.83	0.00				
Current knowledge	0.00	0.03	0.74	0.46				
Information insufficiency	0.00	0.09	2.45	0.01				

time, the Minnesota DNR implemented aggressive deer control efforts that resulted in a 60% decrease in the population, which likely increased negative attitudes toward bTB management and the agency in general.

Our findings pertaining to attitude and social norms exerting a strong influence on risk behaviors of individuals are similar to other applications of the model in the context of zoonotic disease risk perception [e.g., (30)].

TABLE 4 | Sources of information.

	N	Mean	SD
Radio news	718	2.43	0.955
Television news	712	2.33	0.943
Local newspapers	717	2.83	0.981
Statewide newspapers and news magazines	714	2.56	1.005
Internet sources	705	2.30	1.137
Minnesota DNR	716	2.74	1.005
Minnesota board of animal health	704	1.74	0.997
Family, friends, social network	719	2.86	0.888
Public meetings	707	1.65	0.951

Respondents were asked "From what sources did you get information about TB and bovine TB management?"
Responses were "1" (Not at all), "2" (Slightly), "3" (Moderately), "4" (Very Much).

Because the RISP model has been widely applied in other contexts and may be useful to natural resource managers in the future, it is important to understand its operation in an applied setting. This research suggests that using the complete RISP model to explain behaviors after the immediate onset of a threat, or once a threat has been eradicated, may be challenging. Information about the extent of disease was readily available by the time we surveyed hunters, and they were probably better informed about past risks (and the lack of present risk) from bTB than at the time of the initial disease detection. Model variables that are stable across time (e.g., demographics and attitudes) appear to be primary drivers (e.g., explain the greatest amount of variability). In particular, we believe that using the Theory of Planned Behavior (24) might provide a more parsimonious model in cases where the disease has been present for a substantial time, communication has been present, and attitudes, norms, and beliefs are likely the drivers of whether or not people will seek out and use information. In a case where the disease threat is relatively new and most people are unlikely to have had a lot of exposure to information about the threat, information insufficiency might be more of a driver soon after disease outbreak.

CONCLUSIONS, LIMITATIONS, AND IMPLICATIONS

Due to early detection of the disease and aggressive management actions, the prevalence of bTB in Minnesota never reached levels observed in Michigan, where bTB eradication from the wild population of white-tailed deer is unlikely (35). As such, Minnesota provides a case study for successful bTB management (if "success" is measured as no longer detecting positive animals). Although Minnesota received classification as a bTB-free state in 2011, the possibility of future occurrence of bTB or other wildlife disease outbreaks remains. Understanding how hunters perceive bTB and bTB management, as well as what motivates them to attend to information concerning the risks and management of bTB, is integral in creating socially acceptable policy to manage for future occurrences of bTB or similar zoonotic diseases affecting humans and wildlife (3, 35).

This project explores the use of the RISP model in the context of wildlife disease and management. The findings about the operation of the RISP theory in an applied context inform future research and management, indicating that in this instance attitudes and norms exert greater influence on hunters' information seeking behaviors than the RISP framework appears to suggest. Evidence suggests that successful natural resource management and policy implementation requires stakeholder support, especially from hunters and private landowners (12). Communicating zoonotic disease risks to the public, as in the case of bTB, proves challenging for managers (12). The findings from this study might appear to have little practical utility to wildlife management agencies that primarily focus on "scientific management" largely based in the biological sciences. The variables influencing risk perceptions and information seeking behaviors (attitudes and social norms) seem out of the control of managers. As noted by Riley et al. (36), however, effective wildlife management in the twenty-first century might require complex integration of biological and human dimensions information. Such integration is likely to move wildlife disease management decisions and actions away from only attempting to address the biological issues that appear to be under the control of "scientific management" toward also understanding and addressing the psychological and social phenomena associated with the stakeholders for whom agencies are managing the wildlife resource.

When this research is contextualized in this more complex management setting, its benefits to managers are more apparent. Clearly understanding the prevalent attitudes and subjective norms of stakeholder and communities impacted by bTB can assist in developing messages for communicating disease risks as well as management actions. In previous natural research management studies using the Theory of Reasoned Action or Theory of Planned Behavior, careful analysis of the beliefs influencing attitudes and norms have assisted with understanding stakeholders and developing messages and communication strategies. These studies have been conducted in diverse contexts including reintroduction of wolves (37), habitat conservation for endangered species (38), the lethal control of deer populations for conservation (13, 14) and support for limiting use of lead shot (39, 40). Our study demonstrates that the use of similar models holds promise for better understanding what influences stakeholder behaviors related to wildlife disease management. Decisions that clearly take into consideration the impacts to stakeholders may enhance the social acceptance of risk management actions and processing of communication, ultimately bettering relationships with stakeholders and improving policy outcomes.

We also noted several limitations of our study, mostly related to the time between initial detection (2005) and survey implementation (2012). Asking respondents to rate their affective responses when they initially heard about the outbreak limits our findings; there may have been inaccuracies in participants' recollections of their emotions upon hearing of bTB. We also expect that survey participants used hindsight to inform their responses related to perceptions of threats from bTB. We also

asked people to recall in the past how they perceived threats of bTB after it had already been eradicated from the state. Had we surveyed hunters immediately following the onset of bTB, rather than after Minnesota was declared bTB free, respondents' likelihood of reporting perception of a threat from bTB may have been higher. Future research should attempt to minimize the duration between event and research. Additionally, while our response rate was not unusual for such surveys, the fact that most hunters (62%) we contacted did not respond to the survey may indicate a lack of concern, or apathy, about the topic.

AUTHOR CONTRIBUTIONS

DF and LC designed research. DF, LC, and AH performed research and collected data. MC, DF, LC analyzed data. All authors wrote and reviewed the paper.

FUNDING

This work was supported by the US Department of Agriculture/National Institute of Food & Agriculture (NIFA) Award Number: 2010-34427-21107.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AZ declared a past co-authorship with one of the authors AH to the handling Editor.

The reviewer AZ declared a shared affiliation, with no collaboration, with one of the authors, MC, to the handling Editor.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 21 January 2019
doi: 10.3389/fvets.2018.00350

Mycobacterium caprae Infection of Red Deer in Western Austria–Optimized Use of Pathology Data to Infer Infection Dynamics

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OPEN ACCESS

Edited by:
Daniel J. O'Brien,
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Reviewed by:
Graham Nugent,
Landcare Research New Zealand, New Zealand
Paul Cross,
United States Geological Survey, United States

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 29 July 2018
Accepted: 31 December 2018
Published: 21 January 2019

Citation:
Nigsch A, Glawischnig W, Bagó Z and Greber N (2019) Mycobacterium caprae Infection of Red Deer in Western Austria–Optimized Use of Pathology Data to Infer Infection Dynamics. Front. Vet. Sci. 5:350. doi: 10.3389/fvets.2018.00350

Austria is officially bovine tuberculosis (TB) free, but during the last decade the west of the country experienced sporadic TB cases in cattle. Free-ranging red deer are known to be the maintenance host of *Mycobacterium* (*M.*) *caprae* in certain areas in Austria, where cattle can become infected on alpine pastures shared with deer. The epidemiology of TB in deer in alpine regions is still poorly understood. To inform decisions on efficient interventions against TB in deer, a method is needed to better capture the infection dynamics on population level. A total of 4,521 free-ranging red deer from Austria's most western Federal state Vorarlberg were TB-tested between 2009 and 2018. *M. caprae* was confirmed in samples from 257 animals. Based on descriptions of TB-like lesions, TB positive animals were categorized with a newly developed lesion score called "Patho Score." Analyses using this Patho Score allowed us to distinguish between endemic, epidemic and sporadic TB situations and revealed different roles of subgroups of infected deer in infection dynamics. Overall, deer in poor condition, deer of older age and stags were the subgroups that were significantly more often TB positive ($p = 0.02$ or smaller for all subgroups). Deer in poor condition ($p < 0.001$) and stags ($p = 0.04$) also showed more often advanced lesions, indicating their role in mycobacterial spread. TB was never detected in fawns, while hinds were the subgroup that showed the fewest advanced lesions. Analysis of outbreaks of TB and lesion development in yearlings provided some evidence for the role of winter feeding as a source for increased infection transmission. Sporadic cases in TB-free areas appear to precede outbreaks in these areas. These currently TB-free areas should receive particular attention in sampling schemes to be able to detect early spreading of the infection. The Patho Score is a quick, easy-to-apply and reproducible tool that provides new insights on the epidemiology of TB in deer at population level and is flexible enough to relate heterogeneous wildlife monitoring data collected following different sampling plans. This lesion score was used for systematic assessment of infection dynamics of mycobacterial infections.

Keywords: tuberculosis, *Mycobacterium caprae*, red deer, Austria, lesion score, infection dynamics

INTRODUCTION

Mycobacterium caprae (*M. caprae*) is part of the *Mycobacterium tuberculosis complex* (MTBC) and is the causal agent of tuberculosis (TB) of cattle and free-ranging red deer (*Cervus elaphus elaphus*) in the border area between western Austria and southern Germany (1–4). In this area, red deer have been identified as TB reservoir that spreads the pathogen through direct or indirect contact to cattle (5). Transmission of TB between wildlife and farmed animals can occur in both directions.

In red deer, TB is a subacute to chronic disease that is associated with emaciation at an advanced stage, but usually does not lead to marked clinical signs (6). TB is commonly diagnosed by presence of lesions in lymph nodes or organs (7). Tonsils are understood to be the main port of entry (8, 9). The medial retropharyngeal lymph nodes drain the tonsils, which is probably the way these lymph nodes become infected (10–12). Accordingly, medial retropharyngeal lymph nodes are often targeted in early detection and monitoring programs (13, 14). As disease progresses within the host, mediastinal and tracheobronchial lymph nodes, lungs, as well as mesenteric lymph nodes can become affected (15). Deer can also show lesions on pleura, in organs within the abdominal cavity, testicles and udder including their regional or subcutaneous lymph nodes (16).

Lesions indicative for TB in red deer range from pinhead-sized to more than 10 cm (in diameter) large granulomas or abscesses. Lesions develop progressively during the subsequent stages of disease and increase in size and number over time. Thin-walled connective tissue capsules containing creamy yellowish-white pus are typical for advanced stages (2, 15–17). These thin-walled abscesses lead in severe cases of generalized TB to high excretion of mycobacteria and thus an increased infectivity of affected animals (6, 18). TB in red deer was reported to be associated with up to 25% of infected animals without macroscopically visible lesions (2, 12). Nugent (19) identified an area in which even 23 (68%) out of 34 culture positive deer had no visible lesions. There are indications that deer that do not die within a year or two of becoming infected can survive for many years (19). Although the detailed pathogenesis of TB in red deer is not fully understood, there is increasing evidence in literature that species-specific stressors, behavioral and environmental factors as well as genetic factors influence susceptibility to mycobacteria (20, 21).

To better understand the development of slowly progressing diseases such as TB on population level, knowledge of the underlying infection dynamics is decisive: when and where did whom spread infection to whom? Especially in the case of wildlife, it is important to exploit all available information to create a valid overall picture and to be able to better target control measures. Another relevant question is the role of subgroups of animals within the deer population for the maintenance and the spread of TB.

This work aims to characterize dynamics of TB transmission within the red deer population to provide evidence for optimized monitoring and control of TB in alpine areas. We also will be investigating whether qualitative and quantitative criteria of TB-like lesions are a suitable indicator to show and measure infection dynamics of TB in deer. On the basis of readily available data, the impact of population structure, time and space will be investigated retrospectively:

- Population structure: do subgroups of animals within the deer population play different roles for the maintenance and the spread of TB?
- Time: did the infection dynamics of TB in red deer in Vorarlberg change between 2009 and 2018?
- Space: are different patterns of infection dynamics observable in the TB zones?

HISTORY OF *Mycobacterium caprae* IN RED DEER IN VORARLBERG, AUSTRIA

Austria is recognized as an officially bovine TB-free (OTF) country since 1999. Anecdotal observations suggest that TB was present in deer in the most western Austrian state of Vorarlberg prior to 1999: animals with spherical abscesses of the mesenteric lymph nodes were seen which were later referred to as "ball deer". But these cases have never been investigated with laboratory diagnostics. The first confirmed TB case in deer in Vorarlberg was recorded in 2006.

In 2008, TB cases in cattle were reported from the neighboring Austrian state Tyrol, which were linked to infected deer. As a consequence, the first systematic deer monitoring was started in Vorarlberg in 2009 with the aim to assess the risk of TB infection spread to its own cattle population. In the first year of this deer monitoring, *M. caprae* was detected in seven out of a total of 71 examined deer. Since then, TB in deer has been under constant observation. The TB cases are concentrated at a hotspot in two valleys (Klostertal and Montafon north of the river Ill, marked in red as "core area" in **Figure 1**). About 25–30 km north of this hotspot, TB is detected sporadically in deer in the border area with Tyrol and Germany.

In the alpine areas of Vorarlberg, agriculture mainly consists of small cattle farms with 5–20 animals in extensive farming. A special management practice is the annual transhumance of cattle on alpine pastures above 1,600 m for up to 100 days during summer. During summer, deer also prefer sub-alpine and alpine areas at altitudes up to 2,500 m, where cooler temperatures predominate, and nutrient-rich forage is available. In certain areas, this traditional grazing leads to intensified contacts between deer and cattle. In 2010 TB was also confirmed in cattle in Vorarlberg. In consequence, control measures targeting deer were started in 2011 with intensive hunting under adapted conditions, i.e. the statuary close season (no hunting allowed) was shortened and limits on culling of antlerless animals were abolished in defined areas. Control measures were continuously extended and intensified in parallel to the developments of TB in deer and cattle in order to meet the required increasing total kill numbers in accordance with the official hunting plan.

In deer, TB prevalence seemed to have reached its plateau in 2013 (22). In the TB zone with the highest prevalence ("core zone"), 16 (25%) out of 62 deer samples examined were TB

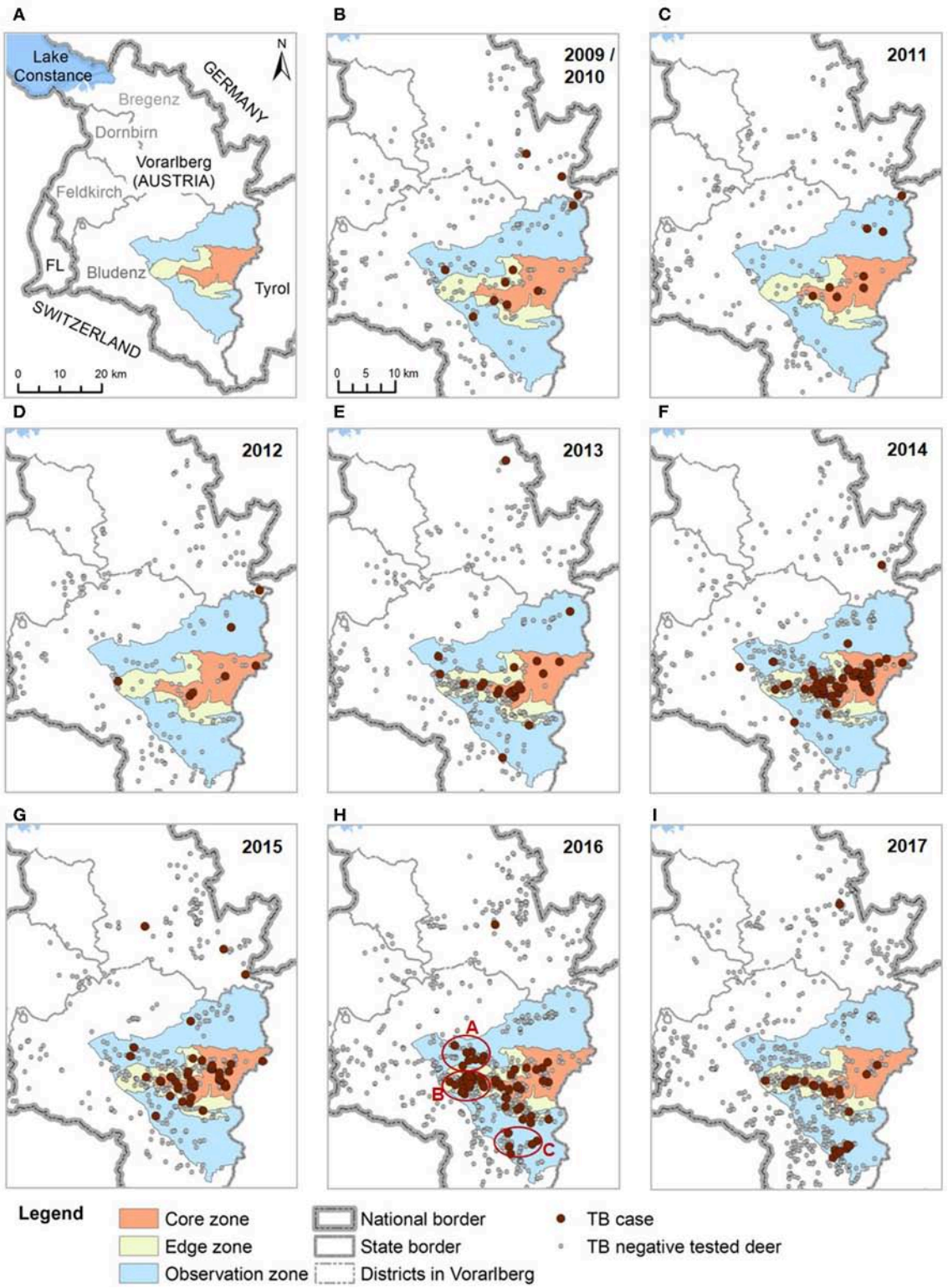

FIGURE 1 | Overview map **(A)** and series of detailed maps of kill locations of TB positive and TB negative deer **(B–I)**, 2009–2017. The core, edge and observation zones form together the TB control area. White areas with kill locations indicate the area outside the TB control area. Cases of the years 2009 and 2010 are shown together in one **(B)**. In 2016 **(H)**, three spots (A–C) with outbreak-like TB are marked in red. FL, Principality of Liechtenstein.

positive in 2013. In the cattle population, TB reached its peak in the winter of 2015/2016 with the detection of *M. caprae* in 30 animals from 13 herds of a total of 9,005 tested cattle from 728 herds (23). In the remaining years between 2013 and 2017, TB was annually confirmed in 4–8 animals from 2 to 7 farms in Vorarlberg (23). The current TB situation does not risk Austria's OTF status yet. The OTF status of a country is based on bovine animals, and a country recognized as OTF will keep this status even though wildlife in the country may be affected, as long as legal conditions are satisfied (Directive 64/432/EEC). However, TB cases in deer require extensive monitoring activities in the cattle population within known deer TB areas. In addition to negative effects on agriculture, hunting and the risk to human health due to this zoonotic agent, annual TB cases led to very high medial and political interest. This interest resulted in part in external pressure for those involved in the control program and reduced their willingness to cooperate in TB control.

Monitoring Tuberculosis in Cattle and Wildlife

All cattle with potential contact to TB-infected deer are annually examined with the comparative intradermal tuberculin skin test between late November and February, and all animals with non-negative skin tests are culled according to legal requirements. Contact animals are traced, and cattle herds are culled if testing indicates a within-herd prevalence of >40%. In addition, all cattle are inspected for TB at the abattoir as part of the national routine TB surveillance. The combination of these measures aims to reduce the risk of undetected TB cases due to imperfect test sensitivity and to ensure that cattle are TB negative in spring before the start of the grazing period. New TB cases diagnosed in cattle in the subsequent testing period led to the conclusion that the main direction of infection is deer-to-cattle, partly followed by spread from cattle-to-cattle within the infected herd. Spoligotyping (24) and mycobacterial interspersed repetitive unit-variable number of tandem repeat typing (25, 26) confirmed for Vorarlberg that all *M. caprae*-positive deer and cattle tested shared the same genotype "Lechtal" (27).

In addition to cattle and red deer, other wildlife species (badgers, foxes, chamois, roe deer) were tested, albeit not systematically. TB could only be detected in a roebuck in 2017, which was hunted in the known TB area (28). Wild boars are rare in Vorarlberg and have not been sampled to date. Based on current evidence, there is no indication of any significant role of other wildlife species in maintenance of TB infection in wildlife populations or for infection transmission to cattle.

Red Deer Management in Vorarlberg

Deer hunting is organized by hunting grounds. Deer hunt is seasonal, with a smaller peak in kills in spring and the main kill season in fall. The fall season accounts for two-thirds of the annual hunting bag. In the winter months between end of December to end of March hunting is generally suspended, with the exception of killing of sick or injured deer. An estimated one third of the deer population is hunter harvested each year, with higher percentages in the TB areas due to control measures in place.

A significant cause for establishment and persistence of TB in deer in Vorarlberg is seen in the marked increase in deer densities in certain regions (29). In the 1970s, a change in deer management practices led to a large increase in deer populations far beyond the natural capacity of deer habitats (30). In parallel, extensive developments in land use, such as growth of settlement areas in alpine valleys, the expansion of infrastructure and increase in tourism have reduced habitat of deer. This meant that deer were forced, against their traditions, to spend the winter at higher altitudes. In order to compensate for limited availability of feed and as strategic intervention to protect avalanche protection forests, winter feeding is nowadays carried out during 140 and 200 days a year (31). Winter feeding not only reduces mortality among weakened animals but will also generate artificially high deer densities around feeding sites. Close contact between animals of different age groups supports direct and indirect transmission of TB (32, 33). Winter feeding of deer is still allowed in Vorarlberg. Since 2017, however, there have been restrictions on choice of feed and more elaborate rules for cleaning and disinfecting feeding sites in spring. Additionally, feeding sites are fenced off with cattle-proof fences during the grazing period (34).

Validated information on deer densities over large-scale administrative areas does not exist for Vorarlberg. However, it is known that densities vary largely across alpine regions with considerable seasonal differences: the highest concentration of deer will be recorded around winter feeding sites on harsh winter days with thick snow cover, with focal concentrations of five up to 300 animals on a small number of hectares. In mild winters, groups of deer at feeding sites will be smaller due to availability of natural feed. In summer, deer are distributed over wider areas and groups of deer grazing together are often small (±10 animals) and will rarely reach group sizes of up to 70–100 animals. Radio telemetry studies showed that the summer habitat of deer in alpine areas can be 1.5–4.5 times larger in size compared to the winter habitat (35).

International Aspects of Infection in Wildlife

Sporadic TB cases in deer in the north of Vorarlberg form a shared deer TB area with Tyrol and Germany (5). In addition, neighboring Switzerland and the Principality of Liechtenstein are at risk of introduction of TB by animal trade and cross-border migration of deer (see **Figure 1A**, for an overview map). Radio telemetry studies showed that some deer cross the border after the snowmelt, spend the summer in a neighboring country and return to their winter habitat in their "home" country in autumn. Through these migratory individuals the deer populations of Vorarlberg, Switzerland and Liechtenstein are in seasonal contact (35). Deer are monitored for TB both in deer TB areas of Austria and Germany, as well as in TB-free border areas of Switzerland and Liechtenstein. Efforts are made by the four countries to increase comparability of their currently not yet harmonized monitoring programs in order to obtain a transnational overview of the TB situation in deer, as well as to develop a common control strategy (36).

MATERIALS AND METHODS

Study Population and Deer Monitoring

The study population consisted of all free-ranging red deer examined from February 2009 to March 2018 in the deer monitoring in Vorarlberg. The total of 4,521 sampled animals of all age groups were hunter harvested (99.6%) or found dead (0.4%). According to the deer population structure and in line with requirements of deer monitoring, younger and female deer were examined more frequently, with 1,297 (48.2%) deer $\leq$2 years and 2,170 (55.5%) females. A total of 172 (4.0%) animals were in poor condition.

Deer monitoring is carried out in all parts of Vorarlberg with deer habitats and distinguishes four zones corresponding to TB prevalence: the area with highest prevalence is the 103 km^2 large "core zone", surrounded by the "edge zone" (77 km^2) and the "observation zone" (346 km^2). Core, edge and observation zones form together the 526 km^2 large TB control area (46.95° N to 47.25° in latitude, and from 09.80° to 10.22° W in longitude) in the district of Bludenz. The fourth zone are the remaining deer habitats in Vorarlberg outside the designated TB control area (1,591 km^2) where TB has so far been detected only sporadically in deer, mainly in the north in the district of Bregenz (**Figure 1**). The boundaries of the zones are largely formed by mountain chains and rivers which allow restricted deer movements between zones. Deer abundance is similar in all four zones, with a variation of areas with high and low deer numbers within every zone (37).

Within the 9-year monitoring period, the size of the TB control area and sample size per zone, split by sex and age group, were regularly adjusted depending on case distributions in previous years and published in the annual official deer monitoring program plan (34). Annual sample sizes ranged between 71 and 940 sampled deer. In the hunting season April 2017 to March 2018 all hunter harvested deer except fawns were sampled in core and edge zones ($n = 211$) in accordance with this plan. In the observation zone at least 25% of the hunting bag had to be examined ($n = 215$). Additionally, all deer found dead and sick deer from the whole TB control area had to be investigated. The area outside the TB control area accounted for 401 samples or 20% of the annual hunting bag of deer $\geq$1 year. The sampled deer do not represent a single random sample.

Sampling and Diagnostic Methods

Trained hunters checked the deer at the kill location for external abnormalities. Subsequently, thoracic and abdominal cavities of animals were opened, and internal organs examined visually, and partly palpated. If no tissue abnormalities were observed, the standard sampling consisted of lung with its tributary lymph nodes (tracheobronchal and mediastinal lymph nodes) and larynx with medial retropharyngeal lymph nodes ("head and thorax" samples). As the entire hunting bag was sampled in core and edge zones, requirements for sample materials were relaxed for antlerless deer: the tissues to be sampled could be reduced to the head with medial retropharyngeal lymph nodes ("head-only" samples).

From deer with visible tissue abnormalities, the carcass including all internal organs had to be presented for examination to an official veterinarian. Deer found dead and deer in poor condition were as a rule sampled by veterinarians. In addition to standard sample materials, all parts of the carcass with gross lesions were required to be submitted to the Institute for Veterinary Disease Control, Austrian Agency for Health and Food Safety (AGES), Innsbruck. The reality of the given field conditions is that the sampling process and sampled tissues were quite heterogeneous.

Submitted sample material was pathomorphologically examined and all gross lesions were recorded. Lymph nodes with no visible lesions were dissected into 2–4 mm thick slices to detect even small granulomas. Tissue samples with lesions were cultured for 12 weeks at 37°C and MTBC species differentiation was performed by PCR. The analytical protocol to confirm infection with *M. caprae* was described by Fink et al. (5) in detail.

Development of the Patho Score

To allow spatial-temporal analysis and comparison of the pathomorphological lesion descriptions in free text, a lesion score ("Patho Score") was developed (**Table 1**). Based on this score, lesions can be subdivided into six categories (score 0–5) depending on their size, number and distribution in the body. The higher the score, the more advanced stage of TB is observed, with score 0 for non-visible lesions. Each examined animal receives a score for the whole package of submitted sample materials. If the sample material is incomplete, Patho Score tends to underestimate disease progress. The interpretation of the score is based on the hypothesis that TB lesions develop progressively and can be grouped and ordered according to their developmental stage.

The criteria for the Patho Score were: a valid, simple and comprehensible measurement tool with good discrimination,

TABLE 1 | Patho Score for the categorization of TB-like lesions in deer.

Score	Lesion
0	Non-visible lesion
1	Singular or multiple lesions with <5 mm in Retro 1[a]
2	Singular or multiple lesions with 5–10 mm in Retro 1[a]
3	Singular or multiple lesions with >10 mm in Retro 1[a]
4	Lymph nodes at multiple body sites affected and/or an organ is affected[b]
5	Overall picture: severe progressed TB/generalization[c]

[a] *Retro 1, Medial retropharyngeal lymph node with the more advanced lesion. Score 1–3 is based on Retro 1. If both medial retropharyngeal lymph nodes are missing in the sample material, the score for the score levels 1–3 is alternatively based on the lymph node with the most advanced lesion in the submitted sample material.*

[b] *Retro 1 has score level 3 (>10 mm) and additionally, at least one other lymph node is affected (e.g., Retro 2 with the less advanced lesion, tracheobronchial, mediastinal or mesenteric lymph nodes). By definition, samples with affected organ tissue (lung, pleura, liver, udder, etc.) are categorized at least with score 4, even if the sample material does not contain affected lymph nodes. Reason: According to Cornet's law of localization, the regional lymph node is always affected if the organ is affected (except in chronic organ tuberculosis). Samples consisting only of the head can reach a maximum score of 4.*

[c] *Example: "Ball deer" with spherical abscessed of the mesenteric lymph nodes, lymphadenitis, lung TB, chronic organ tuberculosis (various organs), severely abnormal lymph nodes.*

that is able to take into account heterogeneity of sample material and can be applied retrospectively to historical samples. The development of the score was based on a so-called localization principle:

- In deer monitoring, medial retropharyngeal lymph nodes are the only tissues that must be present in all samples, i.e., both in head-only samples (from antlerless deer hunted in core and edge zone) and also in standard head and thorax samples.
- Three score levels (1–3) are based solely on a medial retropharyngeal lymph node ("Retro 1"). The two higher levels (4–5) are based on the overall picture gained from the examination of the entire sample material.
- The size of the lesion has more influence on the level of the score than the number of lesions.

Development of the Patho Score was carried out in several rounds with evaluations by two raters: the pathologist, who had made the pathomorphological assessment of almost all samples, and an epidemiologist. To test the scoring tool, the two raters independently scored 242 TB positive samples based on available historical free text descriptions. The two test results were compared and samples with discrepancies were discussed. After each round, the definitions of the Patho Score were specified with the aim of obtaining the highest possible inter-rater agreement. With the final version of definitions, agreement was reached in 248 out of 257 samples (observed proportionate agreement of 96.5%). Discrepancies occurred with samples of score 4 or score 5, as the definition of score 5 "overall picture of severe progressed TB" is partly subjective. The categorization will thus in a limited number of samples depend on the rating pathologist.

The scoring of the sample takes on average less than 1 min (including documentation). The definitions of the Patho Score are clear and easy to understand. Training of a pathologist who is specifically experienced with TB is therefore considered not necessary.

As addition to the development of the Patho Score, a second pathologist histologically examined a sub-selection of samples in a blinded experiment to verify the character of the macroscopic lesions and to assess feasibility of standardization of scores. This independent evaluation step revealed that confirmation of the pathogen was a prerequisite for inclusion of a sample in the scoring system, since occasionally (especially with mild lesions) other pathogens can cause comparable lesions (e.g., actinomycotic or mycotic granulomas).

TB positive samples that were examined before October 2017 were scored retrospectively. From October 2017 onwards, all fresh samples were scored by the same pathologist on a continuing basis.

Data Collection and Case Definition

For each sampled animal, data on date of kill event, coordinates and hunting ground of the kill location, age, sex, condition and any further comments by the hunter were recorded in a standardized manner [age groups: males: yearling (1 year), stag III (2–4 years), stag II (5–9 years), stag I (≥10 years), females: yearling (1 year), hind (≥2 years), fawn (from birth till April 1st of following year); condition: good (deer appearing healthy), poor (sick or injured deer with clinical signs)]. Diagnostic results and data on submitted sample materials were recorded by AGES. A central database with all collected data was maintained by the Office of the State Government of Vorarlberg.

Animals were considered a case if *M. caprae* was confirmed by bacterial culture and subsequent species determination. All deer without TB-like lesions or with negative results in bacterial culture were considered negative. In one deer *M. microti* was detected (38), which was classified as (*M. caprae*-) negative in this study. *M. bovis* was never detected.

Inclusion criteria for the analysis were: all deer examined in the deer monitoring with a test result according to the case definition, and presence of a description of the submitted sample material. Excluded were deer that did not meet the minimum requirements for sample material: the sample had to contain at least two of the following lymph nodes or organs: medial retropharyngeal lymph nodes, pulmonary lymph nodes or lung tissue. A total of 4,265 (94.3%) samples met the inclusion criteria (**Figure S1**). Of these, 334 samples (7.8%) had suspicious lesions. *M. caprae* was confirmed in 257 (6.0%) samples, with 7–72 cases per year. Only positive cases were scored with the Patho Score. Since information on sample material was missing for one case, reported results are based on 256 of the 257 confirmed *M. caprae* cases.

Data Analysis

The descriptive analysis of the spatial-temporal development of the Patho Score over a period of nine years and the statistical association between animal-specific risk factors for TB status and Patho Score of advanced TB-like lesions were carried out in STATA (39) using Pearson's chi-squared test, Cochran–Mantel–Haenszel test (MH) and Wald test of homogeneity of stratum-specific odds ratio's (OR). The Patho Score was used as an indicator to systematically show and quantify dynamics of infection. For comparisons of mean Patho Score between subgroups, the arithmetic mean of scores was calculated (reported with the 95% confidence interval (CI)). For the adjusted MH test, the Patho Score was reduced to two levels (low scores: 1–3; and high scores 4–5).

For the MH test, the reference categories were fawns (vs. yearlings and adults >2 years for the variable "age"), males (vs. females for the variable "sex"), good condition (vs. poor condition for the variable "condition"), head-only samples (vs. additional sample tissues for the variable "sample tissue" type) and zone outside the TB control area (vs. observation zone, edge zone and core zone for the variable "zone"). For the binary variable sample tissue "head and thorax," "head, thorax and abdomen," "thorax-only," and "other" samples were subsumed under samples with "additional tissue" (**Table 2**). For the variable zone, the score test for trend of odds was applied; the reported OR

TABLE 2 | Body sites examined and location of TB-like lesions.

Body sites examined	Lesion location						Total (%)	
	Retro[a] (%)		Thorax (%)		Other tissue (%)			
Head-only	115	(98.3)	–	–	4	(3.4)	117	(45.7)
Head and thorax	96	(93.2)	24	(23.3)	1	(1.0)	103	(40.2)
Head, thorax and abdomen	11	(57.9)	11	(57.9)	12	(63.2)	19	(7.4)
Thorax-only	–	–	6	(100)	–	–	6	(2.3)
Other	5	(45.5)	4	(36.4)	3	(27.3)	12[b]	(4.3)
Total	227	(88.7)	45	(17.6)	20	(7.8)	256	(100)

Row percentages of lesion locations may exceed 100% due to lesions at multiple locations in the sample material of an animal. The last column presents column percentages.
[a]*Medial retropharyngeal lymph nodes (present in 248 cases, affected in 227 cases).*
[b]*Only affected sample material was described, but overall information on submitted tissues was missing.*

estimate is an approximation to the OR for a one unit increase in the level of zone).

A causal diagram was used to conceptualize links between the three animal-specific *in vivo* recordable variables age, sex, condition, and the two further explanatory variables sample tissue and zone with the outcomes "TB status" and "Patho Score" and for bias assessment (**Figure 2**). Condition was identified as an intermediate variable on the causal path between both age and sex with TB status and with Patho Score. Age and sex were thus not adjusted for condition to avoid overadjustment. Zone influences age, sex, condition and sample tissue type through zone-specific differences in the sampling within the deer monitoring. The number of sampled tissues is influenced by age, sex and condition according to the deer monitoring program plan, but also influences the chance that an individual of a certain age, sex, or condition becomes a case, or receives a high Patho Score.

The spatial data visualization and analysis was done in ArcGIS (40). Analyses with annual comparisons are based on the official period of the hunting year (April 1st–March 31st), e.g., 2017 includes all deer tested between April 2017 and March 2018. February and March 2009 were counted to the hunting year 2009.

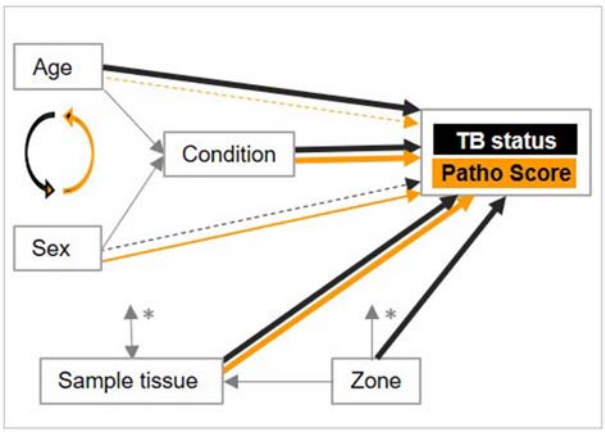

FIGURE 2 | Causal diagram of links between five explanatory variables with the outcomes "TB status" and "Patho Score." Black arrows: links with TB status. Orange arrows: links with Patho Score. For black and orange arrows, thicker arrows indicate stronger evidence for an association with the outcome. Dotted arrows indicate that only one level of the variable appears to be associated with the outcome. Curved arrows indicate interaction between variables. Gray arrows link explanatory variables with each other without any assumptions regarding strength of evidence of an association. *Sample tissue type, age, sex, and condition influence each other in both directions. Zone influences age, sex and condition.

RESULTS

Submitted Sample Material

In a total of 117 (45.7%) out of 256 cases the submitted sample material consisted only of the head or parts of the head including medial retropharyngeal lymph nodes ("head-only," see **Table 2**); 60 (51.3%) of these samples were obtained from hinds and 41 (35.0%) from yearlings. The second largest group were 103 (40.2%) samples consisting of head and thoracic organ tissues ("head and thorax"). See **Table S1** for detailed numbers of deer tested, split by subgroup, TB status and Patho Score.

In 227 (91.5%) out of the 248 samples containing at least one medial retropharyngeal lymph node, this lymph node was affected. Lung or pulmonary lymph nodes were affected in 45 (32.3%) out of 139 samples containing thoracic organ tissues). Other sample tissues with TB-like lesions ("other tissues") comprised parts of the intestine with mesenteric lymph nodes, liver with hepatic lymph nodes, diaphragm with pleura and udder tissue including mammary lymph nodes. Since other tissues were to be presented only in case of visible abnormalities, no valid conclusion can be drawn from these data regarding true frequency of lesions in abdominal organs or other body parts, but they give an overview of the range of lesions and organs affected.

Pathomorphology of Lesions

The pathomorphological abnormalities of TB-like gross lesions corresponded to earlier descriptions on *M. caprae* in red deer in western Austria (4, 5, 16, 41). It could be confirmed that observed lesions predominantly consisted of granulomas and abscesses with creamy pus or caseous cores. With Patho Score 1, lesions were mostly singular, 1–5 mm large granulomas or micro-abscesses in a single lymph node. In 47 (97.9%) of a total of 48 samples with score 1, one or both medial retropharyngeal

lymph nodes were affected. Only in one sample, the medial retropharyngeal lymph node itself showed no alterations, but had a 3 mm abscess of creamy-yellowish pus in its immediate vicinity. The exact localization of this lesion could not be identified due to the conduct of the sampling.

Lesions with score 2 were characterized by singular or multiple abscesses with 5–10 mm of diameter, with creamy, purulent-watery or caseous contents, some of which were encapsulated. Score 3 lesions were grossly similar to lesions described for score 2, but with coalescing abscesses that formed singular abscesses with diameters of up to 80 mm. Lesions with score 4 showed a more differentiated picture: in addition to the increasing sizes of typical abscesses in the lymph nodes, numerous miliary granulomas were observed in lymph nodes or the lungs. Lymph nodes were in some cases very small and of firm consistency. Lesions with score 5 corresponded to generalized TB with advanced lesions in multiple lymph nodes and organs with abscesses up to 200 mm in diameter (**Figure S2**).

Cases with head-only samples received a mean score of 2.4 ± 0.2, and cases with additional tissues had a mean score of 3.2 ± 0.2. Within the group of cases with additional tissues mean scores did not differ significantly after adjustment for condition: for animals in poor condition all carcass parts with gross lesions had to be submitted, leading to more sampled tissues with higher numbers of gross lesions. However, among 19 cases with the maximum range of sampled sites (head, thorax and abdomen, **Table 2**), only one case would have received a lower Patho Score if only the standard sample (head and thorax) would have been presented for pathological examination. This was the only case with TB lesions in the mesenteric lymph nodes but without gross lesions in the medial retropharyngeal lymph nodes or thoracic tissues.

Risk Groups for TB Positivity

Figure 3A shows the (crude) apparent prevalences for deer subgroups split by sex, age and condition. **Table 3** list detailed statistical output for this chapter. In the crude analysis the MH chi-squared test showed very strong evidence ($p < 0.001$) for associations between TB status and the explanatory variables sex, age, condition, zone and sample tissue type. In pairwise adjustments against each other, the MH analysis confirmed the strength of association between condition, zone and the sample material and TB status: deer in poor condition had 6.5 times the odds of having TB. For zone, the score test for trend showed an OR of 2.6 for a one unit increase in zone, with the area outside the TB control area as baseline. The crude OR of 0.6 for sample tissue type was confounded by the differing sampling method in the TB zones. In the low prevalence zone outside the TB control area, only 5.1% of submissions were head-only samples. In the observation, edge and core zones, the percentages of head-only samples were 39.0, 66.1, and 62.7%, respectively. After controlling for zone, deer with additional submitted sample tissues had 1.8 times the odds of TB positivity compared to deer with head-only samples.

The Wald test and the comparison with MH adjusted OR for this bivariate analysis suggested sample tissue type and zone as potential confounders for the association between sex and age with TB status. These confounders were thus controlled for in the following analyses. Sex proved to be a weak indicator for TB status**:** only for the area outside the TB control area, the adjusted MH estimate showed good evidence ($p = 0.01$) for an association between sex and TB status: males had 4.5 times the odds of being TB positive. In the TB control area, age appeared to modify the effect of sex on TB status (and *vice versa*): there was no difference between male and female yearlings ($p = 0.88$). But for adult deer the analysis showed good evidence for an association ($p = 0.02$) between sex and TB status: stags had 1.5 times the odds of being TB positive compared to hinds. The estimated OR was larger for adults with additional sample tissues (OR = 2.2). This association was to a large extent explained by the fact that stags (especially stags II) were more often sick or injured. Out of the 39 cases in adult deer with poor health, 31 (79.5%) were male. After additional adjustment for the intermediate variable condition,

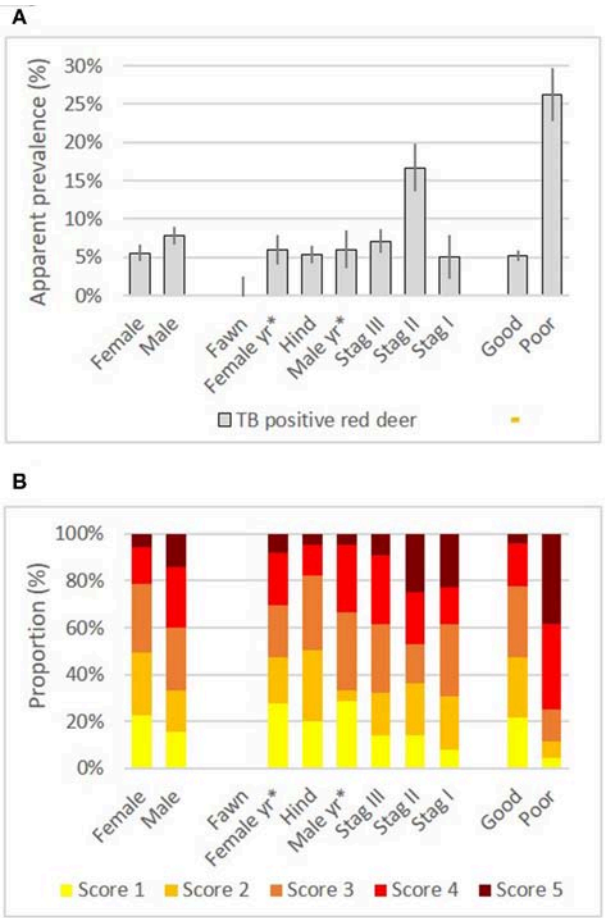

FIGURE 3 | Apparent prevalence with 95% confidence intervals **(A)** and distribution of Patho Scores **(B)**, by sex, sex & age group and condition of TB positive deer ($n = 256$). For better comparability of groups, the Patho Score was normalized in **(B)**. Example how to read the panels: 5.6% of all females were tested TB positive, and of these, 22.3% were categorized with score 1, etc. No sex was recorded for fawns. *yr, yearling, hind: ≥2 years, stag III: 2–4 years, stag II: 5–9 years, stag I: ≥10 years.

TABLE 3 | Models selected to explain the association between TB status and age, sex, condition of deer, number of submitted sample tissues and TB zone of kill location.

Explanatory variable for TB status	Adjusted for	*n* (cases)	chi^2	*p*, MH	OR	95% CI	*p*, Wald test
Age	Crude MH[a]	4,262[b] (257)	18.50	<0.001	1.55	1.27–1.89	
	Zone, sample tissue	3,982[b] (257)	32.87	<0.001	1.90	1.53–2.39	0.94
Age (males only), in area[c]	Sample tissue	1007[d] (125)	6.63	0.01	2.00	1.17–3.45	0.17
Age (females only), in area[c]	Sample tissue	1,296[d] (118)	3.94	0.05	1.52	1.00–2.30	0.78
Sex	Crude MH[a]	3,912 (257)	7.83	0.005	1.43	1.11–1.85	
Sex, outside area[c]	Age	1,611 (14)	5.97	0.01	4.54	1.19–17.16	0.10
Sex (yearlings only), in area[c]	Sample tissue	746 (56)	0.02	0.88	1.04	0.59–1.85	0.94
Sex (adults only), in area[c]	Sample tissue	1,446 (187)	5.05	0.02	1.53	1.05–2.20	0.02[e]
	Head-only	667 (75)	0.27	0.61	0.85	0.47–1.55	Stratum 1[e]
	Additional tissue	779 (112)	10.04	0.002	2.16	1.33–3.52	Stratum 2[e]
	Sample tissue, condition	1,446 (187)	3.14	0.08	1.41	0.96–2.05	0.12
Condition	Crude MH[a]	4,264 (257)	128.27	<0.001	6.48	4.47–9.41	
	Zone, sample tissue	3,983 (257)	100.46	<0.001	6.62	4.32–10.15	0.12
Sample tissue	Crude MH[a]	3,983 (257)	14.76	<0.001	0.56	0.47–0.79	
	Zone	3,983 (257)	16.84	<0.001	1.76	1.36–2.41	0.31
Zone	Crude MH[a]	4,265 (257)	289.42	<0.001	2.64	2.36–2.95	

For each explanatory variable, the table presents the variables adjusted for, number of independent samples (and cases thereof), (pooled) chi-squared statistic, p-value and estimate of the odds ratio of the Cochran-Mantel-Haenszel (MH) test with 95% confidence interval, and p-value of the Wald test for homogeneity of the odds ratios of the stratified analysis. All tests have one degree of freedom.

[a] *Crude MH: MH analysis without adjusting for other variables.*

[b] *Age in three categories: fawns (reference)—yearlings—adults ≥2 years.*

[c] *Area: TB control area, consisting of core, edge and observation zones.*

[d] *Age in two categories: yearlings (reference)—adults ≥2 years, as sex was not recorded for fawns.*

[e] *Stratum-specific odds ratios need to be reported.*

stags had 1.4 times the odds of being TB positive compared to hinds ($p = 0.08$). There was thus only weak statistical support for a controlled direct causal effect of sex *per se* on TB status.

Age *per se* showed to be a good indicator for TB status: After adjusting for zone and sample tissue type, there was even stronger evidence ($p < 0.001$) for an association between age with TB status (adjusted OR = 1.9 vs. crude OR = 1.6). Stratified analysis by sex showed that the odds for TB positivity increased in both sexes with age: stags had 2.0 times the odds of TB positivity compared to male yearlings, and the odds of hinds were 1.5 compared to female yearlings. Out of all subgroups split by sex and age, 5–9 year old stags II showed the highest apparent prevalence of 16.7% (**Figure 3A**). None of the 351 tested fawns was tested TB positive.

Condition was found *per se* to be the most important *in vivo* recordable indicator for TB status. Emaciated, sick or injured deer had after adjusting for zone and sample material around 6.6 times the odds of being tested TB positive compared to deer appearing healthy ($p < 0.001$).

Risk Groups for Advanced Lesions

Figure 3B shows the distribution of the Patho Score for deer subgroups split by sex, age and condition. **Table 4** list detailed statistical output for this chapter. Comparing the crude means of the Patho Score (with levels 1–5), hinds had the lowest mean score (2.5 ± 0.3), followed by yearlings (females: 2.6 ± 0.4; males 2.8 ± 0.5) and stags (stags III: 3.0 ± 0.3; stags II: 3.2 ± 0.4 and stags I: 3.2 ± 0.7). The crude MH analysis showed very strong evidence ($p < 0.001$) for an association between Patho Score (reduced to two levels high/low) and condition and sample tissue type, and strong evidence ($p = 0.002$) for an association between Patho Score and sex. Deer in poor condition, deer with additional sample tissues and males had 10.5, 3.0, and 2.4 times the odds of having a high Patho Score, respectively. There was no evidence for an association between Patho Score and age or zone in the crude analysis.

Sex appears to be a good indicator for advanced lesions in adult deer: Like with TB status, the Wald test indicated interaction between age and sex in respect to their effect on the Patho Score. Adjusting for sample tissue type showed for yearlings no evidence for an association between sex and Patho Score ($p = 0.83$). For adults however, there was good evidence for an association ($p = 0.04$). Stags had 2.2 times the odds of having advanced lesions compared to hinds.

Age is a weak indicator for score 4–5 lesions: Stratified by sex and adjusted for sample tissue type, there was no statistical support for an association between age and Patho Score with males ($p = 0.77$), although the percentage of advanced lesions increased tendentially with age (except the oldest age group of stags I). Out of all age groups of males, stags II were with 17 (45.9%) of 37 submissions the subgroup with the most lesions with scores 4–5. Females showed an opposing trend: there was some evidence for an association between age and Patho Score ($p = 0.06$). Hinds had 0.4 times the odds, or, in other words, female yearlings had 2.4 times the odds of having a high score compared to hinds. Zone did not confound the association between sex and age with Patho Score; the ORs adjusted for zone did only

TABLE 4 | Models selected to explain the association between Patho Score and age, sex, condition of deer, number of submitted sample tissues and TB zone of kill location.

Explanatory variable for Patho Score	Adjusted for	*n*	chi^2	*p*, MH	OR	95% CI	*p*, Wald test
Sex	Crude MH[a]	256	10.14	0.002	2.44	1.38–4.29	
	Sample tissue	256	2.01	0.16	1.55	0.84–2.87	0.17
Sex (yearlings only)	Sample tissue	57	0.05	0.83	0.87	0.25–3.04	0.65
Sex (adults only)	Sample tissue	199	4.25	0.04	2.15	1.02–4.54	0.14
Age[b]	Crude MH[a]	256	0	0.95	0.98	0.52–1.85	
Age[b] (males only)	Sample tissue	135	0.09	0.77	1.17	0.40–3.40	0.31
Age[b] (females only)	Sample tissue	121	3.51	0.06	0.40	0.15–1.08	0.62
Condition	Crude MH[a]	256	47.15	<0.001	10.53	4.55–24.36	
	Sample tissue	256	35.57	<0.001	9.43	3.92–22.69	0.35
Sample tissue	Crude MH[a]	256	14.84	<0.001	3.02	1.67–5.46	
Zone	Crude MH[a]	256	1.59	0.21	0.83	0.62–1.11	

For each explanatory variable, the table presents the variables adjusted for, number of independent samples (and cases thereof), (pooled) chi-squared statistic, p-value and estimate of the odds ratio of the Cochran-Mantel-Haenszel (MH) test with 95% confidence interval, and p-value of the Wald test for homogeneity of the odds ratios of the stratified analysis. All tests have one degree of freedom.

[a] *Crude MH: analysis without adjusting for other variables.*

[b] *Age in two categories: yearlings (reference)–adults ≥2 years, as TB was never detected in fawns.*

marginally differ from the ORs adjusted only for sample tissue type (results not shown).

Condition was again found to be the most important indicator for advanced lesions with scores 4–5. Independent from the levels of age, sex, tissue material or zone, deer in poor condition had 9–10.5 times the odds of showing advanced stages of TB (result shown in **Table 4** are limited to crude analysis and adjustment for sample tissue type). Clinical signs or other abnormalities were recorded for 45 (26.2%) cases; of these 33 (73.3%) received score 4 or 5 (**Figure 3B**). Emaciation was with 12 records the most frequent leading symptom. For another nine deer leg injuries or other injuries were reported. For most cases only non-specific records on the clinical signs were available ("sick," "abnormal behavior"). In general, stags II contributed most to the subgroup of deer in poor condition (19 (42.2%) of 45 cases).

Infection Dynamics in the Years 2009–2017

Geographically, the distribution of TB cases in the core, edge and observation zones remained relatively constant between 2009 and 2012 (**Figures 1B–D**). From 2013 onwards, a redistribution of cases took place: while apparent prevalence decreased in the core zone since 2013, it increased in edge and observation zones in 2013–2016. In the core zone, apparent prevalence was significantly lower ($p < 0.03$) in 2016 and 2017 with 12.0 (±6.8%) and 10.6% (±8.3%) respectively, compared to 2013–2015 with 21.6–27.6% (±4.7–10.0%) (**Figure 4A**). In the neighboring edge and observation zone apparent prevalence was with 10.1% (±2.2%) in 2016 also significantly higher ($p \leq 0.002$) than in 2013 (2.7% ± 2.7%), in 2014 (6.6% ± 3.1%), and also in 2017 (4.8% ± 2.5%). This development was comparable in edge and observation zones and therefore both zones are presented in a joint graph in **Figure 4B**. TB has noticeably spread since 2013, especially in the west and the south of edge and observation zones (**Figures 1E–H**). See **Table S2** for detailed statistics related to apparent prevalences.

Three different patterns of disease occurrence could be identified: endemic disease, epidemics and sporadic cases. These three patterns will be described in more detail.

Endemic Disease

Analyses of the Patho Score showed that all stages of TB occurred together in the core zone. This corresponds to the typical picture of an endemic disease occurrence without much tendency of a change. From 2013 onwards, proportions of all score levels decreased at a fairly similar scale along with a decreasing apparent prevalence (**Figure 4A**) (no *p*-value reported due to several subgroups with zero individuals). There was still evidence for an active infection cycle in 2016 and 2017, which is revealed by 2% of tested deer with score 1, including yearlings. However, deer with score 5 were missing in sample materials in these last two years with lower apparent prevalences.

Epidemic in a Newly Infected Area

The increase of prevalence was no zone-wide evenly distributed phenomenon but was attributable to three newly infected spots in the edge and observation zones that were confirmed in 2016 (**Figure 1H** shows spots A–C). The 2016 hunting year had both the highest apparent prevalence (10.1% ± 2.2%), and also the highest proportion of higher scores (7% of deer with scores 3–5) recorded so far in these two zones.

Spot B will be described as example for epidemic TB in more detail: This spot was a 23 km^2 large hunting ground located west of the core zone in the edge zone. In 2013, a first case of TB was detected in a female with score 2 (**Figure 5**). In 2014, three animals were positive (all three were females with scores 1 or 3), followed by a case of a yearling with score 1 in 2015. In 2016, five cases were shot right at the beginning of the hunting season in April. This unexpected finding led to an intensified hunting and sampling of deer in this area and resulted in a total of 21 cases out of 93 tested deer. The infection could first be detected in

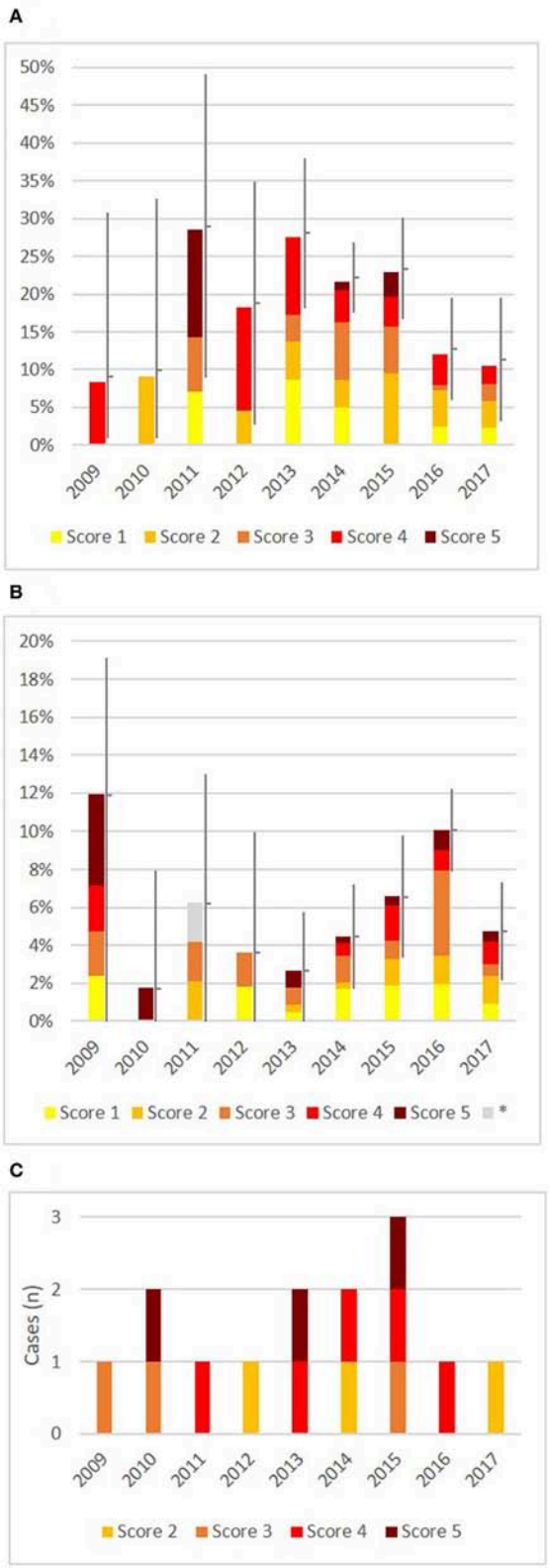

FIGURE 4 | Development of the Patho Score between 2009 and 2017, stratified by TB zones. **(A)** Core zone with in total 136 TB positive deer out of *(Continued)*

FIGURE 4 | 719 tested deer. **(B)** Edge zone and observation zone (joined) with in total 106 TB positive deer out of 1,733 tested deer. *Data on submitted material was missing for one case marked in grey, therefore no score was assigned. **(C)** Area outside the designated TB control area with in total 14 TB positive deer out of 1,815 tested deer. Colors of cases denote the Patho Score. In **(A,B)**, the bars mark the apparent prevalence with 95% confidence intervals; in **(C)** the number of sporadic cases is shown. Up to 2012, the confidence intervals are large due to lower sample size.

yearlings and females in spring and summer. Only from October 2016 onwards scores 4 and 5 were found (in stags). The first deer with clinical signs was a stage II with score 5 in November.

In two outbreak-like spots in the north-western (spot A) and southern observation zone (spot C), TB was confirmed in 2016 with seven and five cases respectively (**Figure 1H**). In both spots the first cases were also detected in March and April. In spot A, the first case was a stag III with score 1, followed by cases with scores 2 and 3 and 11 month later one case with score 5 (no cases detected in 2017). In spot C, the first case was a female yearling with score 5, followed by cases with scores 1 and 3, and seven more cases in 2017 presenting lesions of all five levels of scores.

Sporadic Cases Outside the Designated TB Control Area

Between February 2009 and March 2018, a total of 14 TB cases were detected outside the designated TB control area, with one to three cases each year (**Figure 4C**). Twelve of these cases were recorded in the district of Bregenz (**Figure 6**). Age and sex distribution among these cases showed with nine males and only one yearling a different pattern compared to the TB control area (**Table S3**). With eight (57.0%) cases with score 4 or 5 lesions, advanced TB stages were frequent, and score 1 lesions were not detected so far. Kill locations were up to 30 km away from each other and in different valleys. On the one hand, cases appeared to be independent in time and space. On the other hand, patterns are visible: all deer were hunted between August and November, and kill locations of eight of the 12 cases lie on an imaginary line (cases numbers 1–5 and 7–9, see **Figure 6**).

DISCUSSION

This study attempts to describe infection dynamics of TB in red deer by using patho-scoring as an additional source of information. The study demonstrates that the infection dynamics of TB are associated with individual animal-specific parameters such as sex, age and condition, and environmental characteristics such as vicinity to other TB areas.

Roles of Subgroups in the Infection Dynamic

Three animal-specific parameters are usually recorded by the hunter before the kill: age, sex, and condition. Older animals and stags were significantly more likely to be TB positive. Stags also had higher Patho Scores than hinds. The subgroups with the lowest apparent prevalence were fawns; and hinds had on average the lowest scores. Effect modification between age and

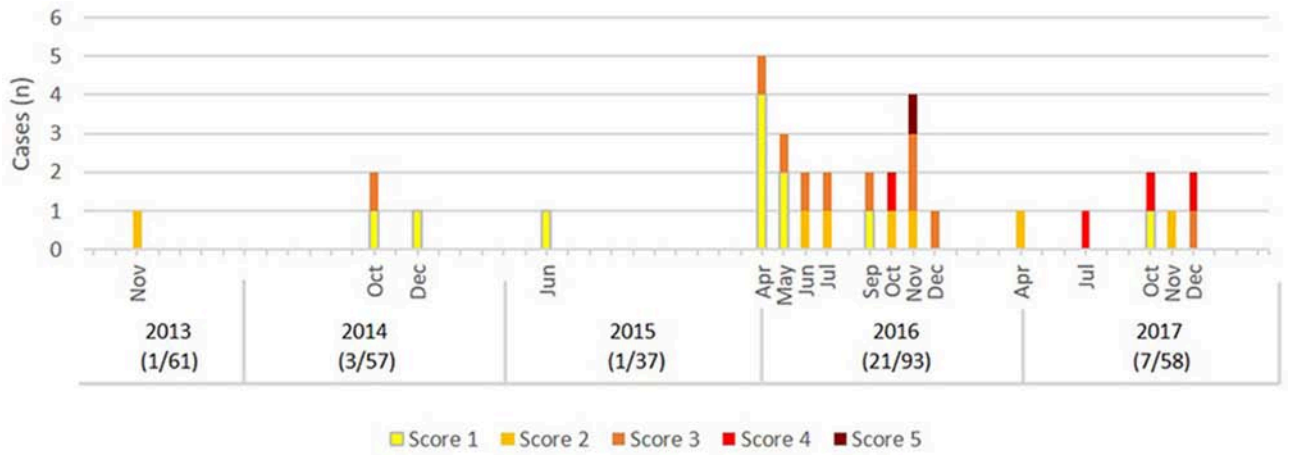

FIGURE 5 | Epidemic curve of TB cases in deer by month of detection for the years 2013–2017, spot B. Colors of cases denote the Patho Score. In Brackets: (number of cases/number of deer tested per year).

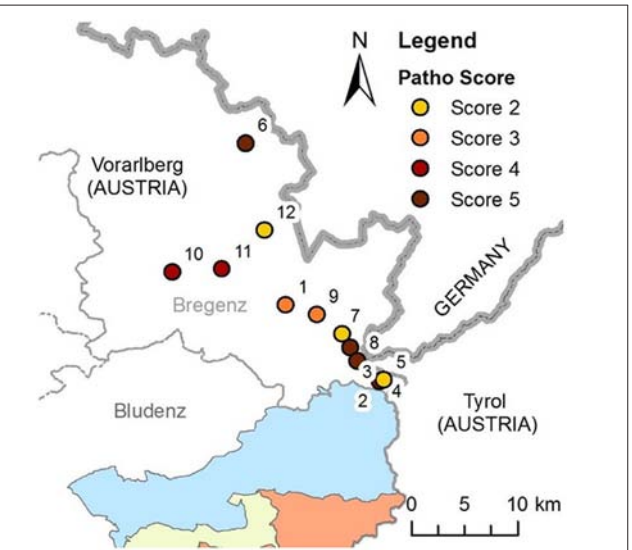

FIGURE 6 | Map of kill locations of 12 TB cases in deer in the area outside the designated TB control area, district of Bregenz. Zones of the TB control area are marked in blue, yellow and red. Case numbers are sorted by kill date: 1: earliest sample from August 2009, 12: latest sample from September 2017). Colors of cases denote the Patho Score (**Table S3** lists the characteristics of cases).

sex on their observed effect on TB status and the developmental stage of lesions could indicate that stags, hinds and yearlings play different roles in the infection dynamics within the infected deer population. These roles for the spread and maintenance of disease of different subgroups should be investigated in more detail to better understand how TB spreads within and between deer populations.

The majority of studies report higher TB prevalence in male deer [reviewed by (42)], including studies from Spain and Michigan (32, 43, 44). In contrast, similar prevalence values were recorded in male and female deer in New Zealand, with a higher prevalence in young males ≤2 years offset by a lower prevalence in older males > 5 years (19). Lugton et al. (12) observed more gross lesions and more cases of advanced TB in male than female red deer, although this difference was not significant. They interpreted this finding not as a higher susceptibility of males to TB *per se*, but attributed this result to the time the stags were shot, which usually fell into the period during which males were in hard antler. During rutting season, stags experience particular stress from aggression and gathering and maintaining a group of hinds, which may influence the development of lesions (12). The results of this study give some support to this conclusion: after adjusting for the (intermediate variable) condition, there was only weak statistical support for a controlled direct causal effect of sex on TB status. Higher prevalences of TB and advanced lesions in stags were largely attributable to higher proportion of sick or injured deer among males compared to hinds.

TB was not detected in fawns in this study. The observation of no or very low numbers of TB positive fawns has been made on several continents (32, 44, 45) and has been interpreted as indication for the limited importance of dams for the infection of their fawns in free-ranging deer. Conversely, for farmed deer, Griffin et al. (46) described an acute outbreak of TB with a prevalence >90% in young fawns accompanied with a prevalence of 60% in breeding hinds. One feasible explanation would be that the TB status of fawns is indeed directly related to the level of exposure from hinds. In Vorarlberg, hinds were one of the subgroups with the lowest prevalence and also had the lowest Patho Scores. The absence of infection in fawns despite 5% of their mothers being infected could also indicate that direct deer-to-deer transmission is very rare, and fawns are only infected in settings with significant indirect environmental transmission. This might not happen in Vorarlberg until winter feeding begins.

Higher figures of infection in older deer are a common finding in TB literature and are attributed i.a. to long exposure time of this long-lived species and chronicity of TB (12, 44, 47). In Vorarlberg, the group of 5–9 year old stags II had both the highest

prevalence of TB positivity and advanced lesions. Over 10 year old stags I were three times less likely to be TB positive compared to stags II, and also less often showed generalized TB (**Figure 3**). The observation that gross lesions are less frequently detected in very old animals was also made by Lugton et al. (12). The authors hypothesized that infected animals may be capable of resolving lesions over time or that susceptible individuals have died, while those remaining have kept the infection under control.

These two different tracks of disease progression could also serve as explanations for the special role of the oldest deer in Vorarlberg: the high numbers of stags II with clinical signs and advanced TB would correspond to the susceptibles that are killed sick or die before they reach the oldest age group. For stags that reach apparently healthy an old age, although they might have been exposed to the pathogen for years, this could indicate some form of natural immunity or clinical latency. An additional explanation could lie in distinct contact patterns. It has been observed that older stags prefer to roam in very small groups of stags or sometimes even become solitary. Such behavior could lead to a limited exposure to the pathogen. To conclude, in case a protective factor could be identified, it would be relevant to investigate whether those "protected" subgroups also show lower infectivity and whether the *in vivo* identification of these animals could be utilized within a selective TB control strategy.

The most important *in vivo* indicator for TB was however the condition. Cases with poor health also had advanced stages of TB (score 4 or the maximum score 5) significantly more often. This relationship was to be expected since the observed clinical signs were likely to be attributable to the disease progress of TB in a considerable number of cases. Research showed that deer with advanced TB can cause massive environmental contamination through the excretion of high numbers of mycobacteria (8, 12, 48). The findings of this study suggest that selective culling that aims at the elimination of potential high shedders should prioritize weak, sick and injured deer, even if no abnormalities indicative for infectious disease are observed. However, to increase the efficiency of a control strategy that includes selective culling, it is advisable to combine findings on risk groups with additional epidemiological information to identify groups of deer with higher exposure or locations with increased risk of environmental contamination, e.g. by targeting groups or areas with earlier detections of deer with scores 4–5.

Patterns of Disease Occurrence

Three different patterns of TB occurrence could be distinguished and characterized: areas with endemic disease, areas with outbreak-like cases, and areas with sporadic cases. This distinction is relevant to assess the infection dynamics in each area and to better inform the selection of targeted prevention and control measures.

Endemic Disease

In the core zone both new infections as well as spreaders with advanced TB were seen, which can lead to further infections. This suggests that an endemic equilibrium has been reached with multiple infection chains occurring in parallel. Surprisingly, only few deer with score 5 were detected in the core zone. In this zone, deer have been intensively hunted for several years as part of TB control and apparent prevalence is declining. The question remains whether absence of cases with score 5 is causally associated with the decline of the apparent prevalence. To exclude biases due to sampling regime or to confirm other potential reasons these developments need to be further monitored.

Epidemic Disease

Outbreak areas showed the typical picture expected for a point source in a previously disease-free population: the first detected cases presented predominantly early stages of lesions. Over time, more advanced disease stages and cases of generalized disease (score 5) were seen, which eventually were accompanied by clinical signs (**Figure 5**). A characteristic of a point source is that all cases are exposed at one point in time or within a limited period of time and location directly or indirectly to the primary case. Whether the pathogen was introduced by a single "super spreader" or by multiple animals serving as co-primary cases cannot be distinguished from the data. Due to the proximity of outbreak areas to endemically infected areas both scenarios are possible.

In all three outbreaks spots first cases were detected in early spring, pointing toward infection spreading during the winter feeding season. In spring, deer tend to browse still close to the location of their feeding site. It would need to be investigated if through early detection of cases in spring deer belonging to the same winter feeding cohort can be identified. Such a classical approach of tracing back could support targeted hunting of deer at higher risk of infection and thereby prevent that TB establishes permanently in a new area. Whether the three outbreak spots already reached a state of endemic infection or whether control measures were successful in limiting further spread will become clearer within the next 1–2 years. Within one year of detection of the first cases both early and advanced stages of lesions have been found in the three outbreak spots. The case of a yearling killed in April with score 5 indicated that TB can quickly progress to stages where infected individuals may cause massive environmental contamination.

Sporadic Cases

Sporadic cases should receive particular attention: so far, all outbreaks in TB-free areas in Vorarlberg were preceded by sporadic cases. This finding contrasts with the situation in the north of Vorarlberg, where sporadic cases have been recorded for nine years without any indication of spread among the local deer population. Deer abundance and management and size of winter feeding sites are comparable in both the TB control area and the area with sporadic cases, and deer densities are estimated to be high enough to support spread in the resident deer population. The north of Vorarlberg is part of the foothills of the alps with lower mountains and better feed availability for deer. Deer are on average heavier and might thus be in a better physical condition compared to deer in the TB control area. However, it is questionable if this physical advantage is sufficient to prevent the establishment of a new TB spot given that the environmental conditions in the north of Vorarlberg resemble those in the TB areas in nearby southern Germany.

Sporadic cases would be expected in various disease scenarios: they could either indicate that TB became established at a very low level in resident deer and is therefore constantly present—or they could be an indicator for a nearby active TB situation from where cases "spill over" into new areas. For the north of Vorarlberg, the results of the deer monitoring rather support the latter, with regular introductions of infected migratory deer from the TB control area in Vorarlberg or neighboring deer TB areas in Tyrol or Germany. Even in the "TB at a low level" scenario deer with advanced lesions of score 4 or 5 would occasionally spend the winter at feeding sites together with critical numbers of susceptibles. This should eventually lead to the detection of additional cases in resident deer.

Sick deer are likely to isolate themselves from their social group. It could be observed that deer with severe TB were commonly found alone and well away from other deer (12). If sporadic cases seen in the north of Vorarlberg are such isolated deer that have migrated from affected areas, this means that even advanced TB is no obstacle for diseased animals to move over long distances. The pattern of sporadic cases being mostly older stags is consistent with results from a telemetry study in the south of Vorarlberg: this study showed that stags more often migrate over long distances up to 30 km across mountains and have a larger mean home range size of 6,400 hectares, compared to females with 2,600 hectares (35). The kill sites of sporadic cases were in distances between 1 and 13 km from the borders to Tyrol, Germany and the TB control area of Vorarlberg. The origin of migratory deer can therefore not be determined based merely on the distance of the kill site to the closest TB area. Cross-border cooperation is needed to better understand the dynamics of TB in this border area. Comparative genomic analyses could provide insights into the relationship of mycobacteria circulating in the different affected regions.

Lesion Presence as Indicator for Disease Progress

At individual animal-level, environmental factors as well as animal-specific factors are understood to influence progression of TB and other diseases within the infected body (20, 21). This implies that the time period to progress from one stage of lesions to the next might vary considerably between infected individuals.

Latency is an important characteristic of *M. tuberculosis* infection in humans (49, 50) described it also as the most frequent expression of *M. bovis* infection in badgers. In cattle, latent TB infections are not considered to be common (51), and *M. bovis* infection of cattle usually results in a slowly progressive disease (52). For red deer, it is not known how often latent TB infections occur and which role they play in infection dynamics. Studies on *M. caprae* in red deer in Austria (4) and Germany (2) and *M. bovis* in red deer in Spain (44, 53) and New Zealand (8, 12, 19) showed that the pathogen could be detected in 22–68% of deer samples without visible lesions. Gavier-Widen et al. (54) argued that this non-visible lesion presentation in animals was likely to include latent cases or merely early-stage infections that do not yet present macroscopic lesions. However, it is uncertain if animals with non-visible lesions would eventually develop progressive disease or if these infections can be cured spontaneously (52, 54). Until host immune mechanisms of red deer are not better understood, inferences on the potential time of TB infection based on lesion need to be made with caution. Especially with adult deer, score 1 lesions cannot be put on a level with recent infections without any supporting epidemiological data. However, lack of gross lesions in the total of 351 tested fawns till December in combination with presence of small lesions detected from April onwards in yearlings of the same birth cohort indicate that these score 1 lesions indeed correspond to recent infections that potentially occurred during the winter feeding period.

Being aware of these open questions, results of this work nevertheless support the approach that an analysis of tissue lesions at population-level is still useful to monitor developments in infection dynamics. The Patho Score allows visualization and quantification of these dynamics. The Patho Score presented in this study focuses on lesions in medial retropharyngeal lymph nodes and discriminates particularly between mild to moderate stages of lesions. At the population level these lesions generally indicate recent infections (11, 33). Predominance of lesions in medial retropharyngeal lymph nodes reported in this study was previously described for *M. caprae* in Austrian red deer by Fink et al. (5) and Nigsch (22). This observation corresponds to results of studies from other countries (11, 15, 53) and supports the conclusion that monitoring programs that focus on the examination of lymphoid tissue of the head are capable to detect a significant portion of TB-infected red deer (53) and are also suitable for early detection of TB.

Applications of the Patho Score

Lesion scores are regularly used in experimental TB challenge studies of cattle, deer and other wildlife, where tissue materials can be sampled under standardized situations (21, 55–57). With naturally infected deer, lesion scores were applied to study infection patterns and effects of TB on deer, to assess the role of deer in perpetuating TB among cattle or to develop sampling protocols (17, 19, 53). In this study, the Patho Score was used to identify risk groups for advanced TB stages and to characterize areas with different patterns of disease occurrence. For many analyses, such as detailed analyses of outbreaks or analyses of sporadic cases, the absolute number of TB cases was too low to obtain statistically significant results. These mainly descriptive analyses are thus considered as exploratory. One strength of the Patho Score is certainly that with the descriptive information it generates, it provides a much more differentiated insight into the TB situation compared to prevalence data alone, at no extra costs. This information can then be used for forming hypotheses to be investigated via more rigorous, multivariable statistical methods in a next step, and for guiding early disease management efforts until those hypotheses are validated.

The overarching goal of deer monitoring is to protect cattle against TB infections from deer. For Vorarlberg, no studies are available on the interaction between cattle and deer, but the main route of infection is assumed to be indirect transmission. The Patho Score helps to identify areas with an increased risk of environmental contamination by deer with advanced stages of

TB, or areas where the risk of infection could increase rapidly due to recent outbreaks in deer. Identification of these high-risk areas is an important prerequisite for targeted measures toward disease prevention in cattle.

Future applications of the Patho Score include comparing infection dynamics of TB in different countries or to support comparison of different monitoring systems, e.g., how successful is the monitoring system to detect very small TB lesions.

Limitations

Deer monitoring and sampling are conducted under field conditions: by definition, the hunting bag is not a simple random sample of the local deer population. On the one hand, some age groups are underrepresented in the hunting bag as red deer management favors a specific age pyramid. On the other hand, the hunting law foresees that obviously sick and injured animals must be harvested for welfare purposes. In the TB control area all sick and injured deer had to be examined and were thus likely to be overrepresented in the sample.

In addition, only tissue material with gross lesions was selected for further examinations to confirm TB. Tissue without lesions was considered TB negative. Lesion-based monitoring tends to underestimate the prevalence. These limitations were known to the authors before the development of the Patho Score. Therefore, the challenge was to develop a valid tool under the given conditions, which is capable to take account of the heterogeneity of the available historical longitudinal data.

A critical task was to assess the impact of missing lymph nodes or other organ tissues in individual samples for the correct categorization with the Patho Score. The association between number of sample tissues and Patho Score was significant. Deer represented with thoracic or abdominal tissues in addition to heads received more frequently a high score of 4 or 5. However, submission of more tissues in addition to the standard sample (head and thorax) has only in one case led to a higher score. The reason for this lies in the definitions for score 4 and 5 (**Table 1**): samples with one affected organ (lung, pleura, liver, udder, etc) are categorized at least with score 4, and one severely affected body site is sufficient to receive score 5. Submission of more abnormal tissues will not necessarily increase a high score.

"Head-only" samples could by definition only reach a maximum score of 4. Sample selection in the current deer monitoring might thus underestimate the proportion of high scores and thereby the proportion of potential super-spreaders among identified cases. However, the amount of submitted tissues and severity of clinical lesions were also causally related: for deer with visible organ abnormalities, deer monitoring required that more tissues including all affected body sites were sampled. The potential bias in selection of sample material was accounted for twofold: firstly, by adjusting for the amount of sample material in the statistical analysis, and secondly by the final interpretation of the Patho Score: in this study, scores 4 and 5 were both interpreted to be more relevant for spreading disease, with score 5 being considered as an advanced stage of score 4. Standardization of sample material (if logistically feasible) would have a positive effect on the overall sensitivity of deer monitoring and furthermore would increase validity of the Patho Score.

Even though deer monitoring will underestimate true prevalence, the authors hypothesize that comparative analyses over time and space remain valid, as sampling mode and diagnostic protocol did not change greatly over the 9-year monitoring period. With lesion-based monitoring the role of animals with non-visible lesions for the infection dynamic could not be investigated. Such an investigation would require culturing of key lymph nodes from all deer, including those with non-visible lesions. However, it can be assumed that deer with gross lesions play at least for pathogen spread a more important role. With 4,521 examined samples virtually the whole range of stages of lesions could be explored. For an external evaluation of validity of the Patho Score, it would be of interest to apply this score on data from other regions to estimate the effect of field conditions.

Recommendations

The assessment of TB-like lesions showed various practical approaches on how to gain better insight into the infection dynamics through the targeted selection of animals to be sampled in early spring to early identify new spots of infection. Identified risk groups for TB and advanced lesions should receive particular attention in infection control programs. Special attention require also sporadic cases in TB-free areas: they do not necessarily indicate that the infection already spreads locally but these sporadic cases appear to be a precursor of outbreaks among resident deer populations. In this context, one relevant question for further research would be: what constellation of animal-specific parameters, lesions, season and other measurable conditions would signal a transition of an area with sporadic cases to an outbreak area or to an area with an endemic level of infection presence?

The next step to draw a holistic picture of the infection dynamics would be to include home range size, habitat selection and deer-to-deer interaction within and between deer populations in this alpine setting in more detail to investigate potential seasonality of infections and to better characterize the role of specific subgroups in maintenance and spread of TB. Furthermore, characteristics of the pathogen should be considered in addition to host-specific, environmental and human interaction related parameters. This could be taken into account in the form of genomic analyses of infection chains between animals or between subpopulations of animals. With the ultimate goal to better understand host-pathogen interactions for this important pathogen. For this task it will be very valuable to link data generated by pathology, diagnostics, epidemiology and systems biology research.

CONCLUSION

This is the first study of *M. caprae* in red deer in the Austrian state of Vorarlberg that describes development of TB and its infection dynamics over the last decade. The study proposes the use of TB-like lesions in a so-called Patho Score as a mirror for infection dynamics. With the Patho Score, a new instrument is introduced to complement monitoring of TB in red deer in western Austria and to systematically visualize and quantify infection

dynamics at no additional costs. This work shows the breadth of application possibilities of this lesion score. The analysis adds some evidence regarding the critical role of winter feeding sites for spread of TB infections in young deer. The identification of geographic areas with differing patterns of disease occurrence demonstrated that TB does spread in Vorarlberg within several geographically connected subpopulations with separate infection cycles. TB spreads only slowly between valleys but migrating infected deer might introduce the agent into new areas.

To the best knowledge of the authors, this is the first study that uses a lesion score for the systematical description of the infection dynamics of mycobacterial disease. Due to the cross-border TB situation, the possibility to systematically compare TB dynamics based on heterogeneous data is an important added value.

DATA AVAILABILITY STATEMENT

Restrictions apply to the datasets: The raw data for this manuscript are not publicly available and are subject to a data protection clause. Requests to access the datasets will be examined on a case to case basis and should be directed to Norbert Greber, veterinaer@vorarlberg.at. Aggregated data supporting the conclusions of this manuscript is contained within the manuscript and the Supplementary Files.

ETHICS STATEMENT

All animal sampling was post-mortem in accordance with the official deer monitoring program plan of the Office of the State Government of Vorarlberg. Wildlife samples came from hunter-harvested individuals that were shot during the legal hunting season, or individuals found dead, independently and prior to our research. According to national legislation (Austrian Tierversuchsgesetz 2012 – TVG 2012, BGBl. I Nr. 114/2012) no permission or consent was required for conducting this type of study.

AUTHOR CONTRIBUTIONS

AN, WG, and NG designed the study. WG and ZB carried out the laboratory work. WG and NG entered and prepared the data for the analysis. AN, WG, and ZB developed the Patho Score. AN performed the analysis. AN, WG, and ZB drafted the preliminary manuscript. All authors participated in the review and the editing of the draft and approved its final version.

ACKNOWLEDGMENTS

In the first place, the authors would like to thank all hunters and official veterinarians from Vorarlberg. Without their support the implementation of the deer monitoring would not have been possible. We would like to acknowledge Hubert Schatz for sharing his expertise in wildlife ecology. We also want to thank Ynte Schukken and Mart de Jong for valuable professional discussions. This study received financial support from the Office of the State Government of Vorarlberg, the Austrian Agency for Health and Food Safety and Wageningen University.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00350/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 30 October 2018
doi: 10.3389/fvets.2018.00262

Infection of Wildlife by *Mycobacterium bovis* in France Assessment Through a National Surveillance System, Sylvatub

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Mycobacterium bovis infection was first described in free-ranging wildlife in France in 2001, with subsequent detection in hunter-harvested ungulates and badgers in areas where outbreaks of bovine tuberculosis (TB) were also detected in cattle. Increasing concerns regarding TB in wildlife led the French General Directorate for Food (DGAL) and the main institutions involved in animal health and wildlife management, to establish a national surveillance system for TB in free-ranging wildlife. This surveillance system is known as "Sylvatub." The system coordinates the activities of various national and local partners. The main goal of Sylvatub is to detect and monitor *M. bovis* infection in wildlife through a combination of passive and active surveillance protocols adapted to the estimated risk level in each area of the country. Event-base surveillance relies on *M. bovis* identification (molecular detection) (*i*) in gross lesions detected in hunter-harvested ungulates, (*ii*) in ungulates that are found dead or dying, and (*iii*) in road-killed badgers. Additional targeted surveillance in badgers, wild boars and red deer is implemented on samples from trapped or hunted animals in at-risk areas. With the exception of one unexplained case in a wild boar, *M. bovis* infection in free-living wildlife has always been detected in the vicinity of cattle TB outbreaks with the same genotype of the infectious *M. bovis* strains. Since 2012, *M. bovis* was actively monitored in these infected areas and detected mainly in badgers and wild boars with apparent infection rates of 4.57–5.14% and 2.37–3.04%, respectively depending of the diagnostic test used (culture or PCR), the period and according to areas. Sporadic infection has also been detected in red deer and roe deer. This surveillance has demonstrated that *M. bovis* infection, in different areas of France, involves a multi-host system including cattle and wildlife. However, infection rates are lower than those observed in badgers in the United Kingdom or in wild boars in Spain.

Keywords: bovine tuberculosis, *Mycobacterium bovis*, surveillance, wildlife, badger, wild boar, France

OPEN ACCESS

Edited by:
Daniel J. O'Brien, Michigan Department of Natural Resources, United States

Reviewed by:
Christian Menge, Friedrich Loeffler Institut, Germany
Carol Geralyn Chitko-McKown, U.S. Meat Animal Research Center (ARS-USDA), United States

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *12 July 2018*
Accepted: *02 October 2018*
Published: *30 October 2018*

Citation:
Réveillaud É, Desvaux S, Boschiroli M-L, Hars J, Faure É, Fediaevsky A, Cavalerie L, Chevalier F, Jabert P, Poliak S, Tourette I, Hendrikx P and Richomme C (2018) Infection of Wildlife by Mycobacterium bovis in France Assessment Through a National Surveillance System, Sylvatub. Front. Vet. Sci. 5:262. doi: 10.3389/fvets.2018.00262

INTRODUCTION

Wildlife can serve as a reservoir for multiple pathogens and may serve as a sentinel of the disease risk to humans and domestic animals. As a result, disease surveillance in wildlife is strongly recommended to provide data on the epidemiological role of wild animals and for the development of adapted disease control measures (1).

Bovine tuberculosis (TB) is a contagious and zoonotic disease caused by *Mycobacterium bovis*, and occasionally by *M. caprae* or *M. tuberculosis* (hereafter referred to as MTBC). This pathogen primarily infects cattle but can be transmitted to a wide range of host mammals, especially numerous wild animals such as Eurasian badgers (*Meles meles*), wild boars (*Sus scrofa*), red deer (*Cervus elaphus*), and roe deer (*Capreolus capreolus*) (2). In the United Kingdom (UK) and in Ireland, the Eurasian badger is considered as TB reservoir, as is the wild boar in Spain. These species are involved in the transmission of *M. bovis* to cattle (3–6).

France is officially declared TB-free since 2001 in the bovine population, because <0.1% of cattle herds being infected annually. However, outbreaks still occur and the number of infected herds has increased since 2004 in certain parts of the country, especially in the South-West: Dordogne, Charente and Pyrénées-Atlantiques (French administrative division called departments) and in the East of France (Côte-d'Or) (7).

In France, TB in wild animals was first detected in 2001 in the Brotonne forest (Normandy) in hunter-harvested red deer exhibiting gross lesions. In 2006, despite control measures (culling), apparent prevalence rates in this forest reached 24% in red deer and 42% in wild boars, the closed environment and high density of wild ungulates were considered major risk factors to explain such high prevalence rates (8). Elsewhere in France, sporadic cases of TB infection have been detected in red deer and/or wild boar in several areas: Côte-d'Or (Burgundy region), Corsica, Pyrénées-Atlantiques, Dordogne, and Ariège in 2002, 2003, 2005, and 2010, respectively. The first cases in wild ungulates were systematically detected by carcass examination in hunter-harvested animals. Since then, event-based surveillance programs (also called passive surveillance) and targeted (or active) surveillance programs for the disease including the badger have been implemented in these areas. TB infection in badgers was initially detected in 2009 in Côte-d'Or (5.7%, $n = 918$ in the 2009–2011 period), then in 2010 in Dordogne and Charente (4.8%, $n = 417$ in 2010–2011). In wild boars, prevalence rates observed in 2008 reached locally 16.5% in Côte-d'Or and 4.4% in 2010–2011 in Dordogne (9). All these cases were detected in the vicinity of cattle outbreaks (10–12).

Increasing concern regarding the status of TB infections in wildlife led the French General Directorate for Food (DGAL) and the main institutions involved in animal health and wildlife management to establish a national surveillance system for TB in free-ranging wildlife: the "Sylvatub" system. This system coordinated by the French platform for epidemiological surveillance in animal health (ESA-Platform), was launched in September 2011. The main aims of Sylvatub are to detect TB in wildlife, to estimate and monitor infection levels in infected areas, to characterize *M. bovis* strains isolated from wildlife and to harmonize surveillance at the national level.

This article summarizes the key data collected on TB infection in France between 2011 and 2017 in badgers, wild boars, red deer, and roe deer. We describe the organization of the Sylvatub system and the findings in terms of TB prevalence in wild boars and badgers, and necropsy data gathered during event-based and targeted surveillance in the four species.

MATERIALS AND METHODS

Stakeholders and Organization of Sylvatub

The organizational structure of the system is shown in **Figure 1** and was described by Rivière et al. (12). Briefly, the DGAL is in charge of the Sylvatub system. Coordination and technical operations are performed by the ESA-Platform (www.plateforme-esa.fr). National governance is ensured by a steering committee and a technical subcommittee, where the different institutions or organizations involved in Sylvatub are represented (**Figure 1**).

The implementation of Sylvatub is based on the regular involvement of national stakeholders [national reference laboratory (NRL) for TB and the national coordinator of Sylvatub] and local stakeholders: veterinary services in charge of Sylvatub coordination, public administration in charge of the environment, hunting federations, associations of "*lieutenants de louveterie*" (historically called wolf-hunter, nowadays being state but volunteer officers in charge of pest control and who supervised badger trapping), trappers associations (volunteers who are duly trained and authorized), French hunting and wildlife agency services, local veterinary laboratories for animal health, cattle breeders, and finally veterinary associations.

Sylvatub: A Risk-Based Surveillance System

Sylvatub targets the wildlife species considered in 2011 to be the most relevant in the TB multi-host system: badgers, wild boars, red deer, and roe deer. Sylvatub's surveillance system components are based on event-based and targeted surveillance. These components are applied according to a risk-based surveillance approach, with three different levels of surveillance, applied at the level of French department (**Table 1**) and defined as follows:

- Level 3, for areas with several outbreaks in cattle and cases in wildlife. This level is applied for at least 4 years, to monitor infection levels in wildlife and to assess the efficacy of control measures;
- Level 2, for areas with sporadic at-risk outbreaks in cattle and/or areas in geographic proximity to high-risk areas. This level is applied for at least 1 year or for as long as necessary to develop a clear understanding of the epidemiological situation. A lower or higher surveillance level is subsequently applied, depending on the results obtained;
- Level 1, elsewhere in the country where no domestic and wild animal has been found infected for a long period of time.

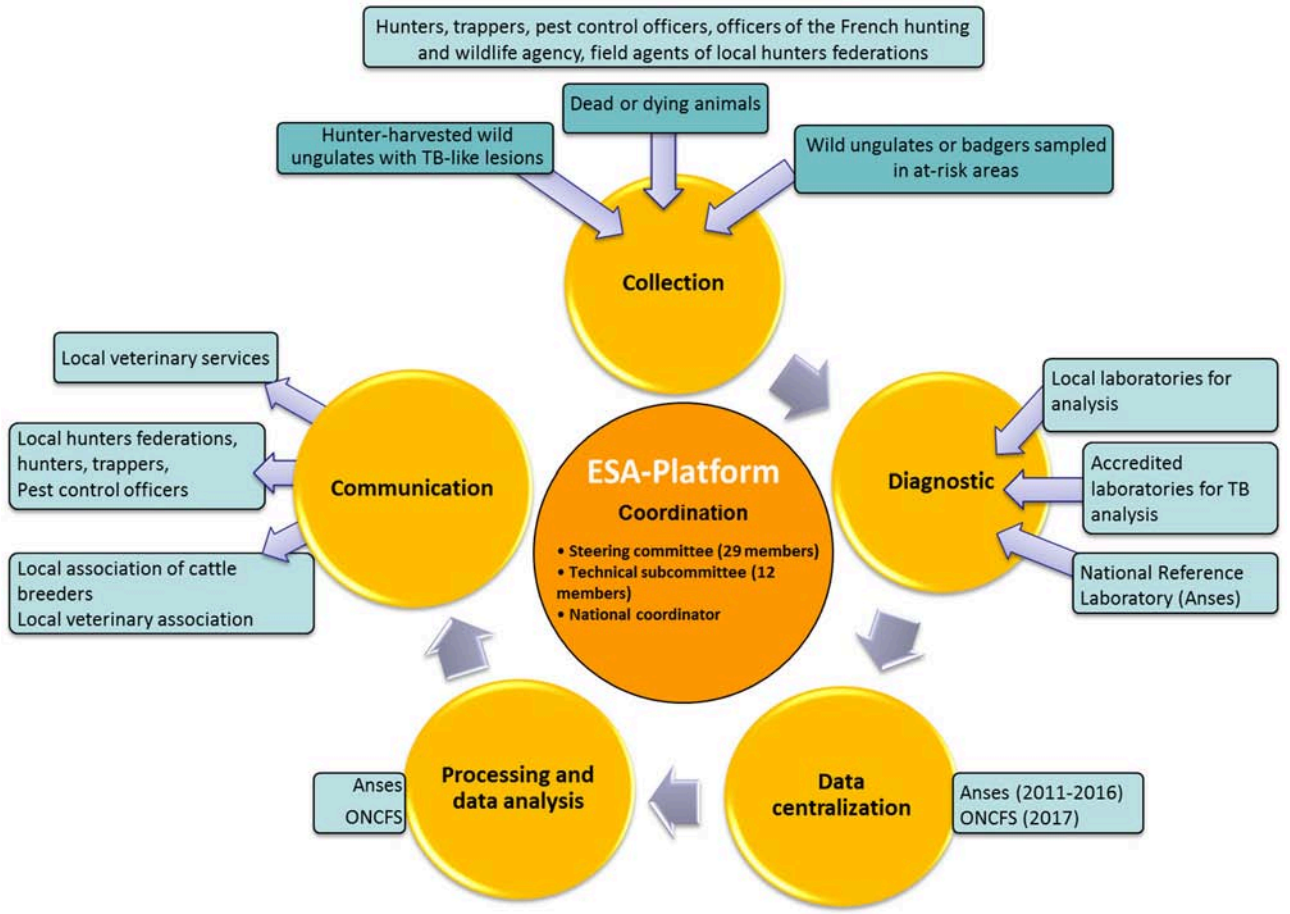

FIGURE 1 | Simplified organization of Sylvatub. *Steering committee members*: French General Directorate for Food (DGAL), Ministry of the Environment (MEDDE), Regional Directorates for Food (DRAAF), Anses, French hunting and wildlife agency (ONCFS), National Hunters Federation (FNC), French association of pest control officers, French association of approved trappers, the French National Federation of Animal Health Defense Associations (GDS France), National veterinary association (SNGTV), Coop de France and French Association of Directors and Managers of Public Veterinary Laboratories of Analyzes (Adilva), Regional veterinary epidemiologists. *Technical subcommittee members*: DGAL, Anses, ONCFS, DRAAF, FNC, GDS France, Adilva.

The local surveillance level is re-evaluated twice a year, to align with the epidemiological situation in cattle and wildlife populations.

Event-Based Surveillance Through Detailed Game Carcass Examination

This surveillance component is applied to all geographic areas, i.e., regardless of the local risk, to hunted wild boars, red deer, and roe deer. It is based on the analysis of animals with macroscopic TB-like lesions detected by hunters during post-mortem examination of all hunted games. The detection of gross lesions is supported by a national network of more than 55,000 hunters trained by the National Hunters Federation (FNC) for food safety purpose. Additionally to this framework, voluntary training courses were organized in the field to train hunters for recognition and reporting of TB-like lesions (internal abscesses or gross lymph nodes) and sampling of affected organs.

Event-Based Surveillance Through the SAGIR Network: Wild Animals Found Dead, Moribund or With Abnormal Behavior

Event-based surveillance on dead and dying wild animals, through the SAGIR network (French hunting and wildlife agency/local hunters federations/FNC), has been implemented in France since 1986 (13). Within the Sylvatub system, dead animals belonging to TB-receptive species (badgers, wild boars, red, and roe deer) collected as part of the SAGIR network are tested for TB (*i*) in the presence of TB-like lesions for level 1 departments, and (*ii*) systematically for level 2 and 3 departments. Moreover, efforts are made to collect and test road-kill badgers in all the level 2 and 3 departments.

Targeted Surveillance

Targeted surveillance may concern badgers, wild boars and/or red deer in level 3 departments depending on the population abundance and distribution, and badgers only in level 2

TABLE 1 | Surveillance methods implemented depending on the estimated risk level.

Surveillance methods	Level 1	Level 2	Level 3
Event-based surveillance:	X	X	X
- Detailed game carcass examination (wild ungulates)			
- SAGIR[(1)] network (wild ungulates, badgers)			
Strengthened event-based surveillance:		X	X
- SAGIR network strengthened (red deer, wild boars, badgers)			
- Road-killed animals (badgers, wild ungulates)			
Targeted surveillance in badgers in at-risk areas or around sporadic bovine outbreaks		X	X
Targeted surveillance in wild boars and red deer in at-risk areas			X

[(1)] Monitoring of dead or dying animals.

departments. This component of surveillance is implemented in "at-risk areas" determined as areas of about seven kilometers in radius around pastures of cattle outbreaks detected in the previous 4 years, and hunting or trapping locations of all infected wild animals. For badgers, at-risk areas are divided in two sub-areas: "infected area" (2 km radius) and "buffer area" (5 km radius around the infected area) to take into consideration badger home range which is smaller than in wild ungulates.

Sample sizes are determined to detect TB infection in at-risk areas, assuming a prevalence of 3%, with a 95% confidence interval. For wild boar and red deer, samples are defined for the whole at-risk area, whereas for badgers, one sample is for the infected area and one is for the buffer area. In large areas where wild populations could be considered as infinite, a sample of 130 animals per species is required. This sample size takes into account diagnostic test sensitivity (estimated at ∼75% for PCR; see below). However, sample sizes are adjusted based on the surface of the area. In practice, samples between 60 and 260 red deer, wild boars, and badgers are programmed annually in each area. These are samples of hunted wild boars and deer or trapped badgers for control measures in infected areas.

Additionally, around sporadic cattle outbreaks outside at-risk areas (in level 2 and 3 departments), systematic TB analysis is conducted on a sample of about 15 badgers trapped within a radius of one or two kilometers depending on the number and localization of badger's setts. These small areas are called "prospecting areas."

Animals are collected even if no macroscopic TB lesions are detected by field stakeholders.

Tissue Collection and Laboratory Investigations

Sample Collection

Wild boars and red deer are collected by hunters, under the supervision of the local hunting federations during the hunting season (generally from August to March each year), and badgers are collected by trappers (accreditation required), under the supervision of pest control officers. In infected areas, where one of the control measures is to reduce badger populations, badger can be trapped, mostly from March to August. Field stakeholders (hunters, trappers, pest control officers) submit animals, organs, or tissues (from a standardized list of samples described in **Tables 2**, **3**) directly to the local laboratory or store them in cooling rooms or freezers for later analysis. Data is collected for each animal on the species, estimated age (juvenile or adult), sex, date, location of collection and body condition (degradation, presence of lesions in the carcass, etc.). Age determination is based on animal size for badgers and/or weight and hunters' knowledge for wild ungulates. Trappers and pest control officers are volunteers but a financial compensation is provided for them.

Tissue samples are taken in local laboratories even if no TB-like lesions are detected except for SAGIR network animals in level 1 department.

Tables 2, **3** shows the field samples collected for testing and changes over time. For wild boars, from mid-2013 until the present time, only the head has been collected and the submaxillary lymph nodes are tested. For badgers, which are relatively small, the entire animal is collected. Analyses at the local laboratory consist of post-mortem necropsy to detect TB-like lesions (caseo-granulomas, mineralized nodules, or purulent abscesses), polymerase chain reaction (PCR) and/or bacterial culture on pooled lymph node samples and on pooled samples with TB-like lesions.

As presented previously, diagnostic schemes differ between surveillance components: either TB testing is performed only if TB lesions are detected (SAGIR event-based surveillance in level 1 departments), or TB testing is performed systematically (for all suspect hunted carcasses and all SAGIR animals in level 2 and 3 departments, as well as for targeted surveillance in level 2 and 3 departments).

Analyses are performed as indicated in **Tables 2**, **3**. The year 2015 marked a diagnostic-methodological transition for badger surveillance as these two diagnostic schemes were used depending on the local laboratory and the time of year. Changing from culture to PCR as a first line test was decided after having demonstrated that in cattle PCR provides a better sensitivity for TB detection without losing specificity (14).

Microbiological Culture

Bacterial culture is performed following the protocol established by the French NRL (NF U 47–104) for isolation of *M. bovis*. Two to 5 g of sampled tissues were crushed with a 4% sulfuric acid solution to decontaminate the tissue. After 10 min, the acid was neutralized by adding a 6% sodium hydroxide solution. After decontamination, the supernatant was seeded on two different media: Löwenstein-Jensen and Coletsos. All seeded media were incubated at 37°C +/– 3°C for three months and exanimated every 2 weeks. If contamination is observed during the first month, samples are decontaminated a second time with 4% sodium hydroxide and then neutralized with 10% sulfuric acid solution.

The isolated *M. tuberculosis* complex (MTBC) colonies are confirmed by DNA amplification (15) targeting the IS*6110*

TABLE 2 | Diagnostic methods used in badgers from 2012 to 2017.

		2012	2013	2014	2015	2016	2017
	Field samples	**Entire carcass**					
Badgers	Pooled samples at local laboratory	- Retropharyngeal, tracheobronchial, mediastinal, hepatic lymph nodes and salivary glands; - Pool of lesions (if present)		- Retropharyngeal, tracheobronchial, mediastinal and hepatic lymph nodes - Pool of lesions (if present)			
	Analysis at local laboratory	Culture; PCR on pool of lesions			Culture; PCR on pool of lesions or PCR, culture on positive PCR pools	PCR; Culture on positive PCR pools	

TABLE 3 | Diagnostic methods used in wild boars and deer for the hunting seasons from 2011 to 2017.

		2011–2012	2012–2013	2013–2014	2014–2015	2015–2016	2016–2017
Wild boars	Field samples	Head, pulmonary system, organs with lesions if present		Head, organs with lesions if present			
	Pooled samples at local laboratory	Cephalic and pulmonary lymph nodes; Pool of lesions (if present)		Submandibular lymph nodes; Pool of lesions (if present)			
	Analysis at local laboratory	Culture; PCR on pool of lesions				PCR; Culture on positive PCR pools	
Deer	Field samples	Head, pulmonary and digestive systems, organs with lesions if present					
	Pooled samples at laboratory	- Retropharyngeal, tracheobronchial, mediastinal and mesenteric lymph nodes; - Pool of lesions (if present)					
	Analysis at local laboratory	Culture; PCR on pool of lesions				PCR; Culture on positive PCR pools	

sequence present in all species of MTBC (16), and *M. bovis* is confirmed by spoligotyping (see below).

Tissue PCRs

DNA extraction is performed on a pool of lymph nodes (retropharyngeal, pulmonary and mesenteric) and on organs with gross lesions when present, after mechanical lysis using an LSI MagVet™ Universal Isolation Kit (Life Technologies) with a KingFisher™ Flex automate (Thermo Scientific), following the manufacturer's instructions. The LSIV and MAX™ MTBC Real-Time PCR kit (Life Technologies), which targets IS*6110*, is used. A volume of 5 µL of the extracted DNA is mixed with 20 µL of reaction mix, and the reaction is carried out at 50°C for two min (1 cycle), followed by one cycle of 10 min at 95°C and 45 cycles of 15 s at 95°C, and one min at 60°C. Results are interpreted following the manufacturer's recommendations and by comParison with negative and positive controls. If DNA amplification is positive, *M. bovis* or any other MTBC species is confirmed by spoligotyping (see below).

Spoligotyping

Spoligotyping is performed as described by Zhang et al. (17), using TB-SPOL kits purchased from Beamedex® (Beamedex SAS, Orsay, France) on Bio-PLex 200/Luminex 200®. Molecular typing is performed either on MTBC isolates or directly on PCR-positive sample DNA. The presence or absence of the 43 spacer sequences contained in the DR locus is represented in a binary code of 43 entries. Spoligotypes are named according to an agreed international convention (www.mbovis.org).

Data Analysis

An infected animal is defined as an animal with an analytical result demonstrating *M. bovis* (or if it had been found *M. tuberculosis* or *M. caprae)* by molecular diagnosis or by bacterial culture.

All results presented in this article come from the Sylvatub national database.

Results are presented by calendar year for badgers and by 12 month period starting with the beginning of the wild boar hunt (August to the end of July). The Sylvatub system was set up in September 2011; as a result surveillance findings for wild ungulates are presented from the 2011–2012 hunting season to the 2016–2017 hunting season and from 2012 to 2017 for badgers.

To simplify, at-risk areas are renamed with numbers as follows, by chronological order of detection of TB in wildlife: 1: Brotonne-Mauny forest (Seine-Maritime); 2: Côte-d'Or; 3: Dordogne/Charente/Charente-Maritime/Haute-Vienne/Corrèze/Gironde; 4: Dordogne/Lot; 5: Béarn (Pyrénées-Atlantiques/Landes/Gers); 6: Ardennes/Marne; 7: Marne (Reims mountain); 8: Loir-et-Cher (Sologne); 9: Lot-et-Garonne; 10: Pays Basque; and 11: Ariège/Haute-Garonne (**Figure 2**).

In this paper, apparent prevalence rates are indicated based on the diagnostic test used (culture or PCR). Furthermore, results were aggregated into two periods of 2 years each to calculate prevalence rates with more precision: P1 (2013 and 2014 for badger surveillance; the 2012-2013 and 2013-2014 hunting seasons for wild ungulate surveillance) and P2 (2016 and 2017 for badger surveillance; the 2015-2016 and 2016-2017 hunting seasons for wild ungulate surveillance). In P1, the

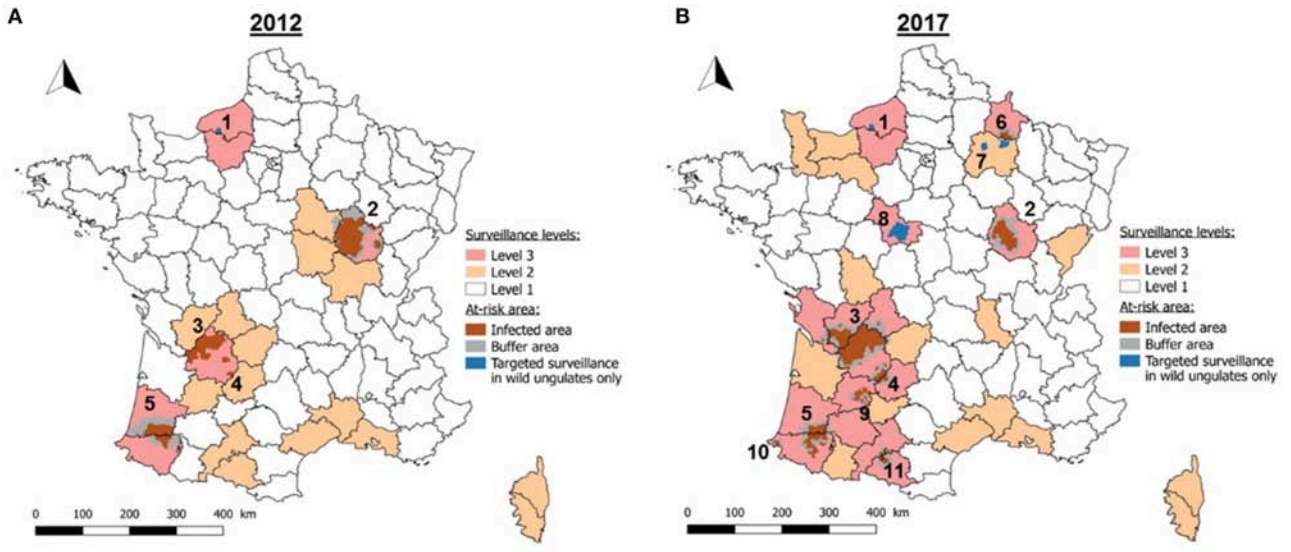

FIGURE 2 | Changes in surveillance levels and at-risk areas between 2012 **(A)** and 2017 **(B)** (*1*: Brotonne-Mauny forest; *2*: Côte-d'Or; *3*: Dordogne/Charente/Charente-Maritime/Haute-Vienne/Corrèze/Gironde; *4*: Dordogne/Lot; *5*: Béarn; *6*: Ardennes/Marne; *7*: Marne (Reims Mountain); *8*: Loir-et-Cher (Sologne); *9*: Lot-et-Garonne; *10*: Pays Basque; *11*: Ariège/Haute-Garonne).

diagnostic test used was sample culture, whereas in P2, PCR was used. We focused on areas where sampled animals were most numerous and where infection was confirmed to present and compare these results. To compare results in badgers between event-based and targeted surveillances, we focused in the at-risk area 3 because it is the only area where the number of analyzed badgers was sufficient for each of these surveillance components.

Regarding the number of analyzed animals, we counted only those with an interpretable analysis result (positive or negative). Apparent prevalence and 95% confidence intervals were calculated using exact binomial tests and *p*-values using the Fisher's exact test. Data analysis was performed using LibreOffice Calc (version 5.2) and R Studio software (version 3.3.1). Maps were generated using QGIS software (version 2.16.3).

RESULTS

Functioning Results

Since its implementation in 2012, Sylvatub has been gradually strengthened due to the larger number of areas where wild animals have been found infected, the enlargement of infected areas affecting cattle, and the detection of sporadic cattle outbreaks in new areas. This is reflected in the increase of number of departments at level 2 and 3 (from 21 in 2012 to 32 in 2017) (**Figure 2**). The surveillance levels for the departments were re-evaluated by the steering committee 10 times between 2011 and the end of 2017.

In 2012, there were five distinct at-risk areas. Since then, six other at-risk areas have been defined, with a total of 11 areas in 2017. Targeted surveillance was implemented for wild boars and red deer in three at-risk areas (numbered 1, 7, and 8), for wild boars and badgers in four at-risk areas (numbered 9, 5, 10, and 11) and for the three species in four at-risk areas (numbered 2, 3, 4, and 6) depending on the populations abundance and distribution.

In the meantime, size of these at-risk areas was expanded for five areas (3, 4, 6, 10, and 11), reduced for two areas (2 and 5) and remained approximately stable for the others (see details in **Supplementary Table 1**). The surface area of mainland France classified as at-risk was 14,397 km² in 2012 and 20,434 km² in 2017.

Event-Based Surveillance

The number of wild boars, red and roe deer collected by event-based surveillance increased from 77 in 2011–2012 to 134 in 2015–2016. In total, 316 wild boars, 197 roe deer, and 98 red deer have been collected since 2011. The number of dead or dying badgers collected per year (SAGIR and road-killed badgers) has increased from 70 badgers in 2012 to 582 in 2015. This number has been stable since 2015 with about 580 badgers collected per year, most of them being road-killed badgers.

Targeted Surveillance

An average of 296 red deer (min: 226; max: 380), 1,420 wild boars (min: 1,078; max: 2,175) and 1,881 badgers (min: 1,447; max: 2,239) were analyzed per year between 2011 and 2017.

TB Infection in Wildlife

TB Infection in Badgers

Event-based surveillance

Among the 2,491 badgers collected by event-based surveillance in 45 departments, 2,397 were analyzed (2,372 with an interpretable

analysis result), and 89 were found infected with *M. bovis* (**Figure 3**). In all, 84 of these infected animals were found in the vicinity of cattle outbreaks (75 infected badgers in infected areas, five in buffer areas and four in prospecting areas). Five infected badgers ($n = 716$) were from outside but very close (<3.5 km) to at-risk areas (areas 3 and 9). In infected area 3, where badgers collected by event-based surveillance are numerous, apparent prevalence rates seems to be stable between P1 (8.2%; 95% CI: 4.2–14.2%) and P2 (9.6%; 95% CI: 6.8–13.1%).

Targeted surveillance

From 2012 to 2017, 378 badgers ($n = 10,184$) were found infected with *M. bovis* by targeted surveillance in at-risk areas 2, 3, 4, 5, 6, 9, 10, and 11. In all, 340 of these badgers ($n = 6,870$) originated from infected areas and 27 ($n = 3,314$) from buffer areas (**Figure 4**). Targeted surveillance has not been implemented in at-risk areas 1, 7 and 8, where badger populations are very small.

In infected areas, apparent prevalence rate observed with culture (from 2012 to 2014) was on average 4.57% ($n = 3,198$) and with PCR (2016-2017) on average 5.14% ($n = 2,412$) (**Supplementary Table 2**). In buffer areas, apparent prevalence rate observed with culture was on average 1.33% ($n = 1,508$) and with PCR on average 0.41% ($n = 1,217$) (**Supplementary Table 2**). Regarding results in the four main infected areas (areas 2, 3, 5 and 6) for the two periods (P1 and P2), prevalence in badgers was significantly lower in P1 in infected area 3 than in the three other areas ($p < 0.001$; $p = 0.009$; $p = 0.0351$, respectively). In P2, prevalence was higher in infected area 5 than in areas 2 and 3 ($p = 0.02$; $p = 0.013$, respectively) (**Table 4**).

In area 2, apparent prevalence was higher in P1 than in P2 ($p = 0.008$) (**Table 4**).

The targeted surveillance in prospecting areas has detected two sites of infection: in Ardennes in 2013, five infected badgers were detected from a total sample of 37 badgers collected close to four bovine TB outbreaks and, in 2015, in the Pays Basque (Pyrénées-Atlantiques), two infected badgers were detected from a sample of nine badgers, also sampled on the outskirts of four bovine TB outbreaks. As a result of these findings, infected and buffer areas were defined in these two departments and other cattle outbreaks were discovered nearby.

Males were found to have significantly higher infection rates (4.95%, $n = 3,394$) than females (2.02%, $n = 4,213$) ($p < 0.001$).

In infected area 3, prevalence were significantly higher (in P1 and P2) in badgers collected by event-based surveillance (mainly road-killed badgers) than by targeted surveillance (mainly trapped badgers) ($p = 0.006$ and $p = 0.01$, respectively) (**Table 5**).

TB Infection in Wild Boars and Deer

Event-based surveillance

Among the 323 free ranging wild boars collected from 2011–2012 to 2016–2017 in 53 departments, 258 were analyzed, 241 had an

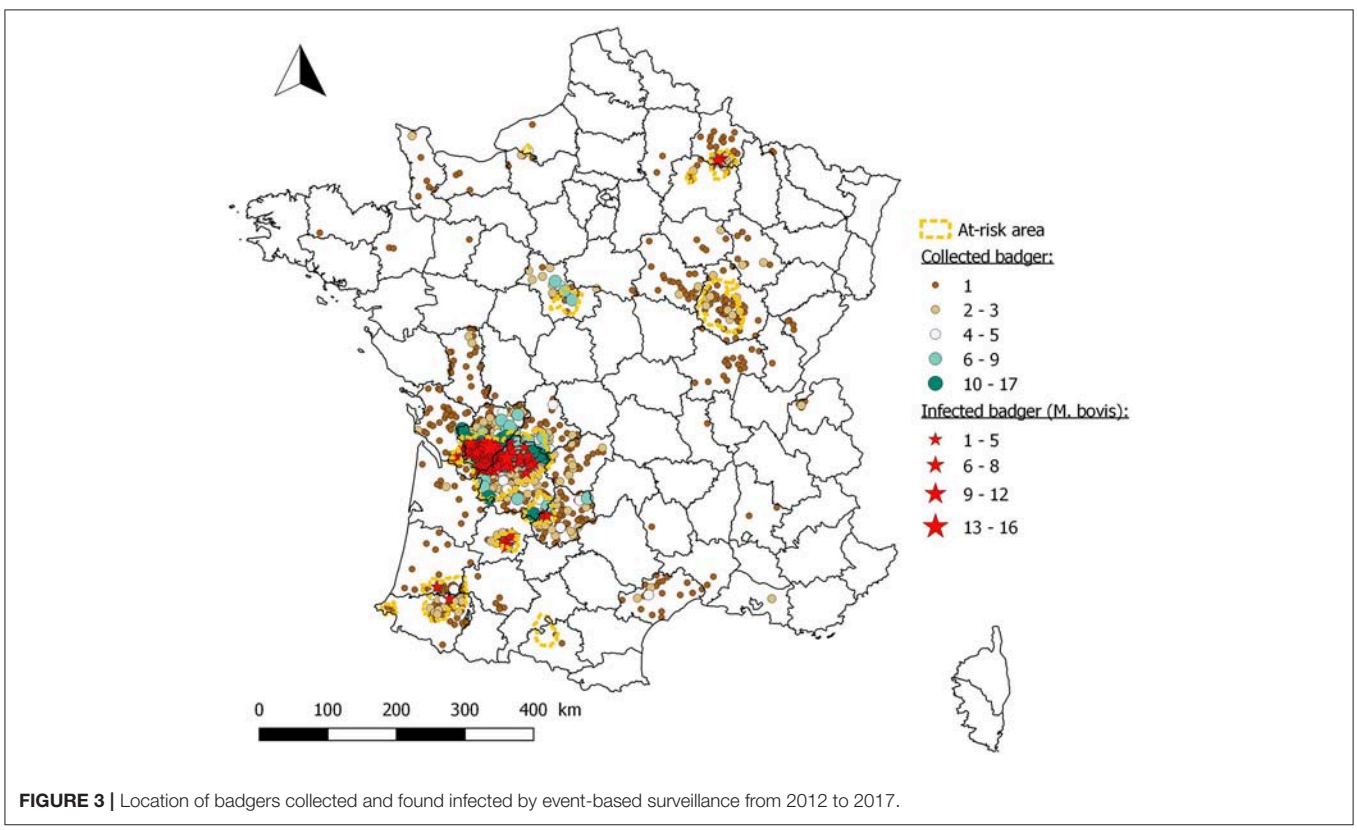

FIGURE 3 | Location of badgers collected and found infected by event-based surveillance from 2012 to 2017.

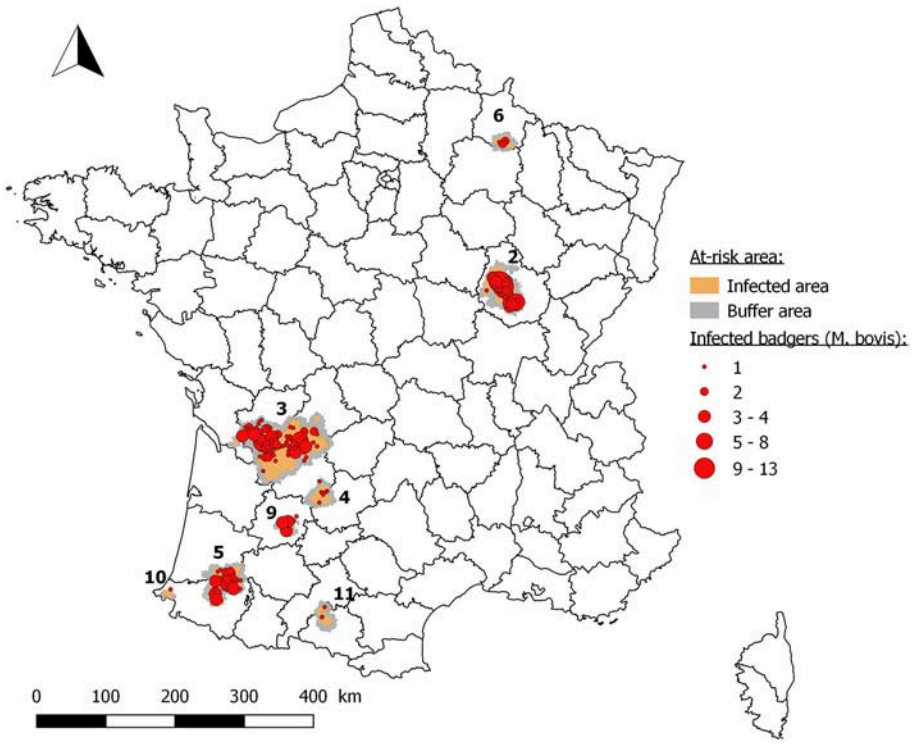

FIGURE 4 | Location of infected badgers collected by targeted surveillance from 2012 to 2017 (*2*: Côte-d'Or; *3*: Dordogne/Charente/Charente-Maritime/Haute-Vienne/Corrèze/Gironde; *4*: Dordogne/Lot; *5*: Béarn; *6*: Ardennes/Marne; *9*: Lot-et-Garonne; *10*: Pays Basque; *11*: Ariège/Haute-Garonne).

TABLE 4 | Apparent prevalence rates in badgers collected by targeted surveillance in the four main infected areas of France between 2013-2014 (Period 1: P1) and 2016-2017 (Period 2: P2) [percentages are given with 95% confidence intervals (CI); in brackets number of infected/analyzed animals].

No. of the infected area (full name of the area)	P1 (2013–2014) *Culture*	P2 (2016–2017) *PCR*
	Apparent prevalence rates [95% CI] (No. of infected badgers/no. analyzed)	
2 (Côte-d'Or)	8.1% [6.3–10.3%] (61/751)	4.2% [2.6–6.2%] (22/528)
3 (Dordogne/Charente/Charente-Maritime/Haute-Vienne/Corrèze/Gironde)	2.7% [1.7–4.1%] (22/805)	5.3% [4.1–6.8%] (61/1143)
5 (Béarn)	5.9% [3.9–6.8%] (26/439)	7.9% [5.2–11.2%] (27/344)
6 (Ardennes/Marne)	6.7% [3.1–12.4%] (9/134)	3.1% [0.4–10.7%] (2/65)

TABLE 5 | Comparison of apparent prevalence rates in badgers obtained by event-based surveillance and by targeted surveillance for two periods in infected area 3 [percentages are given with 95% confidence intervals (CI); in brackets number of infected/analyzed animals].

Type of surveillance component	P1 (2013–2014) *Culture*	P2 (2016–2017) *PCR*
	Apparent prevalence rates [95% CI] (No. of infected badgers/no. analyzed)	
Event-based surveillance	8.2% [4.2–14.2%] (11/134)	9.6% [6.8–13.1%] (35/365)
Targeted surveillance	2.7% [1.7–4.1%] (22/805)	5.3% [4.1–6.8%] (61/1143)

interpretable analysis result and 29 were found to be infected in six departments (Dordogne, Charente, Côte-d'Or, Haute-Corse, Corse-du-Sud, and Loir-et-Cher). Ten came from at-risk areas (level 2 and 3 departments), 18 came from Corsica and one wild boar found infected in January 2015 in Loir-et-Cher, a livestock TB-free area since 1986 (**Figure 5**). This wild boar was found in open forest, although the area is surrounded by private game parks. *M. bovis* was also isolated from a wild boar in a closed game park in the Reims Mountain (Marne) in 2012 (18). Data for this case were not integrated in Sylvatub database because it was not a free ranging wildlife animal.

A total of 107 red deer and 190 roe deer have been collected through event-based surveillance since 2011 from 35 and 48 departments, respectively. Two red deer submitted by hunters both in 2016 were found to be infected in areas 2 and 3, and five infected roe deer were detected in area 3 in 2012, 2013, 2015, and 2016. For more details on roe deer surveillance in France see Lambert et al. (19) (**Figure 5**).

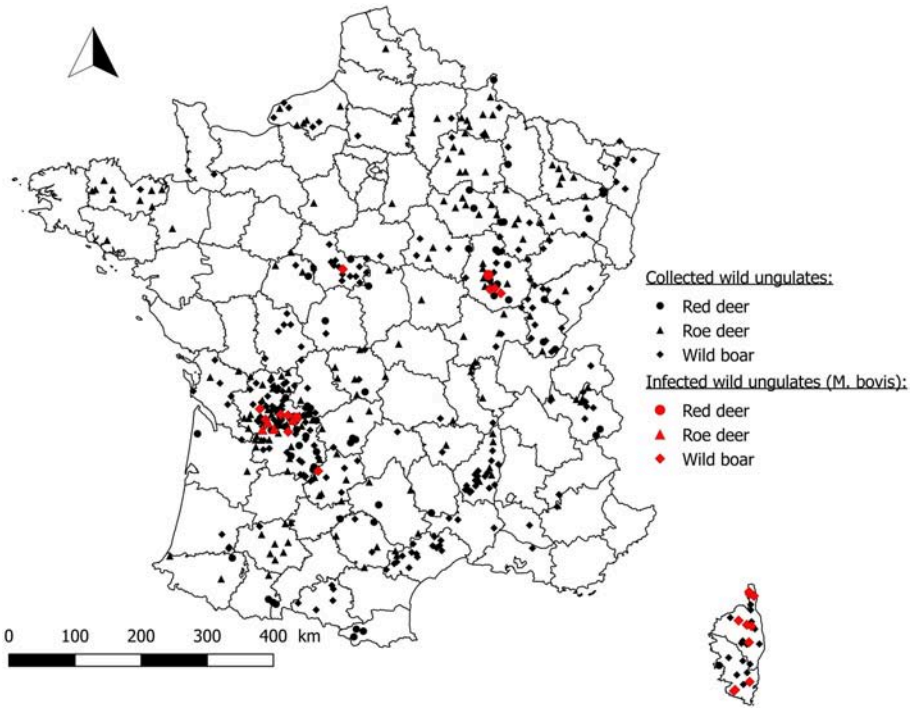

FIGURE 5 | Location of wild ungulates collected by event-based surveillance from 2011 to 2017.

Targeted surveillance

Targeted surveillance on wild boars was implemented in 11 at-risk areas with 7,634 wild boars analyzed since 2011. In total, 180 wild boars were found infected with *M. bovis* in seven at-risk areas. In at-risk areas where infection in wild boars was known, apparent prevalence rate observed with culture (from 2011 to 2015) was on average 3.04% ($n = 3{,}786$), and with PCR (2015–2017) on average 2.37% ($n = 2{,}536$) (**Supplementary Table 3**). Prevalence in the main at-risk areas was between 1.5 and 4.3% in P1, and between 0.5 and 4.4% in P2 (**Table 6**). No infected wild boar has been found in areas 6, 7, 8, and 10 (**Supplementary Table 3**). In the Brotonne-Mauny forest (area 1) surveillance in wild boars has been implemented since 2001 due to the detection of infection at high prevalence rates in deer and wild boars (20). From that time point onwards, one to five additional infected wild boars were found each year between 2011 and 2017 (among about 200 wild boars analyzed/year) (**Supplementary Table 3**). Furthermore, following the discovery of one infected wild boar in Loir-et-Cher (Sologne), 986 wild boars were analyzed from 2015 to 2017 in open forest and in 12 game parks, but no additional wild boar was found to be infected.

Infection in males and in females was similar (2.4%, $n = 2{,}947$ and 2.5%, $n = 2{,}753$, respectively) ($p = 0.80$).

Targeted surveillance on red deer was implemented in seven at-risk areas, where 1,817 red deer carcasses have been examined including 1,491 analyzed since 2011. Six red deer were found infected in area 2. Results by year and by area are detailed in **Supplementary Table 4**.

M. bovis Strains Isolated in Wildlife

In total, nine genotypes of *M. bovis* have been identified in wildlife in France (**Figure 6**). Some of these genotypes are found in different at-risk areas (SB0120 in areas 2, 3, and 6 and in the Corsica region, SB134 in areas 1, 2, and 11), but their variable number tandem repeat (VNTR) profiles are different (**Table 7**). We should note the presence of two different genotypes in two areas: SB0120 and SB0134 in area 2, and SB0120 and SB0840 in Corsica. In area 5, the two genotypes SB0821 and SB0832 are in geographic proximity, but nevertheless in different sectors.

Gross Lesions and TB Infection

Lesions in Badgers

Among 357 badgers out of 13,620 (2.6%) necropsied showed TB-like lesions. Of these, 95 were found to be infected. Furthermore, only 21.5% of the 442 infected badgers showed TB-like lesions. Lesions were mostly found in the cephalic lymph nodes (retropharyngeal and submandibular) and the pulmonary tractus (lung, bronchial and mediastinal lymph nodes). In total, eight infected badgers had TB-like lesions on at least two internal organs and at least two lymph nodes. 16.2% of infected badgers (47/291) collected by targeted surveillance showed TB-like lesions, whereas in infected badgers collected by event-based surveillance, 31.0% (22/71) showed TB-like lesions ($p = 0.04$).

Lesions in Wild Boars

In all, 526 wild boars out of 7,838 (6.7%) collected by targeted surveillance showed TB-like lesions, and 77.8% of the infected

TABLE 6 | Apparent prevalence rates in wild boars in at-risk areas of France where infection has been found in wild boars in the 2012-2013-2014 period and the 2015-2016-2017 period [percentages are given with 95% confidence intervals (CI); in brackets number of infected/analyzed animals].

No. of the at-risk area (full name of the area)	P1 (2012–2013 and 2013–2014) *Culture*	P2 (2015–2016 and 2016–2017) *PCR*
	Apparent prevalence rates [95% CI] (No. of infected wild boar/no. analyzed)	
1 (Brotonne-Mauny forest)	1.5% [0.6–3.2%] (6/401)	1.3% [0.4–2.9%] (5/394)
2 (Côte-d'Or)	3.1% [1.7–5.1%] (15/483)	2.2% [1.0–4.2%] (9/404)
3 (Dordogne/Charente/ Charente-Maritime/Haute-Vienne/Corrèze/Gironde)	4.1% [2.4–6.4%] (17/419)	2.7% [1.7–4.1%] (21/770)
4 (Dordogne/Lot)	4.3% [1.9–8.2%] (8/188)	3.2% [1.6–5.7%] (11/341)
5 (Béarn)	2.1% [0.9–4.2%] (8/373)	4.4% [2.4–7.4%] (13/295)
9 (Lot-et-Garonne)	/	4.2% [1.6–8.9%] (6/143)
11 (Ariège/Haute-Garonne)	/	0.5% [0–2.9%] (1/189)

/: Targeted surveillance on badgers not required in the area.

wild boars showed TB-like lesions. Most of the TB-like lesions were found in the submandibular lymph nodes.

Lesions in Deer

Among the eight red deer found infected since 2011, six came from targeted surveillance and two from event-based surveillance (submitted by hunters because of visible TB-like lesions). Five red deer showed gross TB-like lesions: in the pulmonary system for three red deer, in retropharyngeal lymph nodes for three, in mesenteric lymph nodes for one and in the liver for one. Gross TB lesions were also observed in infected roe deer (19).

DISCUSSION

Organization of the Surveillance System

Surveillance of TB in wildlife on a national scale in France, coordinated by the Sylvatub system, has been implemented gradually since 2011 thanks to the strong contribution of stakeholders at the national or local levels. Thanks to the involvement of hunters, event-based surveillance through detailed game carcass examination has often enabled us to detect first cases of TB in wild ungulates before targeted surveillance is deployed. It has help to detect TB as early as possible.

However, the main challenge for Sylvatub lies in the involvement of volunteer actors (hunters, trappers, pest control officers) and local veterinary services in such a complex multi-partner network. The targeted surveillance recommended in the Level 2 and 3 departments required a particularly strong involvement of local volunteers to collect tissue samples of wild ungulates and to trap badgers, explaining why some targeted plans have only been partially implemented in some areas. And these volunteers are, for the large majority, not those who are directly impacted by TB in cattle (cattle breeders) but could be affected by negative consequences if infection is found in wildlife, as for example, the implementation of density control measures of wild ungulates. Furthermore, in some departments, surveillance has been renewed for many years, which leads to a weariness of local actors. It should also be noted that the cost of Sylvatub is important for the government since it amounts to about 1 million euros per year.

Sampling and Diagnostic Protocols

For each surveillance component, sampling was opportunistic and not performed randomly: for targeted surveillance it was based on trapper and hunter activity, and for event-based surveillance on different field actor interventions. Some areas or sectors were overrepresented whereas others were underrepresented depending on the number of volunteers, the intensity of their activity and the possibility to hunt. Moreover, samples collected were not strictly homogeneous from 1 year to the next, even within the same area.

For event-based surveillance, the collection of carcasses or the reporting of animals with TB-like lesions strongly depend on the awareness of actors in the field. We can reasonably expect that this awareness is generally greater for in the departments affected by TB (level 2 and 3 departments) which constitutes a selection bias.

In addition, wildlife surveillance must deal with unknown parameters such as densities, distribution, or the social behavior of animals. Surveillance must therefore adapt as well as possible to this variability and propose reasonable, realistic and attainable objectives but prevalence estimates are certainly biased. For example, it is difficult to set badger surveillance objectives without full knowledge of their densities and distribution. In practice, the samples planned in at-risk areas therefore had to be adjusted to the size of the area. We have set the design prevalence for sample size calculations at 3%. This threshold was chosen to be able to detect a relatively low prevalence while minimizing the sample size and therefore the cost. It could have affected the ability to detect TB in areas where TB is only present at prevalence below 3%, particularly in deer where prevalence seems lower than in the others species.

The amount of time dedicated to post-mortem examinations and their thoroughness influence the detection of visible lesions (5, 21). Here, wild ungulates were not always examined in a standardized way by hunters in the field. Even though training is provided, detection of lesions suggestive of TB by hunters in the field is less sensitive than the necropsy performed in the laboratory. Nevertheless, in some laboratories, necropsy of badgers or ungulates is sometimes performed without thoroughly inspecting all lymphatic nodes and organs. In addition, for wild ungulates, only organ blocks (head, lungs and mesenteric apparatus depending on the species and year) are transmitted to the local laboratories as part of targeted surveillance. For

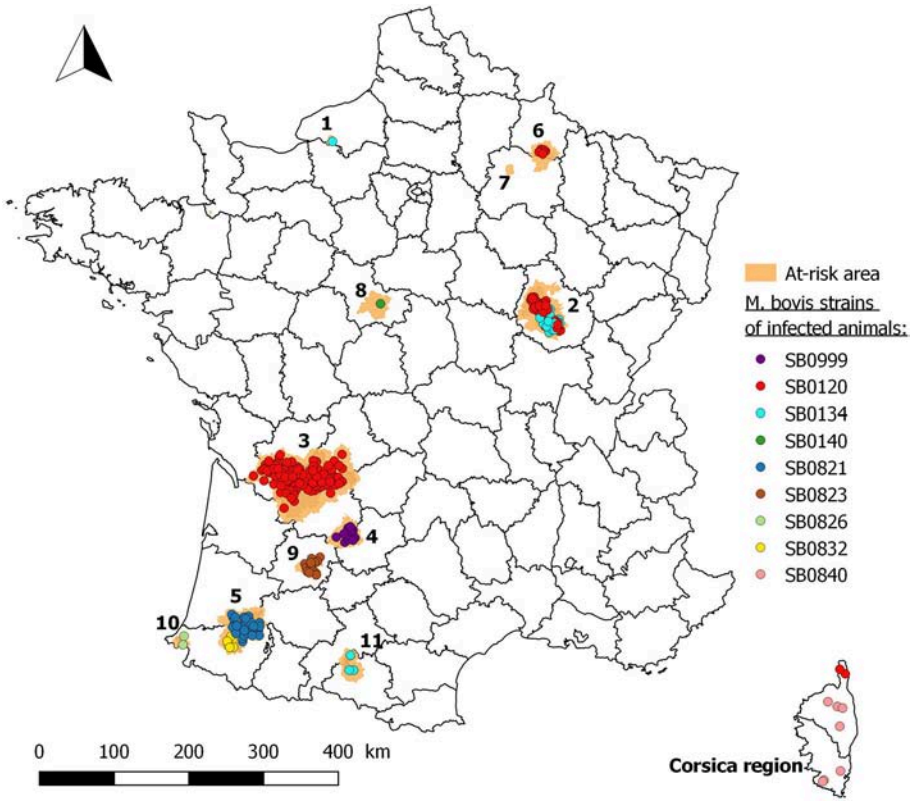

FIGURE 6 | Location of *M. bovis* strains in wildlife in France (*1*: Brotonne-Mauny forest; *2*: Côte-d'Or; *3*: Dordogne/Charente/Charente-Maritime/Haute-Vienne/Corrèze/Gironde; *4*: Dordogne/Lot; *5*: Béarn; *6*: Ardennes/Marne; *7*: Marne (Reims Mountain); *8*: Loir-et-Cher (Sologne); *9*: Lot-et-Garonne; *10*: Pays Basque; *11*: Ariège/Haute-Garonne).

all these reasons, the frequency of lesions has certainly been underestimated.

Since 2011, the protocol for sampling biological tissue and the composition of analyzed pools of tissues have changed. Before 2014, for badgers, salivary glands were sampled and mixed with lymphatic nodes. For wild boars, since 2014, only the cephalic lymph nodes have been sampled and analyzed whereas before the pulmonary lymph nodes were also analyzed. The purpose of these changes was to concentrate the tissue pools with the organs most likely to be contaminated by *M. bovis,* in order to increase diagnostic sensitivity. Unfortunately, the impact of these measures could not be assessed (22, 23).

Moreover, the type of analysis used for TB diagnosis changed from 2015: bacterial culture was used from 2011 to 2014 and then, from 2015, PCR was deployed. The year 2015 was a year of transition with the use of culture or PCR, depending on the local laboratory. Sensitivities were estimated in cattle populations by Courcoul et al. (14): on average 87.7% [82.5–92.3%] for PCR vs. 78.1% [72.9–82.8%] for culture. Culture sensitivity is probably lower when used for wild animals due to field conditions and the potential contamination and degradation of samples and because only a limited range of tissues are collected and analyzed from each wild animal. Furthermore, pooled samples are analyzed for wildlife, whereas cattle samples are analyzed separately. All together these factors lead to a decreased culture sensitivity estimated by about 30–40% and PCR sensitivity by about 10–20% (Boschiroli, personal communication) compared to those in cattle.

In order to calculate prevalence more accurately, we grouped the results into two periods: P1 (2012–2014—use of culture) and P2 (2016–2017—use of PCR), and we calculated apparent prevalence. These prevalence rates do not take into account the sensitivity of the two analytical methods. For comparison of results in badgers, the year 2012 was not taken into consideration because it was the first year of operation of the Sylvatub system with a protocol that was not always fully implemented. The year 2015 was also ruled out because of the use of both analytical methods, depending on the local laboratory.

M. bovis Infection in Wildlife

Distribution of Infected Wild Animals

All infected wild animals detected since 2012 have been found in the vicinity of cattle outbreaks (in at-risk areas up to 10 km from recent cattle outbreaks pastures), except for one wild boar found in Loir-et-Cher (Sologne), an area without known TB infection. The molecular profile of the *M. bovis* strain (SB0140; VNTR profile: 7 5 6 3 10 3 4 7) found in this wild boar was identical to that of a bovine animal slaughtered in Vendée (western France)

TABLE 7 | Genotype of *M. bovis* strains in wildlife in France.

No. of the at-risk area or region (full name of the area)	Spligotype	VNTR profile
1 (Brotonne-Mauny forest)	SB0134	7 4 5 3 10 4 5 10
2 (Côte-d'Or)	SB0120	5 5 4 1 11 4 5 6
		5 5 4 3 11 4 5 6
	SB0134	6 4 5 3 6 4 3 6
		6 5 5 3 6 4 3 6
3 (Dordogne/Charente/Charente-Maritime/Haute-Vienne/Corrèze/Gironde)	SB0120	5 3 5 3 9 4 5 6
4 (Dordogne/Lot)	SB0999	6 4 5 2 8 2 4 7
5 (Béarn)	SB0821	6 5 5 3 11 2 5s 8
	SB0832	6 5 5 3 11 2 4s 8
6 (Ardennes/Marne)	SB0120	5 3 5 6 11 4 6 8
8 (Loir-et-Cher)	SB0140	7 5 6 3 10 3 4 7
9 (Lot-et-Garonne)	SB0823	6 5 5 3 11 2 5s 6
10 (Pays Basque)	SB0826	6 6 3 3 10 2 5s 8
11 (Ariège/Haute-Garonne)	SB0134	6 5 5 3 6 4 5 6
Corsica region	SB0840	7 4 5 3 8 2 5s 4
	SB0120	4 5 5 3 11 4 5 7

in 1997. This profile is currently unknown outside of France, which appears to indicate that the infection originated within the country. However, cattle monitoring revealed no cases in herds located or grazing within 5 km of where the infected wild boar was found (844 cattle tested using the comparative intradermal tuberculin test, all negative) (24). Moreover, targeted surveillance in wild boar and red deer populations from open and closed areas (game parks) in a perimeter of 12 km around the index case has been implemented since the 2015–2016 hunting season without any infected animal having being discovered. There is no clear evidence about the origin of this case to date but it seems essential that the greatest vigilance be given to game parks and associated game movements. Infected wild boars were found in all at-risk areas except in the area of Ardennes-Marne (area 6) which is the only area where no infected wild boars were found despite 4 years of targeted surveillance.

Infected badgers were mainly found in infected areas. A few were found in buffer areas (2–7 km from recent cattle outbreaks pastures) and only five from TB-free areas surrounding buffer areas. These five infected badgers were collected by event-based surveillance very close to at-risk areas (<3.5 km), and four of these five badgers were collected in 2012 and 2013 when perimeters of at-risk areas were not well defined. The discovery of these badgers has led to strengthened surveillance in cattle and subsequent detection of bovine outbreaks. However, the absence of TB detection in TB-free areas should be interpreted with caution due to the limited sampling size. In the UK, infected badgers were also detected between 1972 and 1993 in TB-free areas. In Ireland, this prevalence was estimated to be 15% in places where TB had not been reported in cattle for 6 years (25).

All isolates obtained from infected wild animals exhibited the same genotypes that had already been found in isolates from cattle outbreaks in the same regions. In areas where two different genotypes are isolated in wildlife, we observe exactly the same two genotypes in cattle. Strains with the SB0120 or the SB0134 spoligotypes are present in cattle and wildlife in different regions in France albeit presenting different VNTR profiles (26, 27). These results highlight the epidemiological relationship between wildlife and cattle, and evidence that *M. bovis* infection spreads within a multi-species system in these areas as also observed in the UK, Ireland and Spain (28–31). The current risk is that complex reservoirs of *M. bovis* including one or more wild populations and the environment are locally constituted. Badger and wild boar are at the moment the two species most found infected (see sanitary results) and thus worrying in terms of maintenance community. But recent finding on red foxes in France (32) and in other regions of continental Europe (33) raises questions about the epidemiological role of foxes, and have motivated ongoing investigations in different endemic areas.

Location of Lesions

The location of lesions in badgers observed in France is consistent with previous studies carried out in the UK and in Ireland: the thoracic cavity (lungs and lymph nodes) and cephalic lymph nodes were the most common sites (3–5, 34, 35). As in these two countries, the presence of visible lesions only on lymph nodes was the most frequent finding.

With regard to wild boars, lesions are generally smaller and confined to the lymphatic nodes of the head (mainly submandibular lymph nodes) and are therefore less visible to hunters at the time of carcass examination. Systematic inspection of these lymph nodes in the laboratory or by a trained person is therefore essential.

The fact that the majority of infected deer have TB-like lesions and that these lesions are generally located in the pulmonary system makes it possible to suggest that surveillance by careful hunter examination and reporting observed lesions, remains a relevant and sensitive surveillance modality.

Prevalence of *M. bovis* Infection

Concerning deer, we have observed only a few *M. bovis*-positive cases since 2012 (eight red deer and five roe deer) in France, all of them from Côte-d'Or and Dordogne/Charente (areas 2 and 3) which are infected areas with large deer populations, the presence of numerous cattle-TB outbreaks, confirmed infection in badgers and wild boars. These observations suggest a more minor role of deer in the interspecific transmission of *M. bovis* in France compared to other species such as wild boars or badgers. These results are similar to those obtained in others European countries, especially in the Czech Republic, in Hungary where sporadic cases were observed in red deer or in Poland

and Italy in roe deer (36, 37). In Austria, red deer infected with *M. caprae* have been found since 1999 (38). In the UK, a large-scale study was conducted in 2007 and revealed a prevalence of 1.02% in red deer ($n = 196$) and 1.02% in roe deer ($n = 885$) (39).

The epidemiological situation of the Brotonne-Mauny forest (area 1) is special for France because of the very high prevalence level in red deer which were considered a TB maintenance host in the early 2000s (8, 9). This situation was also observed in southern Spain ($P = 27.4\%$, $n = 95$ in the Doñana National Park) (40) and in Portugal between 2009 and 2013 ($P = 38.3\%$, $n = 115$) (33).

The wild boar is considered a key maintenance host for tuberculosis in Spain with prevalence >50% in areas with high-density populations, such as in the Doñana National Park or in large hunting parks (22, 41). In Portugal, prevalence rates ranging from 6 to 46% have been observed (bacterial culture on a pool of lymph nodes) (42). Infected wild boars are commonly discovered in Germany, Italy and in several countries of Central Europe (43, 44).

In France, prevalence in wild boars is mostly lower [on average 3.04% in the 2011–2015 period (with culture) and 2.37% in the 2016–2017 period (with PCR)] than that observed in badgers in the same areas. This epidemiological situation is very different from that observed in south and central Spain where the wild boar is considered a key maintenance host for tuberculosis with prevalence >50% in areas with high-density populations, such as in the Doñana National Park or in large hunting parks (22, 41). In Portugal, prevalence rates ranging from 6 to 46% have been observed (bacterial culture on a pool of lymph nodes) (42). Infected wild boars are also commonly discovered in Germany, Italy and in several countries of Central Europe (43, 44).

Finally, outside the Sylvatub system, TB was also detected in 2012 in 7.3% of a wild boar population from a game park in the Marne, a cattle TB-free area (18). This detection raised questions on disease surveillance in captive wild animals, especially in game parks.

The prevalence observed in badgers in French infected areas was lower than that found in the bovine infection areas in the UK and Ireland. The prevalence rates estimated by culture in the UK during the randomized badgers culling trial, carried out between 1998 and 2006, varied widely (1.6–37.2%) depending on the *post-mortem* and culture methods used, with an average of 16.6% (3). In Ireland, 19.5 to 26.1% of the badgers analyzed by culture in the four study areas were found to be positive (45). A more recent study, in areas where there is high prevalence of bovine TB based on detailed *post-mortem* examination, histopathology, and culture in each specimen, reported a prevalence of 36.3% (4). Analysis of prevalence rates in ∼5,000 badgers in Ireland revealed a decrease in the overall prevalence from 26 to 11% between 2007 and 2011 (46). In parallel, it should be noted that the herd prevalence in cattle in the UK and in Ireland is higher than in France (5–6 vs. 0.04%), and that all the analyzed badgers in these countries originated from areas with the highest prevalence in cattle (47).

In the north of Spain, in the provinces of Galicia and Asturias, where TB prevalence in cattle is between 0 and 4.3% depending on the district, badger prevalence rates between 6 and 7% are observed (28), which is similar to rates observed in some areas of France.

It is not possible to draw any conclusion on the relationship between the prevalence of TB in cattle herds and in surrounding badgers population due to important methodological differences. However, it would be also interesting to follow the relationship between the trends observed in some areas in cattle associated with disease control measures and the trend in the prevalence of these wild animal populations. Although the present dataset does not allow precise time comparison between P1 and P2 due to variations of sampling patterns and method for analyses, the ongoing surveillance may contribute to constitute consistent time series that may be analyzed for that concern. Nevertheless, in Côte-d'Or (area 2), if we considered that the apparent prevalence in P1 is underestimated because of the average sensitivity of the culture compared to that of the PCR, one can hypothesize that the prevalence has decreased in badgers from P1 to P2.

In Dordogne/Charente (area 3), where both event-based and targeted surveillance in badgers have been effective, we observe that badgers collected by event-based surveillance (mainly road-killed badgers) were more infected than those trapped. This could be explained by a lower vigilance of infected badgers and therefore increased collisions with vehicles. Moreover, movements of infected badgers are higher (48) and these infected badgers occupy larger home ranges (49, 50). These elements point to a greater probability of detecting infection by collecting road-killed individuals than by trapping.

Finally, we observed a higher prevalence in male than in female badgers, which corroborates several studies (51, 52). This has been interpreted as an influence of the aggressive behavior of males for the defense of the territories, and the attendance of several social groups during the rut period, resulting in a higher rate of infection in male badgers (52).

CONCLUSION

The implementation of a wide and important surveillance system as Sylvatub relies on the involvement, at the national or departmental level, of the main organizations involved in wildlife surveillance and field volunteers without whom this surveillance would not be possible. The detection of numerous cases of TB in free-ranging wildlife occurs in areas of cattle outbreaks with the same profile of *M. bovis* strains showing evidence that, in main endemic regions of France, TB circulates between cattle and wildlife. The current risk is that complex reservoirs of *M. bovis* including one or more wild populations and the environment are locally constituted. It is also worrisome to note the increase in the number of areas in which infected badgers or wild boars are found, partly due to a better surveillance over time. However, prevalence observed in France in badgers and wild boars are lower than those observed in the UK and in Ireland for badgers or in Spain for wild boars, and only sporadic cases have been detected in red deer and roe deer.

Wildlife surveillance contributes to the implementation of control strategies in wildlife and in cattle by allowing defining at-risk areas. It also allows adapting surveillance in cattle keeping in mind the multi-host aspect of the disease as well as targeting prevention actions and to follow their long-term efficiency. This information, complemented by scientific investigations and researches, are needed for conducting biosecurity measures in wildlife (control of artificial feeding, management of hunting waste, banning game release, wildlife populations reduction in highly infected TB areas), and in livestock (management of water points, protection of the food and barns for example). Convinced that wildlife can be an additional local factor of TB spread and maintenance, the French ministry in charge of agriculture has decided to make Sylvatub sustainable.

In parallel, evolvement of sampling strategies have been discussed in the aim to improve surveillance. In buffer areas, targeted surveillance will be replaced by *M bovis* detection in road-killed badgers. Another main development will concern wild boar in at-risk areas: serological testing for the detection of antibodies directed against *M. bovis* have been proposed as suitable tools for TB screening in wild boar populations (18, 53). ELISA method has been tested in comParison to PCR and culture in different areas in France with encouraging results (Richomme and Boschiroli, personal communication). It will be used as an alternative method to monitor the *M. bovis* exposure level in wild boars in the next years.

ETHICS STATEMENT

Samples were collected from animals trapped with appropriate permits, hunted legally during the hunting season with appropriate permits, shot legally because of severe debilitation, or found dead. All the samples included in this study were obtained from animals analyzed within an official context relating to TB surveillance in free-ranging wildlife. All sampling procedures complied with national and European regulations, and no specific ethics approval was therefore required.

AUTHOR CONTRIBUTIONS

JH, AF, ÉR, CR, M-LB, ÉF, LC, FC, IT, SP, and PH: Sylvatub conception and monitoring; ÉR: Data analysis; ÉR, M-LB, CR, and SD: Paper writing; CR, M-LB, SD, JH, ÉF, AF, LC, PJ, IT, SP, PH, and FC: Manuscript critical revision.

FUNDING

Samples collection and analysis were funded by the DGAL.

ACKNOWLEDGMENTS

The authors would like to thank the local state veterinary services, the local hunting federations, the local services of the National Game Hunting and Wildlife Agency, the local laboratories for analysis, the local Animal Health Defense Associations, and Julie Rivière, the first coordinator of the Sylvatub system from 2011 to 2012. We are grateful to all the trappers and their associations, Pest control officers and their associations and hunters of wild ungulates without whom the surveillance could not have taken place. Thank to Craig Stevens for his very useful comments on this article and his careful reading.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00262/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 12 November 2018
doi: 10.3389/fvets.2018.00277

Roll-Back Eradication of Bovine Tuberculosis (TB) From Wildlife in New Zealand: Concepts, Evolving Approaches, and Progress

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The New Zealand government and agricultural industries recently jointly adopted the goal of nationally eradicating bovine tuberculosis (TB) from livestock and wildlife reservoirs by 2055. Only Australia has eradicated TB from a wildlife maintenance host. Elsewhere the disease is often self-sustaining in a variety of wildlife hosts, usually making eradication an intractable problem. The New Zealand strategy for eradicating TB from wildlife is based on quantitative assessment using a Bayesian "Proof of Freedom" framework. This is used to assess the probability that TB has been locally eradicated from a given area. Here we describe the framework (the concepts, methods and tools used to assess TB freedom and how they are being applied and updated). We then summarize recent decision theory research aimed at optimizing the balance between the risk of falsely declaring areas free and the risk of overspending on disease management when the disease is already locally extinct. We explore potential new approaches for further optimizing the allocation of management resources, especially for places where existing methods are impractical or expensive, including using livestock as sentinels. We also describe how the progressive roll-back of locally eradicated areas scales up operationally and quantitatively to achieve and confirm eradication success over the entire country. Lastly, we review the progress made since the framework was first formally adopted in 2011. We conclude that eradication of TB from New Zealand is feasible, and that we are well on the way to achieving this outcome.

Keywords: bovine tuberculosis, eradication, TB, possums, disease freedom, wildlife surveillance

OPEN ACCESS

Edited by:
Daniel J. O'Brien, Michigan Department of Natural Resources, United States

Reviewed by:
Simon More, University College Dublin, Ireland
Graham John Hickling, University of Tennessee, Knoxville, United States

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *10 August 2018*
Accepted: *18 October 2018*
Published: *12 November 2018*

Citation:
Nugent G, Gormley AM, Anderson DP and Crews K (2018) Roll-Back Eradication of Bovine Tuberculosis (TB) From Wildlife in New Zealand: Concepts, Evolving Approaches, and Progress. Front. Vet. Sci. 5:277. doi: 10.3389/fvets.2018.00277

INTRODUCTION

In 2016 the New Zealand government and agricultural industries jointly adopted the ambitious goal of nationally eradicating bovine tuberculosis (TB) from livestock and from all wildlife reservoirs by 2055 (1, 2). *Mycobacterium bovis*, the cause of TB, undoubtedly first arrived in New Zealand with imported cattle in the 1800s (3). By the mid-1900s it had spread into wildlife, and the disease became widely established in a highly susceptible and ubiquitous maintenance host, the introduced brushtail possum (*Trichosurus vulpecula*) (4), from which it often spills over to a number of other wildlife hosts, including feral pigs and wild deer (5) and feral ferrets (6).

Although diagnostic testing and removal of test-positive animals, coupled with slaughterhouse carcass inspection and livestock movement control to prevent further outbreaks, has reduced TB levels in livestock in many developed countries (7), the disease has been difficult to fully eradicate, especially in countries where TB is also independently cycling in wildlife reservoirs, such as badgers in Great Britain, wild boar and red deer in Spain, African buffalo and other species in South Africa, cervids (white tailed deer and elk) in North America, and brushtail possums in New Zealand (8). An exception is the successful eradication of TB from introduced water buffalo in Australia, where the "wildlife host" was a semi-domesticated or feral bovid with much the same TB epidemiological dynamics as cattle (9).

The main wildlife host in New Zealand (the brushtail possum) is very different from cattle: it is a small, nocturnal, and predominantly arboreal marsupial that is widespread and can occur at high densities (>20/ha) (4). Although it is a comparatively rapidly fatal disease for individuals, high-density possum populations can independently maintain TB (10) and can readily transmit TB to cattle (11). As a result of TB becoming widespread in possums in some parts of New Zealand in the 1970s, management of TB in New Zealand since then has therefore necessarily involved not only conventional management of the disease in livestock (12) but also efforts to break the TB cycle in possums though severe reductions in local possum density ("control") (3). In this review we first very briefly summarize the c. 50-year history of TB management in New Zealand since it became both a livestock and wildlife problem, and then describe the key concepts and tools that have recently been developed to help achieve and confirm the new (2016) goal of national TB eradication.

We then focus more specifically on the concept of roll-back eradication. TB is established in wildlife in four main areas of New Zealand, which in total covered about 40% of the country in 2011. As the name implies, roll-back eradication entails locally eradicating TB from wildlife at the fringes of those four main areas and, over time, shrinking the size of each area from the outside in.

The key tool underpinning this concept is a Bayesian "Proof of Freedom" (PoF) framework, which is used to quantify the probability that TB is absent from possums in a specific area (P_{free}). When that probability is considered high enough, an area is declared free of TB in wildlife and active management of TB in wildlife there ceases, with the management resources redirected to other areas where possums (and other wildlife) are still likely to be infected.

The PoF framework utilizes a number of information streams, including assessments of how effective efforts to reduce (control) possum densities have been, and infection surveillance data, not only from possums themselves but also from other TB hosts that can be infected by possums. We describe the background to the PoF framework (the concept of combining theoretical prediction of P_{free} with empirical TB-possum surveillance data), and how it was first implemented in 2011. We then summarize recent innovations, as follows:

i. Simultaneous use of possum control efficacy data as well as TB surveillance data for updating the prior probability of freedom (13)
ii. Use of livestock as additional sources of data (sentinels) for detecting TB in wildlife (14)
iii. Use of decision theory to determine the optimal "stopping threshold" probability for declaring a particular local area free of TB (15)
iv. A description of how the progressive roll-back based on local areas can be scaled up to eventually confirm eradication success over the entire country (16).

Lastly, we review actual roll-back progress since 2011, and assess the likely accuracy of the P_{free} estimates given the lack (thus far) of any "post-freedom" failures (i.e., local re-emergence of TB in wildlife).

The review is based largely on the published work of the authors and our colleagues within Manaaki Whenua—Landcare Research, and builds on the comprehensive set of reviews about the epidemiology and management of TB in New Zealand wildlife in a 2015 special issue of the *New Zealand Veterinary Journal*. However, we also cite four reports documenting research that has not yet been published; these are available online via the DOIs appended to their citations.

We use "eradication" to refer to the complete or absolute absence of *M. bovis* from New Zealand livestock and wildlife, with negligible chance of re-invasion (except perhaps in human immigrants). Declaration of national eradication will signal the end of the programme. The term "TB freedom" is used in this paper specifically to denote a lesser but still high level of confidence that *M. bovis* is actually absent from wildlife in a given local area, either because wildlife there were never infected or because the disease has been eradicated. An area designated as free of TB can contain infected livestock if that infection is known to have not been caused by wildlife. The declarations of local-area freedom in wildlife therefore differ conceptually from the international standard for declaring national TB freedom in bovids and cervids, which explicitly permits a low level of continued infection in livestock (17). We also note that, for convenience, the term "disease" is used throughout this paper to encompass the presence of subclinical *M. bovis* infection as well as the presence of actual symptoms of disease (**Appendix**).

MANAGEMENT OF TB IN NEW ZEALAND

Since about 1995, management of TB in New Zealand has been conducted by a non-government agency (OSPRI, formerly TBFreeNZ, and even earlier the Animal Health Board). OSPRI represents a public–private partnership between government and the agricultural industries, and is responsible for implementing a formal National Pest Management Plan (NPMP) for TB (18). The initial NPMP in the mid-1990s aimed simply to try to prevent TB spreading further in wildlife. Then, in revisions in 2004 and 2011, it adopted more ambitious goals of not only reducing TB levels in livestock but also locally eradicating TB from possums and other wildlife (18, 19). By 2016 the national cattle herd TB annual period prevalence had been reduced to 0.09% (20), below

the 0.2% threshold stipulated by the OIE (17) for declarations of whole-country TB freedom.

That success led to a fourth iteration of the NPMP, which adopted not only an ultimate goal of national eradication by 2055, but also intermediate goals of disease elimination from farmed livestock by 2026, and TB freedom in wildlife by 2040 (21). The long, 39-year timeline to eradication reflects the immensity of the problem: by 2004 TB was believed to be potentially established in wildlife in 10.5 million ha of New Zealand (c. 40% of the country), which encompassed not only farmed areas but also large tracts of remote, mountainous, and/or heavily forested lands, often occupied by high densities of possums (3). The scale of the problem was such that it was never economically feasible to immediately apply possum control over the whole of the affected area, so the eradication campaign has been, of necessity, centred on progressive reduction or "roll-back" of the areas thought to contain infected wildlife, termed vector risk areas (VRAs).

THE CONCEPT OF ROLL-BACK ERADICATION

The progressive roll-back concept is based on local TB management units within the VRAs, called vector control zones (VCZs), of which there were about 700 in 2011, with a typical size of 10,000–15,000 ha (but ranging from <1,000 ha to one of over 100,000 ha). The history of possum population control, livestock surveillance (herd test-and-cull and slaughterhouse inspection), and wildlife TB surveillance (necropsy) is recorded for each VCZ, and after 5–20 years of management an effort is made to quantitatively assess the probabilities that both livestock and wildlife are free of TB. When those probabilities are considered high enough, the VCZ is declared free of TB, and most of the management resources (funding) for that VCZ are then shifted to still-possibly-infected VCZs.

The broad theory and concepts underpinning this local PoF approach for wildlife are described in detail by Anderson et al. (22), but, briefly, are as follows.

- The effectiveness of possum control is assessed by field monitoring of possum relative abundance (or by inference from the known typical effectiveness of the control techniques)
- A spatially explicit model of TB dynamics in possums (23). is then used to predict the probability that TB could still be present given that level of control.
- Using Bayesian logic, this "prior" probability is then updated with empirical TB surveillance data. These data are based on necropsies of possums, or of spill-over hosts (such as pigs) that act as sentinels of TB in possums, to calculate a "posterior" probability of TB freedom P_{free} (22); that is, the probability of TB freedom given negative surveillance.
- Decisions on whether or not to declare the area free are then based on the estimated posterior probability.

The approach was first developed and used formally in 2011, and 174 VCZs totalling 2.05 million ha were declared free using this process in the subsequent 7 years (Crews, OSPRI, unpubl. data).

THE INITIAL (2011) POF FRAMEWORK FOR POSSUMS

The TB Freedom Concept

The original concept underpinning the PoF framework for roll-back eradication (24) was simply that local management units (i.e., VCZs) can be quantitatively declared free of TB in possums (i.e., at some arbitrarily specified minimum level of confidence, usually, thus far, 95%) if:

(i) There is sufficient theoretical evidence (prediction) indicating that enough control has been applied to break the TB cycle in possums
(ii) This prediction was backed up by empirical field surveillance data indicating a low probability of continued TB presence in possums.

For this, Bayes' rule was formulated as:

$$P_{free} = \frac{Prior}{1 - (SS\,(1 - Prior))}$$

where P_{free} is the estimate of the "posterior" P_{free} required for decision-making, Prior is the measure of belief that an area is free of TB in wildlife based on historical control effort, and SS is a measure of surveillance sensitivity (formally defined below) describing how much effort has been made to find TB in possums without success. This simplified version of Bayes theorem assumes perfect specificity; i.e., surveillance is always negative when TB is not present in possums.

In operational terms, this usually translated into conducting intensive possum control for at least 5 (and often 10 or more) years and then implementing 2–3 years of field surveillance in an effort to detect any remaining TB in the residual possum population (25).

Theoretical Prediction of TB Freedom Based on Control Effectiveness

Because VCZs vary greatly in topography, habitat, possum density and TB history, the number of years of control (duration) and efficacy of control (percentage reduction in possum density) result in wide variation in control histories between VCZs, which is amplified by frequent changes in funding priorities. Prediction of whether a given control history is likely to have succeeded in eradicating TB is based on early modeling indicating TB has very little chance of persisting in possum populations that reduced to well below 40% of carrying capacity for 10–15 years (26). This was subsequently supported by field data (11), and a spatially explicit individual-based version of the Barlow model (the "SPM") (23).

The SPM includes parameters representing both possum population dynamics (e.g., birth rates, mortality, density dependence, dispersal) and the epidemiological dynamics of TB in possums (e.g., transmission rates, TB-induced mortality). It is used within the PoF framework to simulate the effect of population control on reducing TB prevalence (23). To initialize these simulations, TB managers summarize the "control history" for the VCZ of interest, using (as far as possible) field measurements of the relative abundance of possums, most

commonly a standardized index of trapping success (25). For unmonitored control operations, conservative estimates of control efficacy are assumed based on monitored outcomes at similarly managed sites. At least 100 simulations of the control history are then run with the SPM, with the prevalence of TB 30 years before the first control operation usually assumed to be 2.5% (based on the 2–5% prevalence typically recorded in unmanaged long-infected possum populations (4). The proportion of simulations in which TB is predicted to disappear is then used as a Bayesian "prior" P_{free} at the end of the series of control operations.

When the PoF framework was initially implemented, many of the VCZs being assessed had been under some form of possum control for more than two decades due to the strategic goal of the previous NPMP being one of ongoing TB suppression rather than eradication. When those long control histories were simulated in the SPM, the model would often predict eradication in every simulation (i.e., $P_{free} = 1.0$). As the predicted P_{free} exceeded the desired >95% minimum level of confidence, such VCZs could have been declared free on the basis of the model predictions alone, but TB managers required additional supporting empirical data from surveillance.

Requirement for Empirical Possum-TB Surveillance

The reason TB managers required additional information is that there is uncertainty about the accuracy of the SPM predictions. Not all SPM parameters have been formally validated, so it was accepted that some were likely to be wrong; for example, it was originally assumed that infected possums lived for about a year after becoming infected (23), but recent evidence indicates a much shorter duration of infection (27). Further, the accuracy of the control histories is often suspect as a result of data gaps. It was therefore decided by OSPRI that, as an operating principle, declarations of freedom would always require a minimum level of empirical post-control surveillance. To achieve this, a default maximum-permissible prior P_{free} of 0.9 was prescribed; in other words, if the SPM predicted (based on simulations of the duration and intensity of historical possum control) a prior of >0.90, it would be reduced to 0.90. In addition, at that time a posterior P_{free} of 0.95 was prescribed as the desired threshold ("stopping rule") for declaring a VCZ free of possum TB. The gap between the maximum-permissible prior (≤0.90) and the stopping rule (0.95) meant that some surveillance was always needed.

The empirical TB surveillance required under this operating principle is obtained through necropsy surveys of possums or sentinel species. The surveys aim to quantify the surveillance sensitivity (SS), or the probability of detecting a TB-positive animal if the disease were actually present in a specified number of possums [the design prevalence, P*; (28)]. In principle, P* should be set at one possum if the goal is confirming TB absence at the time of the survey. If the prior P_{free} is predicted to be at (or above) the maximum permitted level (0.95), and $P^* = 1$, then 53% of the possum populations would need to be tested (with perfect test sensitivity) to increase the posterior P_{free} to the 0.95 stopping rule for declaring local Tb freedom. More pragmatically P* is now routinely set at 2, on the assumption that possum densities in the surveillance phase will almost always be well below the disease maintenance threshold, so TB is much more likely to die out rather than persist. That reduces the amount of field surveillance required by about 40%.

Once surveillance has been completed (and assuming no TB has been found in possums), the SPM-predicted prior P_{free} is updated annually using the surveillance sensitivity data obtained that year. If the posterior P_{free} exceeds the 0.95 stopping rule, the VCZ can be declared free of TB. If not, further surveillance is usually undertaken. However, in recognition that both the prior and the SS estimates are based on assumptions that may not all be valid, other qualitative factors (such as historical levels of infections, infection in neighboring VCZs, ease of remedying false declaration) are taken into consideration.

Possum-TB Surveillance in Practice—Alternative Sampling Units

(i) Possums as the sampling unit: The amount of surveillance required under the maximum-prior and stopping-rule settings above is large, usually equating to the equivalent of necropsying at least a third of the residual low-density possum population. Surveys of TB prevalence in possums had traditionally been conducted by capturing possums in leg-hold traps set for three or more nights, necropsying them, and conducting mycobacterial culture of tissues most likely to be infected, an approach believed to detect TB in about 95% of infected possums (29). Given negative surveillance (no TB detected), the SS could in theory then be calculated as a joint function of diagnostic test sensitivity and the proportion of the population sampled. However, the latter requires a precise estimate of local possum population size, which would be prohibitively expensive to routinely obtain. In addition, because surveys are usually conducted when possum densities are very low, much of the trapping effort results in empty traps. Such empty traps would not contribute to a conventional SS calculation based on number of possums necropsied, but failure to capture a possum at a particular site indicates a high probability that possums (and therefore TB) are absent from that site.

(ii) Traps and detection devices as the sampling unit: To circumvent the problem of not knowing possum population size, and to make use of the information provided by empty traps, a novel spatially explicit data-modeling approach to disease surveillance was developed (22), in which a VCZ is divided into 1 ha grid cells, and the cell rather than the individual possum is used as the sampling unit. Using data from all set traps (empty and captures), and estimated parameters for other studies on possum home range size and probabilities of trapping, this method estimates a VCZ-level SS (22).

To describe how this is done, assume that a trap is placed within the home range of a TB-infected possum, and that if that infected possum is captured it is necropsied and tested for TB. The probability of detecting TB given that TB is present (SS) is the product of (1) the probability of trapping the infected possum, and (2) the probability that the diagnostic test (mycobacterial culture) returns a positive result. By considering the trapping and

diagnostics as two independent "tests" conducted in series, this allows us to include traps that do not capture possums (22, 30); i.e., the product can be applied to the trap whether or not it captures a possum, provided a diagnostic test is always performed whenever a possum is captured. This spatially explicit approach to estimating SS readily accounts for non-random sampling (so it does not require representative sampling).

A further extension of the ability to use empty traps (rather than possums) as sampling units involves the use of detection devices to reduce the trapping effort required. The detection devices [peanut-butter-lured chewcards (31)] are far lighter and easier to deploy than traps, and do not need to be checked daily, so they are used to cheaply identify the few small areas where possums are still present. Traps are then deployed only at those positive detection sites, and all possums captured are necropsied and tested for TB. The probability of detecting TB in this system (given TB presence) is the serial product of the probability of detection, the probability of capturing a possum in traps set at detection sites, and the probability of a positive diagnostic test. Although deploying traps only at detection sites results in a lower SS than if traps were deployed everywhere, the much lower cost of deploying chewcards and trapping only at detection sites makes this approach more cost effective, but still only affordable in readily accessible areas.

(iii) Spill-over hosts as the sampling unit: The high cost of direct possum surveillance led to the use of other spill-over host species as sentinels for TB presence in possums (32). By making data-based assumptions about sentinel home range size and the probability of a sentinel becoming infected when its home range overlaps with that of an infected possum, the surveillance sensitivity provided by these sentinels can also be estimated in a similarly spatially explicit way (22). Pigs, in particular, are highly sensitive sentinels because they very readily become infected in the presence of infected possums (33), have homes ranges that are much larger than those of possums (34), and survive in an infected state for far longer than possums (35). So where pigs can be readily obtained, surveying pigs can sometimes provide much cheaper possum-TB surveillance than would surveying possums themselves.

RECENT INNOVATIONS

Combining Surveillance and Final Control

One shortcoming of the sequential "control-then-survey" approach outlined above is that it is only affordable in easily accessible farmland. There are many less accessible areas within VRAs where ground-based control and subsequent surveillance would be prohibitively expensive. Aerial poisoning provides an affordable alternative to ground control of possums in these areas (25), and sentinel pigs can sometimes provide the required level of surveillance at an affordable cost, but there are many areas where they do not.

A new approach for such difficult areas partially reverses the control-then-survey paradigm by conducting surveillance in conjunction with a final aerial control operation (13). That final operation will have been preceded by one or more earlier aerial poisoning operations, so the prior P_{free} (as predicted by the SPM) will already be high at the time of the final operation. A low level of direct possum TB surveillance is undertaken within a mark-recapture framework, involving trapping and marking (radio-collaring) and releasing possums just before the control operation and then, after the aerial poisoning, recapturing possums by searching for, recovering, and necropsying the killed possums.

Provided no TB is detected, the likelihood of no TB being detected in the survey for each possible number of TB possums in the population (i.e., 0, 1, 2, 3,…, up to N: the pre-control population size) is calculated (**Figure 1A**). The efficacy of the control operation is determined from the percentage of radio-collared possums killed, and from that the probability that at least one TB possum would have survived if 0, 1, 2, 3,…, N infected possums were actually present (**Figure 1B**). The two probability distributions are then combined to estimate the probability that any infected possum could have survived undetected for each possible prevalence value (**Figure 1C**). Despite never knowing the number of TB possums in the population before surveillance and final control, we can use the maximum of the curve in **Figure 1C**, which corresponds to the worst-case scenario. The inverse of this can be further combined with the prior P_{free} to calculate the posterior P_{free}.

The concept was successfully demonstrated in the Hauhungaroa Range in 2016/17 (13). This c. 80,000 ha area historically had some of the highest recorded levels of TB infection in wildlife, with almost all pigs and at least a third of the wild deer infected in the 1990s (4, 5). By 2016 all parts of the area had been under intensive control for 10–22 years, and the estimated P_{free} was 0.9. About 7% of the possum population (N = c. 4000) was necropsied, with no TB detected. Control efficacy was extremely high, with 99.6% of 241 radio-collared possums killed, resulting in a <4% probability that any infected possum would have survived undetected, which when combined with the prior $P_{free} = 0.9$ results in a posterior $P_{free} > 0.99$ (**Figure 1C**).

The main advantage of this approach is the greatly reduced amount of surveillance needed, although that is partially offset by the need to obtain precise estimates of control efficacy (% kill) and the proportion of the population sampled. The other main advantage is that it enables faster declarations of freedom.

Balancing Control and Surveillance Effort and Optimizing the Stopping Rule

The total costs of possum control and possum-TB surveillance depend on a number of factors (such as possum carrying capacity, ease of access, etc.), most of which have wide cost ranges. We modelled and compared management options to demonstrate that the optimal balance between the two activities necessary to achieve and verify eradication of TB from New Zealand wildlife varied greatly between VCZs (36). This work provided managers with a simple cost- and risk-evaluation framework they could use to identify the most expedient and economical ways of achieving

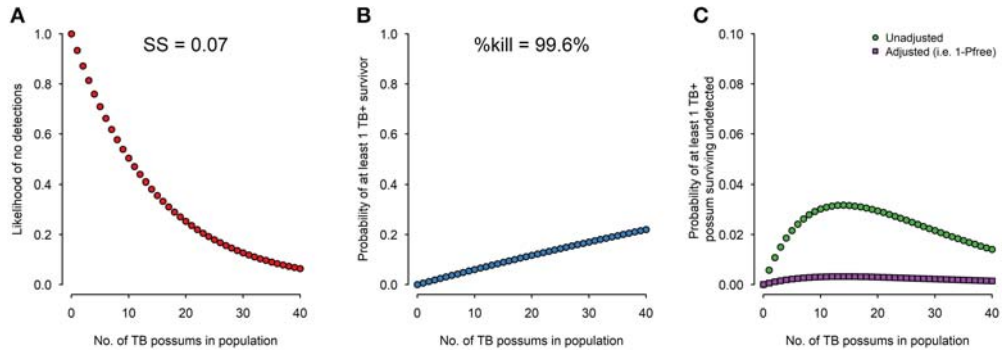

FIGURE 1 | For the range of 0–40 TB possums in the population before surveillance and final control, **(A)** surveillance sensitivity (the likelihood of no detections) from a survey of 7% of the population with 95% diagnostic sensitivity; **(B)** TB survival probability (probability of at least one TB+ survivor) given a recorded control efficacy (% kill) of 99.6%; and **(C)** the probability of at least one TB possum surviving undetected which is shown both unadjusted (i.e., based solely on the evidence from the 2016/17 operation) and adjusted by the prior probability of freedom derived from the history of previous control, and is the complement of P_{free}.

and quantitatively verifying TB eradication from possums in a particular VCZ.

The initial stopping rule (posterior $P_{free} > 0.95$) was chosen subjectively by TB managers and their stakeholders (e.g., farming organizations, governmental funding bodies) to represent what they considered to be an "acceptable" level of risk of disease persistence. Our recent decision-theory modeling (15) indicates how the choice of stopping rule could be better optimized for each VCZ by explicitly including costs of surveillance and potential re-control costs.

If the posterior P_{free} are accurate, and if all VCZs are declared free as soon as they reach 0.95, it follows that 5% of VCZs will be falsely declared free of TB. TB managers therefore expect that in up to 5% of declared-free VCZs, TB will re-emerge in possums after possum control ceases, but will possibly not be detected for many years: where that occurs, potentially expensive re-control will obviously be required.

A higher stopping rule will result in a lower *expected cost* of re-control (the actual cost of re-control multiplied by the probability of incurring that cost). However, the cost of surveillance to achieve that higher target will increase. Conversely a lower stopping rule will result in a higher expected cost of re-control (due to an increased chance of incurring the actual re-control cost), but a lower surveillance cost due to stopping earlier. The optimal stopping rule for a VCZ will be the one that minimizes the total expected cost (expected costs of surveillance and re-control combined).

Our analysis of the total expected costs indicates that where surveillance is relatively expensive compared with re-control, it will usually be more cost-effective to stop earlier than 0.95 at an increased risk of incorrect declaration [**Figure 2A**; (15)]. Conversely, where re-control is much more expensive than surveillance, it should be better to carry out more surveillance and choose a stopping threshold that is higher than 0.95 in order to mitigate the risk of incurring expensive re-control (**Figure 2B**).

This analysis has been used to develop a decision-support framework that provides guidance on how to optimize the economics of TB eradication, with the aim of eliminating the inefficiencies arising from relying on a single, predetermined, arbitrary stopping rule. Further work is now underway to see how best to include socio-political costs (the loss of credibility associated with incorrectly declaring an area free of TB), and therefore the risk profiles of decision-makers (risk averse vs. risk takers).

Livestock as Sentinels

Having expanded TB-possum surveillance options from surveying possums themselves to using data from traps and detection devices, and/or using spill-over hosts as possum-TB sentinels, we next explored the option of also using livestock as sentinels. Livestock are tested annually within all VCZs (and at longer intervals in areas designated as being free of TB in wildlife), and all livestock sent to slaughter are subject to rigorous inspection. The primary purpose of this testing and inspection is to determine TB levels in the livestock, but the same data can be used (at very little extra cost) to assess the likelihood TB is present in sympatric possums.

This might, at first sight, seem problematic because cattle are themselves maintenance hosts, so the occurrence of TB in a herd could be caused by recrudescence of latent in-herd infection or transfer of infection between herds by livestock movement rather than by transmission from wildlife. Identifying between-farms movement of livestock as the cause of a new outbreak in livestock (and therefore ruling out wildlife as the source) is facilitated by New Zealand's National Animal Identification and Tracing system, which is also managed by OSPRI. If, however, there is no detection of TB in livestock within a VCZ for many years, that obviously indicates there is no transmission from any source, including from possums.

We therefore developed an analytical technique to objectively use livestock as sentinels for TB in possums as an additional source of possum-TB surveillance information. For this, the spatially explicit modeling approach used to estimate SS from point-source data [i.e., the known kill locations of wildlife sentinels; (22)] was adapted to take into account the fact that the location of an individual cow or deer (and therefore the negative

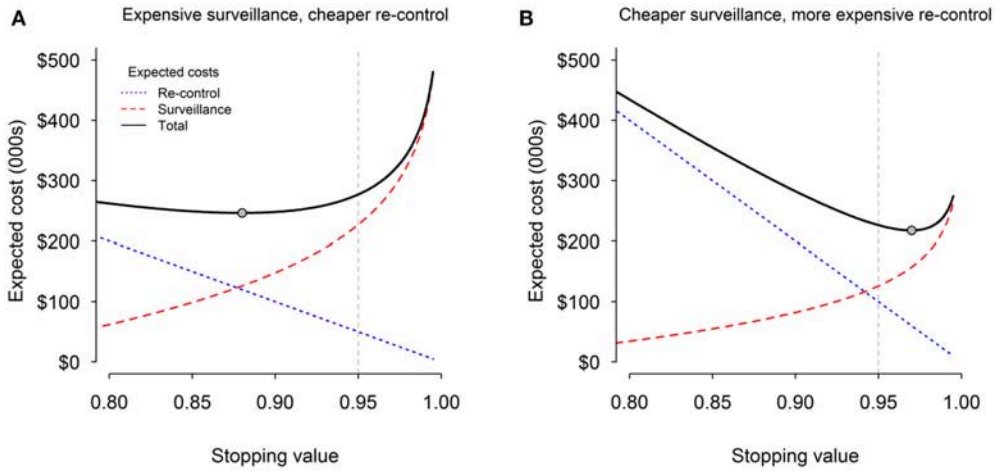

FIGURE 2 | Cost of surveillance (red dashed line), expected cost of re-control (blue dotted line), and total combined expected cost (black solid line) for **(A)** expensive surveillance and cheaper re-control; and **(B)** cheaper surveillance and expensive re-control. The gray circles indicate the point associated with the minimum total expected cost. The vertical dashed gray line indicates the current default stopping threshold of 0.95.

result of any TB test or slaughterhouse inspection) could not be localized to a single point. Instead, the surveillance data for the herd as a whole have to be spread evenly over the entire area in which they were grazed (14).

Because the probability of an individual cow becoming infected by a single infected possum somewhere on the same farm is believed to be very low (24), the SS provided by testing or inspection of a single cow is inevitably low. However, that poor individual sensitivity is offset by a large amount of livestock testing and slaughterhouse inspection data, available at very little cost because livestock are intensively surveyed annually within VRAs to confirm (or not) that the herds themselves remain free of TB (12). Thus, ongoing negative surveillance outcomes from livestock surveillance provide very-low-cost surveillance of TB in possums, reducing the amount of wildlife surveillance required.

Although not yet implemented, we envisage that in VCZs where herds were clear of TB before the end of the control phase, the livestock data will also be taken into account in identifying the prior P_{free}. In addition, we believe the use of livestock as possum-TB sentinels will provide a crucial low-cost form of "post-freedom assurance surveillance," particularly for on- and near-farm areas. The aim of such assurance surveillance is to provide the earliest possible detection of local eradication failure (i.e., persistence and re-emergence of TB in possums). It typically relies on passive (unfunded) rather than active (planned and funded) surveys. A key point is if TB does re-emerge, the numbers of infected possums will progressively increase over time, which will substantially increase the sensitivity of livestock surveillance in detecting the presence of TB.

Scaling Up From VCZs to National Eradication

To date, the roll-back eradication process has focused on achieving and declaring TB freedom at the VCZ level. This is done in a spatially strategic way to minimise the risk of reinvasion into VCZs previously declared free of TB. It may be tempting to simplistically conclude that the entire country will be free of TB once all VCZs have been declared free in this way. However, the declarations are probabilistic rather than certain. Given the 0.95 stopping rule used, there is a probability of up to 0.05 that the VCZ declared free was still infected. This error rate is compounded across all c. 800 VCZs so that the overall probability of total eradication from the country will be very close to zero (e.g., $0.95^{800} \approx 0$). This is not a bad result, because the bioeconomic optimisation modeling indicates that it is economically sensible to take some risks and be prepared to fail in some of the VCZs and have to re-initiate control and surveillance in them (**Figure 2**).

To account for this failure rate across VCZs in the context of the goal of declaring eradication from the entire country, the operational and decision processes can be divided into two stages (16). Stage I ("achieving freedom") covers the initial efforts to eliminate TB from a given VCZ and the operational decision to declare that VCZ free of possum TB. Stage II (the "assurance" phase) requires (as noted above) ongoing but very-low-cost surveillance to either (i) quickly detect TB in cases where the declaration of freedom was false, or (ii) provide broad-scale SS data that can be used to calculate a probability of eradication at the level of whole regions, whole islands, or the whole country.

As outlined above, continued TB testing of livestock and slaughterhouse inspection is likely to provide such quantifiable assurance surveillance in on- and near-farm areas. Away from farmland there is currently mostly only limited passive and unquantified surveillance provided by recreational or commercial hunters, who might notice and report infection in any grossly infected pig or deer or ferret they kill, so consideration may need to be given to encouraging and quantifying the sensitivity of this kind of surveillance (or to funding low-intensity surveillance of sentinels and possums in high-risk areas with limited or no such passive surveillance).

Once all VCZs in a region, an island or the nation have been declared free, and TB is no longer being detected in them, the Stage II surveillance sensitivities across all VCZs will be aggregated to calculate a whole-area probability of eradication. Only when that exceeds some very high threshold (e.g., 0.99) will we be able to confidently declare that TB has been eradicated from New Zealand.

PROGRESS TOWARD ERADICATION

In the 7 years since the PoF framework was first formally adopted, 174 VCZs have been declared free, with all but 15 of those declarations based at least partly on the estimated posterior P_{free} (OSPRI, unpubl. data). A majority of these are farmed areas, and given the roll-back approach, most are at the former fringes of the VRAs where TB was generally not as well established in possums as in more central parts of VRAs. Nonetheless, the total does include several of the worst-affected forest areas in which TB was long established in wildlife at high levels, including the Hauhungaroa Range mentioned above. In total, over 2.05 million ha has now been declared free using the PoF framework, about 20% of the total area designated VRA in 2011.

By 2018 these 174 VCZs had been free for an average of 3.8 years, equating to 694 years of VCZ freedom. If TB was still present in a declared-free VCZ, we expect that it would re-emerge and be detected (on average) within 4–5 years of being declared free (at least where high numbers of cattle are TB tested and/or slaughtered annually). If so, and if up to 5% of declarations were false, we would have expected the detection of re-emergent TB in 5–10 VCZs by now. There have been none (OSPRI, unpubl. data).

The lower-than-expected failure rate partly reflects the fact that many of the VCZs were not declared free until the posterior P_{free} was substantially above the 0.95 stopping rule (**Figure 3**). This is largely because until recently the PoF process was very largely retrospective: control and surveillance were conducted according to a fixed standard schedule (25), and only on completion of that were the data analyzed. That resulted, in many instances, in far more surveillance being done than was strictly necessary. To help avoid that in future, we have developed an online decision-support tool (https://landcare.shinyapps.io/JESS), which enables managers to determine, for any given prior $P_{free,}$ the minimum amount of surveillance needed to reach the stopping rule.

A second possible reason for the low failure rate is the conservative setting, by OSPRI, of a maximum prior P_{free} of 0.90 even when the SPM predicts that a given control history would have eradicated TB in 100% of simulations. If the model predictions were accepted as accurate, the posterior P_{free} estimates would have been higher (and therefore the expected failure rate lower).

Another possible reason for the low failure rate is that the SPM may be predicting that eradicating TB from possums is more difficult than it actually is, biasing the prior P_{free} estimates low. A converse point is that the low failure rate provides some validation of the SPM predictions: if the SPM was falsely providing overly optimistic predictions of the probability of eradication, there would have been more failures observed than expected.

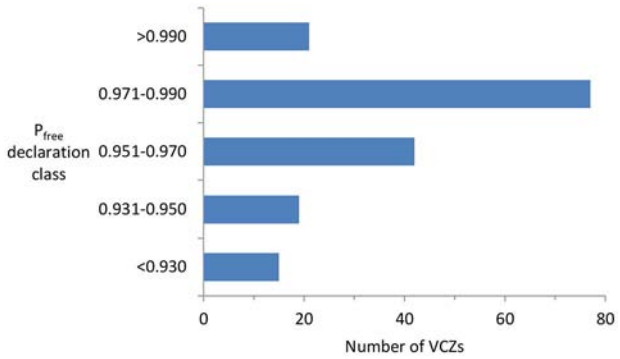

FIGURE 3 | Frequency distribution of the number of VCZs declared free between 2011 and 2018 in relation to the Pfree estimates (calculated using a design prevalence of 2, and grouped into five classes) at the time of declaration. The "Pfree declaration class <0.930" represents VCZs declared free on a largely qualitative basis rather than a quantitative one.

There are some indications that the SPM was indeed biased when applied to areas in which possum carrying capacity was well below average. In such areas the SPM often predicted TB would not persist even without control, despite evidence that TB had actually been detected in possums in some such areas (Crews, OSPRI, unpubl. data). To remedy that mismatch between model prediction and reality, the SPM has been revised by changing the possum–possum contact-rate function from one based on distance between home range centres to a more realistic one based on home range overlap (37), resulting in a greater amount of control being needed than previously for the model to predict eradication in areas of poor possum habitat.

Whatever the reason, there is evidence that the prior P_{free} estimates being used are conservative. In 2015, key TB managers were asked to subjectively assign prior P_{free} estimates for all VCZs in which TB surveys of possums had recently been conducted (38). There were 133 surveys in VCZs that had been under possum control for many years and that had prior P_{free} estimates in the range 0.70–0.95. Using conventional probability theory, the surveillance sensitivity estimates from these surveys were used to determine the probability that those surveys would have detected TB if it were actually present as frequently as the managers' prior P_{free} suggested it should be. Collectively, these 133 surveys should have resulted in 13 detections of infected possums if the P_{free} estimates were accurate, but again there were no detections. The implication is that there was far less infection in long-managed populations than managers believed, based on their experience with the PoF framework.

DISCUSSION

The evolution of the TB eradication programme in New Zealand (3) is the product of an adaptive management (39) effort in which management decisions are evidence based, and new research questions and developments are shaped by management

outcomes and needs. New Zealand's TB management agency, OSPRI, has historically funded, and continues to fund, robust and innovative science to support their desire for evidence-based decision-making. There is a strong focus on continual improvement, with a constant appetite for exploring new methodologies in order to achieve TB freedom ever more cost-effectively and ever more quickly.

A key factor in the continued success of the components of the "TBfree" programme that are specifically aimed at eliminating TB in wildlife has been the strong partnership and close working relationships over more than two decades between the end-user (OSPRI) and researchers at New Zealand's main terrestrial environment research institute (Manaaki Whenua—Landcare Research). This relationship, and the research findings that have flowed from it, has resulted in wide-ranging changes in operational strategies and activities. In particular, the PoF framework has become an integral part of TB management, with the posterior P_{free} increasingly recognized as the ultimate management performance metric. We believe that the challenges and successes of this collaborative experience will be instructive for other countries aiming to manage or eradicate TB from very large areas.

With less than a quarter of the area believed to contain infected wildlife declared free so far, there is clearly still an immense amount of management (and research) to be done. However, the success and progress to date, as well as the development and implementation of new methodologies and smarter decision-making tools, means that New Zealand is well on the way to eliminating TB in both livestock and wildlife, and is well on track to achieve the goal of disease eradication by 2055.

AUTHOR CONTRIBUTIONS

GN, AG, and DA reviewed and summarized their own bodies of work and collectively integrated those summaries into the overall review. KC (and other OSPRI staff) provided data on eradication progress, and also reviewed the whole document.

ACKNOWLEDGMENTS

We acknowledge the insight and guidance provided over the last decade by Dr. Paul Livingstone, former Eradication and Research Manager at OSPRI. We also acknowledge the contributions of numerous colleagues and co-workers in both Manaaki Whenua—Landcare Research and OSPRI in helping develop, test, and implement the new theory, tools, and systems summarized in this review.

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Conflict of Interest Statement: Most of the research and development described in detail here was conducted the Manaaki Whenua—Landcare Research authors in collaboration with, and with part or full funding from OSPRI, New Zealand's TB management agency, of which author KC is Head of Programme (Disease Management).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX: LIST OF ABBREVIATIONS AND DEFINITIONS USED IN THE TEXT

Assurance surveillance	Possum-TB surveillance undertaken after an area has been declared free, usually using unplanned, low-cost, "passive" methods (hunter observations from possums, deer and pigs, and livestock testing or slaughterhouse inspection data collected for other purposes).
Control	Reduction in possum density by lethal trapping or poisoning.
Control	efficacy Effectiveness of possum population reduction (% kill).
Control history	Summary of the duration (span of years) and intensity (control effectiveness or efficacy).
Disease	A term of convenience used to encompass the presence of subclinical *M. bovis* infection as well as the presence of actual symptoms of disease.
Eradication	Complete or absolute removal of *M. bovis* infection from all animals in an area with negligible chance of re-establishment.
Freedom	High but not absolute probability of absence of *M. bovis* infection from all animals in a specified area at a specific time.
Max prior	Maximum permissible prior: a subjective precautionary prescription of the maximum value that can be ascribed to the prior (defined below).
NPMP	National Pest Management Plan: a national plan required under New Zealand biosecurity legislation, first developed in the mid-1990s and revised and updated in 2005, 2011, and 2016.
P_{free}	Probability of absence of *M. bovis* infection from possums in a specified area at a specific time.
PoF	Proof of Freedom: a Bayesian belief-updating framework in which a quantitative estimate of the belief (confidence) that an area is free of TB at a given time (the "prior") is updated at a later time by the new information gathered between the two times to produce a new estimate (the posterior). The updating takes into account the possibility of re-introduction of new infection.
Posterior	A quantitative probabilistic estimate of the belief (confidence) that an area is free of TB at a given time that is derived by updating an initial prior belief with new empirical evidence of TB absence.
Prior	A quantitative probabilistic estimate of the belief (confidence) that an area is free of TB at a given time
P∗	Design prevalence: the specified surveillance target.
Re-control	Additional control required when an area is falsely declared free of TB, resulting in eventual to re-emergence of the disease and a need to again reduce possum densities in a further effort to break the TB cycle in possums.
Sentinels	Spill-over hosts of TB that can become infected by transmission from possums, but which do not independently maintain the infection, either because they are largely end hosts (pigs, deer, and ferrets in most places), or because they are subject to effective TB management (livestock).
SPM	Spatial Possum Model: an individual-based, spatially explicit simulation model of the eco-epidemiological dynamic of TB in possums, which is used to predict the likely effect of historical possum control on TB prevalence in possums.
SS	Surveillance sensitivity: the probability of finding an *M. bovis* infected animal in a particular survey sample of possums or sentinels if n TB possums were actually present in the area surveyed, with n/N (the population size) being the design prevalence P∗.
Stopping rule	The desired or prescribed level of confidence required before an area can be declared free of wildlife TB.
Surveillance	Empirical survey of animal disease status (through necropsy and mycobacterial culture of wild animals, or TB testing and/or slaughterhouse inspection of livestock).
TB	Bovine tuberculosis, caused by infection with *Mycobacterium bovis*.
TB possum	A possum with *M. bovis infection*.
VCZ	Vector control zones: formally defined areas, typically of 10,000–20,000 ha, used for planning possum control and surveillance, and forming the primary spatial management unit.
VRA	Vector risk area: an area considered to have a non-zero probability of containing infected wildlife (see https://ospri.co.nz/our-programmes/tbfree/about-the-tbfree-programme/wildlife-and-pest-management/vector-risk-areas/). "Vector free" areas (all of the non-VRA land) can contain infected livestock provided there is high confidence that the infection originated in other livestock elsewhere.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 06 September 2018
doi: 10.3389/fvets.2018.00214

Association of *Fasciola gigantica* Co-infection With Bovine Tuberculosis Infection and Diagnosis in a Naturally Infected Cattle Population in Africa

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OPEN ACCESS

Edited by:
Michele Ann Miller, Stellenbosch University, South Africa

Reviewed by:
Kimberly VanderWaal, University of Minnesota Twin Cities, United States
Brianna R. Beechler, Oregon State University, United States

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *07 May 2018*
Accepted: *15 August 2018*
Published: *06 September 2018*

Citation:
Kelly RF, Callaby R, Egbe NF, Williams DJL, Victor NN, Tanya VN, Sander M, Ndip L, Ngandolo R, Morgan KL, Handel IG, Mazeri S, Muwonge A and Bronsvoort BMdeC (2018) Association of Fasciola gigantica Co-infection With Bovine Tuberculosis Infection and Diagnosis in a Naturally Infected Cattle Population in Africa. Front. Vet. Sci. 5:214. doi: 10.3389/fvets.2018.00214

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, remains a major livestock and public health problem in both high and low-income countries. With the current absence of an effective vaccine, control in cattle populations is reliant on regular testing and removal of positive animals. However, surveillance and control are hampered by imperfect diagnostic tests that have poorly described properties in naturally infected populations. Recent research in cattle co-infected with the temperate liver fluke, *Fasciola hepatica*, has raised concerns about the performance of the intradermal skin test in high fluke incidence areas. Further, recent studies of parasitic co-infections have demonstrated their impact on Th1 and Th2 responses, concurrent disease pathology and susceptibility to mycobacterial infections. Here we report for the first time the association of co-infection with the tropical liver fluke, *Fasciola gigantica*, with the presence of bTB-like lesions and the IFN-γ response in naturally infected African cattle. After adjusting for age and sex we observed a complex interaction between fluke status and breed. Fulani cattle had a higher risk of having bTB-like lesions than the mixed breed group. The risk of bTB-like lesions increased in the mixed breed group if they had concurrent evidence of fluke pathology but was less clear in the coinfected Fulani breed. Further, we observed a slight decline in the IFN-γ levels in fluke infected animals. Finally we explored factors associated with IFN-γ false negative results compared to the presence of bTB-like lesions. Fulani cattle had a higher risk of having a false negative result compared to the mixed breed group. Further, the mixed breed cattle had an increased risk of being false negative if also co-infected with fluke. Interesting, as with the risk of bTB-like

lesions, this association was less clear in the Fulani cattle with weak evidence of a slight decrease in risk of having a false negative test result when fluke pathology positive. This interesting interaction where different breeds appear to have different responses to co-infections is intriguing but further work is needed to confirm and understand more clearly the possible confounding effects of different other co-infections not measured here, breed, management or exposure risks.

Keywords: bovine tuberculosis, *M. bovis*, co-infection, *F. gigantica*, fasciolosis, Cameroon, diagnostic tests, interferon-γ

INTRODUCTION

In natural populations, individuals are usually infected with multiple pathogens, also known as "co-infections," rather than single infections (1). In the presence of multiple co-infections, the immune response observed to an individual pathogen, across a population, is variable. This has been shown to depend on the combination of infections and their differing interactions with the host immune system and other infections (2). Like many infectious diseases, *Mycobacterium bovis* infection has been studied in isolation until relatively recently. Co-infections with *Fasiola hepatica* have been implicated as a potential reason for poor bTB diagnostic test performance and disagreement between tests (3). More specifically, co-infections with *F. hepatica* have been shown to down-regulate the Th1 responses (with a resultant dampening of the IFN-γ response), with subsequent predominance of Th2 responses, in order for the parasite to survive and reproduce (4–8).

Bovine tuberculosis (bTB), caused by the bacterium *M. bovis*, is both a major veterinary and public health disease of cattle and other livestock. It is an important zoonosis (9, 10) causing pulmonary and extra-pulmonary disease in people and is responsible for an estimated 3% of human tuberculosis globally (11) amounting to an estimated 147,000 zoonotic cases per year, of which 70,000 are in sub-Saharan Africa (www.who.int/tb/areas-of-work/zoonotic-tb/en/). In many high-income countries, such as the United Kingdom and New Zealand, compulsory bTB "test and slaughter" programs coupled with compensation have been successful in reducing transmission of *M. bovis* in livestock populations (12–14). Diagnostic testing involves detection of immune responses in the early stages of infection, such as dominant Th1 responses (15), to remove bTB positive animals as soon possible. Ante-mortem diagnostic tests, such as the single intradermal comparative cervical test (SICCT) or the interferon-γ (IFN-γ) assay, are based on detecting the Th1 immune response to *M. bovis* (16). However, the variable sensitivity of the SICCT (55.1-93.5%) and the IFN-γ assay (73-100%), which rely on detecting this Th1 response, particularly in late stage disease when a Th2 immune response dominates, can lead to false negative cattle persisting within the population (17) resulting in continuing transmission and larger outbreaks.

Although the co-infection relationship has yet to be fully elucidated, various studies have demonstrated that *F. hepatica* co-infection is associated with a reduced Th1 immune response (3) and a reduced mycobacterial burden (18), which potentially leads to the underestimation of bTB prevalence (19). This is particularly important when using the IFN-γ assay to detect bTB positive cattle. IFN-γ is a cytokine which is produced as part of the Th1 immune response to *M. bovis* infection (20). It has been demonstrated that *F. hepatica* infections down-regulate IFN-γ pro-inflammatory cytokine responses in favor of Th2 cytokine induction and an IgG1 response (21). When using the IFN-γ assay to detect bTB infected animals, the presence of *F. hepatica* co-infection can lead to a reduction in IFN-γ response below the diagnostic test cut-off leading to false negative results (22). However, the extent of bTB misdiagnosis using the IFN-γ assay in bTB endemic cattle populations co-infected with other *Fasciola* species, remains unquantified. In addition, although similar immune evasion and modulation strategies to *F. hepatica* have been identified in bovine *F. gigantica* infections, the effect of co-infection with *F. gigantica* on bTB immune responses has been minimally investigated (23).

This paper reports the first study of co-infection with *F. gigantica* (23) in bTB infected cattle under natural conditions in a tropical African population. Bovine tuberculosis (24–26) and *F. gigantica* (27–29) infections are endemic in cattle populations in Cameroon and are currently poorly controlled, providing an opportunity to study their interaction within a natural transmission setting. The data used for this analysis were a subset of data that were generated from a larger study of *M. bovis* epidemiology in Cameroonian cattle (26, 29, 30). We describe the association of *F. gigantica* co-infection with the presence of observable bTB-like lesions and diagnostic test results using the IFN-γ assay.

METHODS

Abattoir Cross-Sectional Study

Data were collected at the Ngaoundere municipal abattoir in the Adamawa Region, a major cattle-producing area of Cameroon (**Figure 1**). The details of study design and sample collection are reported elsewhere (26) and were based on collection of bTB-like lesion material for culture. In brief, based on previous estimates of bTB-like lesions from the North West Region of Cameroon (31) we assumed a prevalence of lesions of ∼5% and calculated a target sample size of ∼1000 cattle to ensure recovery of at least 25 isolates assuming a 50% recovery from culture. This would allow the within abattoir prevalence of 5% to be estimated with a precision of ±1.3% at 95% confidence. During sampling, cattle were cast for slaughter by the butchers, after which the

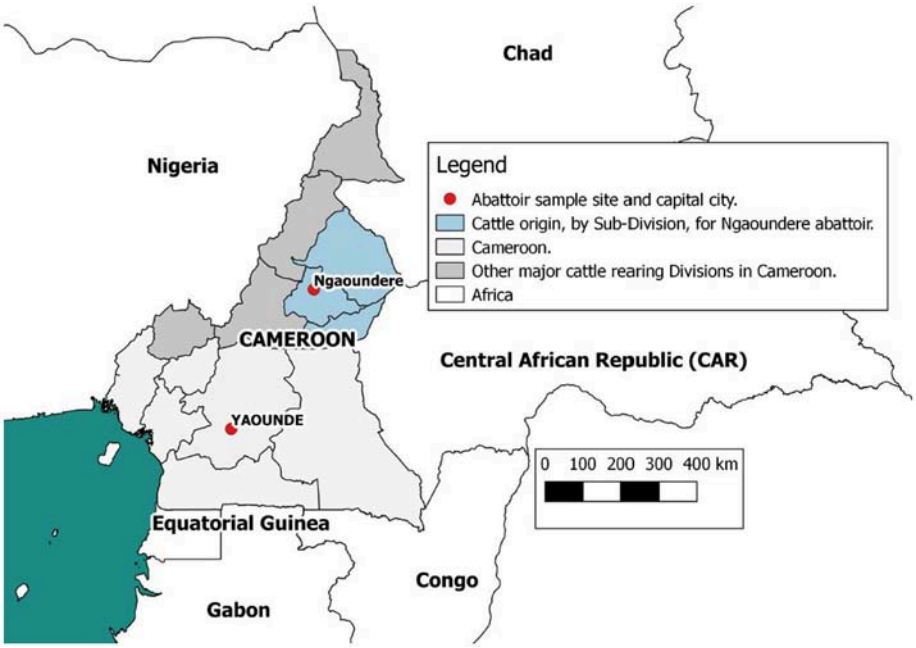

FIGURE 1 | Map of Cameroon showing the location of the Ngaoundere abattoir and the catchment areas of cattle feeding into it. This figure was generated using QGIS 2.2 (www.qgis.org) and shp files obtained from the GADM database of Global Administrative Areas (www.gadm.org).

research team tagged the animal, collected a heparinized blood sample and recorded animal-level data on owner/butcher, sex, breed as reported by the butcher, dentition score (DS) as an estimation of age (32) and market of origin as reported by the butcher. Post mortem meat inspection was carried out by local Ministry of Livestock, Fisheries and Industrial Agriculture (MINEPIA) inspectors who examined the carcass and offal for presence of granulomatous bTB-like lesions and evidence of liver damage/cirrhosis. Once identified by the veterinary inspectors, the research team collected up to 3 macroscopic bTB-like lesions from different anatomical sites per animal into sterile 25ml universal tubes using forceps and scalpel blades. Lesion grades were also recorded following identification and tissue samples taken (33, 34). Matching numbered tags issued by the research team were used to link animal data, blood samples, meat inspection of the carcass, offal (including the liver) and head. In addition to the tissue samples for culture from animals with lesions, a number of animals classed as non-lesioned by the meat inspectors were randomly sampled (using random number generator www.Random.org) and a single retropharyngeal lymph node per animal collected for culture as controls.

Tissue samples (lesioned lymph nodes) were stored in the vapor phase in liquid nitrogen dry shippers (Taylor-Wharton) and shipped to the Tuberculosis Reference Laboratory (TBRL) Bamenda. Upon arrival at the TBRL the samples were stored at -80°C until processed. Heparinised blood samples were stored in a coolbox at the abattoir (ranging between 10°C to 26°C) and then taken to the lab and kept at room temperature prior to being stimulated in the IFN-γ assay (Bovigam®) described below. Animal data recorded on paper in the abattoir, was transferred to a relational Microsoft Access database where the results could be linked back to individual animals.

Diagnostic Tests

Fasciola Pathology at Meat Inspection

All carcases were inspected for evidence of *F. gigantica* infection by MINEPIA meat inspectors. The meat inspectors examined the liver systematically to identify gross pathology associated with *Fasciola* infection by slicing down the common bile duct with an additional 1–2 slices through the liver parenchyma. Once an animal was identified to have gross fasciolosis related pathology, the liver was graded by a member of the research team and scored 0–3 (35). A score of 0 = no visible pathology; 1 = low grade pathology with minimal damage to the parenchyma of the liver through migratory fibrotic/ cirrhotic tracts from the parasite, thickening of bile ducts with a few *F. gigantica* parasites noted in bile ducts; 2 = moderate grade pathology with *F. gigantica* species parasites found in the bile ducts and up to approximately half the liver having evidence of fibrosis/ cirrhosis; 3 = severe grade pathology with the majority of the liver is noted to have extensive fibrosis/ cirrhosis without having to cut the surface of the liver. For this analysis the score was converted into a presence (positive) or absence (negative) of *F. gigantica* pathology for subsequent analysis.

Mycobacterial Culture and Typing

The tissue samples were prepared and cultured as previously described (26) following the World Organization of Animal Health (OIE) guidelines with minor modifications. Briefly, samples were processed, inoculated into a Mycobacterial Growth

Indicator Tubes (MGIT) and incubated for 8 weeks on the BACTEC MGIT 960 automated culture system (Becton, Dickinson and Company, 1 Becton Drive, Franklin Lakes, NJ, USA) following the manufacturer's instructions. A further 2 cultures were prepared by inoculating 0.1 ml (2 drops) of prepared sample onto each of two Lowenstein Jensen (LJ) slopes (one supplemented with pyruvate and the other with glycerol). These were observed weekly for up to 12 weeks. A smear was made with 3% formal saline from any observed growth on the LJ media and any MGIT indicated positive tube. The smears were heat-fixed, stained by the Ziehl-Neelsen (ZN) method (36) and microscopically observed for the presence of acid fast bacilli (AFB). All acid fast bacilli were typed using the Hain GenoType® MTBC assay and GenoType® Mycobacterium CM/AS kit (Hain Lifescience®,GmbH, Nehren, Germany) (26). Animals were classed as confirmed bTB cases (as opposed to having a bTB-like lesion) if one or more lesions were positive by one or more culture methods confirmed by the Hain Genotype® test.

Interferon-Gamma Assay

The IFN-γ ELISA (Bovigam®) was conducted as per published protocol (37, 38). Briefly within 6–12 h of collection three aliquots of heparinised blood, per animal, were incubated with either avian PPD, bovine PPD (Prionics® Lelystad Tuberculin PPD) or PBS for 24 h at 37°C in a portable polystyrene egg incubator (http://www.theincubatorshop.co.uk) run in the field. Following incubation samples were centrifuged at 300g for 10 minutes, the plasma was aliquotted and stored at −20°C in a portable travel freezer (Waeco CF50 12V/240 fridge freezer)). Plasma samples were transported at −20°C to the LEID and the IFN-γ ELISA was conducted as per the published protocol. The acceptable averaged negative bovine OD value was <0.130 and positive bovine control was >0.700. Animals with a bovine stimulated sample optical density of ≥0.1 above that of the avian PPD sample were classified as test positive and interpreted as the animal being infected with *M. bovis*.

Statistical Analysis

The proportions of cattle with bTB-like lesions, a positive IFN-γ result and liver fluke pathology were calculated and various co-infection definitions were explored using *Fasciola* pathology and one of the bTB outcomes (positive IFN-γ, bTB-like lesion or *M. bovis* culture positive).

Multivariable logistic regression (MLR) models were developed to explore the association between fluke infection and bTB status using a number of different definitions including IFN-γ, bTB-like lesion and *M. bovis* culture results. Animal-level explanatory variables (breed, dentition score and sex) were always included in the models as fixed effects to control for confounding. Model selection was based on the AIC and the best model was selected using the lowest AIC and ΔAIC (39). MLR models were constructed using the *brglm* function in the *brglm* package (40) with AIC and ΔAIC calculated using the *modavg* function from the *AICcmodavg* package (41). Predicted probabilities and their standard errors were calculated from each model for specific covariate patterns using the *predict* function and used to produce 95% confidence intervals for plotting.

Some variables were simplified due to small numbers of observations in some categories. The dentition score (DS) was simplified from number of permanent teeth to a binary age category based on the approximate relationship between age and eruption of permanent incisors in cattle. DS <2 was categorized as "<3 years old" and DS of ≥2 was categorized as "≥3 years old." The breed variable was simplified to "mixed" (collapsing mixed breed, where the butchers were unsure of the breed cross and Gudali to a single category) or "Fulani" (collapsing Red and White Fulani to a single category).

RESULTS

Summary Statistics

During a 4 week period in August 2013, 935 cattle were examined at the Ngaoundere abattoir. The details of bacterial culture results have been presented elsewhere (26). A total of 173 records were dropped due to missing data (at random) due to the hectic nature of the sampling in the abattoir environment which resulted in occasional failure to collect a blood sample or link samples to a carcass. A further 30 animals from one day were dropped from this analysis due to missing IFN-γ results giving a final dataset of 732 animals.

During the sampling period 10.7% (78/732) of animals had visible bTB-like lesions observed. The proportion of animals with evidence of liver fluke infection was 49.6% (363/732) and the proportion of animals positive by the IFN-γ assay was 6.6% (48/732). The distribution of the prevalances of bTB-like lesion, liver fluke pathology and IFN-γ based on animal dentition (as a measurable proxy for age) are given in **Figure 2**. Exploratory bivariate relationships between the variables of interest (age, breed, sex, lesion status, fluke status and IFN-γ status) were checked prior to inclusion in the multivariable models (**Figure 3A**). Breed and fluke status were strongly associated with presence of visible bTB-like lesions and breed was strongly associated with IFN-γ status. There was an association between breed and the ordinal distribution of fluke pathology scores (χ^2 test p-value <0.001) but the odds ratios did not change across pathology scores above zero so the effect is captured by collapsing the fluke score into a binary variable (**Figure 3B**).

Association of *F. gigantica* Co-infection With Bovine Tuberculosis-Like Lesions

A multivariable logistic regression (MLR) model of the association between visible lesion and fluke status was developed (**Table 1**). This suggests a complex interaction between breed and *F. gigantica* pathology status with the probability of observing visible TB-like lesions in cattle in this setting. Fulani cattle had a higher risk of having observable bTB-like lesions than the mixed breed group. However, the risk in the mixed breed group increased if they also had fluke pathology. This association ith fluke pathology was less clear in Fulani cattle. For example, an adult, female, mixed breed animal, that had no fluke pathology had a ∼ 5.2% (95% CI: 2.5–7.8) probability of having a TB-like lesion compared to ∼ 27.7% (95% CI: 16.8–38.4) for a Fulani animal (**Figure 5A**). The presence of fluke increased this risk in mixed breed animals to ∼ 12.0% (95% CI: 7.7–16.3) while the

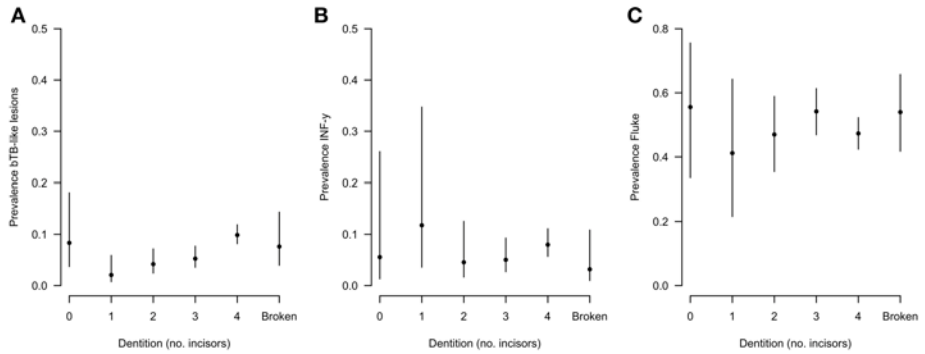

FIGURE 2 | Distribution of **(A)** lesion, **(B)** fluke, and **(C)** IFN-γ prevalences based on animal dentition for Ngaoundere abattoir ($n = 732$).

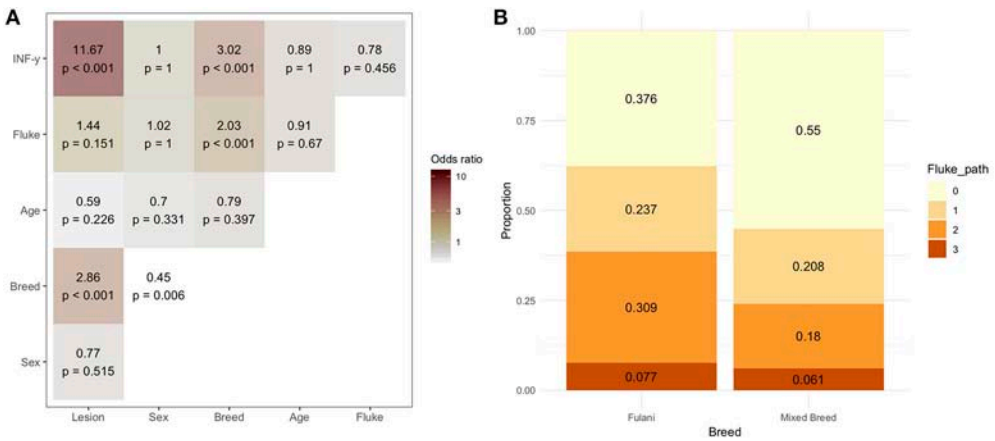

FIGURE 3 | (A) Bivariable odds ratios and p-values for pairwise associations of variables of interest for inclusion in multivariable models. **(B)** Proportion of Fulani ($n = 194$) and mixed breed ($n = 538$) classified cattle sampled in Ngaoundere abattoir by *F. gigantica* pathology score. Legend: Lesion, lesion status (positive/negative); sex, male/female; Breed, Fulani/mixed; Age, <3 years/≥3 years; Fluke, fluke pathology status (positive/negative); IFN-γ, IFN-γ test positive/negative.

risk declined (although the evidence is weaker) in Fulani cattle to ∼ 15.6% (95% CI: 8.8–22.2). The age and sex terms were included to control for confounding but do not appear to be important for this model.

Impact of *F. gigantica* co-infection on IFN-Gamma Responses

The association between IFN-γ result and *F. gigantica* pathology status was investigated. The raw PPD-B minus PPD-A difference in ELISA OD readings were explored in the subset of animals which were confirmed *M. bovis* culture positive (n = 53). The raw difference is plotted, stratified by *F. gigantica* pathology status in **Figure 4** where there is some evidence of a dampening of the IFN-γ response with a smaller variance in the fluke pathology positive group. When the outlying high value for the fluke pathology positive group is removed the variances are statistically significantly different (Mann-Whitney test $p < 0.001$, $n = 52$).

A multivariable linear regression model of the raw PPD-B minus PPD-A difference was developed (with the outlying value dropped) and after accounting for age, sex and breed there was a small mean decrease in the difference of −0.02 (−0.04 to 0.00) in OD value in the fluke pathology positive animals (**Table 2**). A multivariable logistic regression model for IFN-γ binary test

TABLE 1 | Multivariable logistic regression model for the presence of TB lesions at slaughter ($n = 732$).

Variable	Levels	Odds ratio	95% CI
Sex	Female	1	
	Male	1.17	0.50–2.47
Age	≥3 years	1.00	
	<3 years	0.60	0.25–1.26
Breed	Mixed	1	
	Fulani	7.00	3.36–14.95
Fluke	Negative	1	
	Positive	2.51	1.32–4.98
Breed*Fluke		0.19	0.07–0.51

*Key: Lesion, TB lesion result (Positive or negative); Sex, Sex of cattle (Male or Female); Age, Age of cattle by dentition score (<3 years or ≥3 years); Fluke, F. gigantica pathology score; Breed, Breed of cattle (Mixed breed or Fulani breed); *Interaction between variables.*

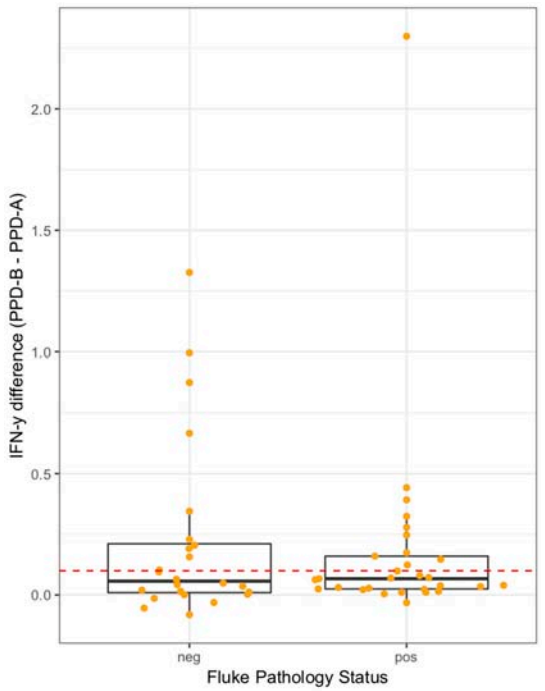

FIGURE 4 | IFN-γ response (PPD-B minus PPD-A) stratified by *F. gigantica* pathology status (neg, no pathology; pos, evidence of fluke pathology) in the subset of *M. bovis* culture positive cattle ($n = 53$). Individual cattle; Orange circles. Dashed red line IFN-γ recommended test cut-off of 0.1.

TABLE 2 | Multivariable regression model for the raw IFN-γ PPD-B minus PPD-A difference ($n = 731$).

Variable	Levels	Coef	95% CI
Sex	Female	1	
	Male	–0.01	–0.46 to 0.02
Age	≥3 years	1	
	<3 years	–0.01	–0.31 to 0.02
Breed	Mixed	1.00	
	Fulani	0.02	0.13 to 1.46
Fluke	Negative	1	
	Positive	–0.02	–1.92 to 0.01

Key: IFN-γ, IFN-γ assay result (PPD-B - PPD-A); Sex, Sex of cattle (Male or Female); Age, Age of cattle by dentition score (<3 years or ≥3 years); Fluke, F. gigantica pathology score; Breed, Breed of cattle (Mixed breed or Fulani breed).

TABLE 3 | Multivariable logistic regression model for the raw IFN-γ PPD-B minus PPD-A difference (n=731).

Variable	Levels	Odds ratio	95% CI
Sex	Female	1	
	Male	0.91	0.28–2.29
Age	≥3 years	1	
	<3 years	0.99	0.38–2.22
Breed	Mixed	1.00	
	Fulani	3.18	1.73–5.85
Fluke	Negative	1	
	Positive	0.62	0.33–1.13

Key: IFN-γ, IFN-γ assay result (Positive or negative); Sex, Sex of cattle (Male or Female); Age, Age of cattle by dentition score (<3 years or ≥3 years); Fluke, F. gigantica pathology score; Breed, Breed of cattle (Mixed breed or Fulani breed).

status and fluke pathology status was also developed including age, breed and sex as potential confounders (**Table 3**). Both these regression models give some weak support for an association between the IFN-γ result and *F. gigantica* pathology status, with fluke pathology positive cattle more likely to have a lower OD value difference and to be IFN-γ negative. The association using the binary test results suggest an adult, female, mixed breed cow had a ∼ 5.6% (95%: 3.1–8.1) probability of testing positive which dropped to ∼ 3.5% (95%: 1.7-5.6) if infected with liver fluke compared to a Fulani, adult, female animal which had a ∼ 16.3% (95%: 8.7–24.0) of testing positive which dropped to ∼ 11.1% (95%: 5.9–16.2) if fluke pathology positive (**Figure 5B**).

Factors Associated With a False Negative IFN-γ Test Result When Compared to TB-Like Lesion

Using the subset of results where the IFN-γ test result was negative ($n = 684$) a new variable was generated where a IFN-γ test result was classified as a false negative if there was an observed bTB-like lesion ($n = 54$) and true negative if there was no lesion observed ($n = 630$). A multivariable logistic regression analysis to explore the association with fluke pathology was conducted including sex, age and breed as confounders. In addition, for this model the presence or absence of non-tubercular mycobacteria (NTM), based on the Haines typing from cultured samples from lesions, was also included as a known potential confounder for being lesion positive but test negative. The final model is given in **Table 4**. Again the best fitting model includes an interaction between fluke pathology status and breed. The baseline probability of being a false negative IFN-γ test result in adult, female, non-tubercular mycobacterium negative (NTM), fluke pathology negative mixed breed cows was ∼ 4.3% (95%: 1.9–6.7) which increased to ∼ 10.4% (95%: 6.3–14.5) if they had fluke pathology. In comparison IFN-γ test negative Fulani cattle had a ∼ 16.0% (95%: 6.3–25.6) probability of being a false negative which declined to ∼ 8.5% (95%: 3.1–14.0) if fluke pathology positive (**Figure 5C**). An NTM infection also was also associated with a large increased risk of giving a false negative IFN-γ result.

DISCUSSION

It is well recognized, though still poorly studied, that co-infecting pathogens can have a range of synergistic or antagonist effects. For example, (42) showed African buffalo co-infected with strongyle nematodes had a dampened Th1 response which facilitated bTB invasion and establishment of infection. Interactions between bovine tuberculosis and the temperate liver fluke *F. hepatica* have been shown in the United Kingdom (3, 19)

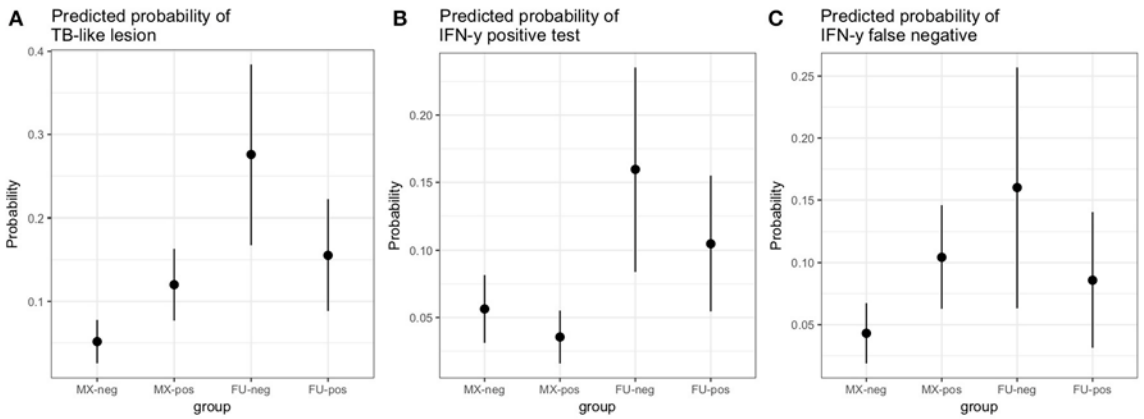

FIGURE 5 | Predicted probabilities for an adult female animal by breed (MX, mixed breed; FU, Fulani) and fluke status (neg, no fluke pathology; pos, evidence of fluke pathology) based on the logistic regression models described in the results section.

TABLE 4 | Multivariable logistic regression model ($n = 684$) for being IFN-γ false negative result conditioned on having a negative IFN-γ test result and using observable TB-like lesions as the true state (gold standard).

Variable	Level	Odds ratio	95% CI
Sex	Female	1	
	Male	0.97	0.31–2.46
Age	≥3 years	1	
	<3 years	0.68	0.24–1.57
NTM	Negative	1	
	Positive	15.29	3.32–75.7
Breed	Mixed	1	
	Fulani	4.23	1.68–10.44
Fluke	Negative	1	
	Positive	2.59	1.28–5.49
Breed*Fluke		0.19	0.06–0.64

*Key: Lesion, TB lesion result (Positive or negative); Sex, Sex of cattle (Male or Female); Age, Age of cattle by dentition score (<3 years or ≥3 years); Fluke, F. gigantica pathology score; Breed, Breed of cattle (Mixed breed or Fulani breed); NTM, non-tubercular mycobacterium; *Interaction between variables.*

but less is known about the interactions of bovine tuberculosis and the tropical fluke *F. gigantica*. Despite the ecological, genetic and antigen differences between these two species, both species appear to evade and modulate the host immune response to infection (43). Here we have examined the association between both bTB-like lesion occurrence and the Bovigam (*M. bovis specific*) IFN-γ response in a naturally *F. gigantica* infected cattle population in Africa.

The levels of fluke infection were very high in this abattoir population with nearly 50% of animals showing liver pathology consistent with fluke infections and/or adult fluke identified in the bile ducts. Previous studies have identified an association with visible bTB-like lesions and being fluke positive in slaughter cattle populations such as in Zambia (44). Further, in experimental infections in mice it has been shown that mice infected with *M. tuberculosis* have higher bacterial loads and TB lesion pathology when co-infected with the trematode *S. mansoni* (45). It is proposed that the fluke infection suppresses the Th1 response resulting in a reduced level of IFN-γ. In cattle, previous studies have reported that co-infection results in a lower bacterial load but no qualitative or quantitative differences in tuberculous lesions of *M. bovis* (18).

In the present study, we observed a complex interaction between cattle breed and liver fluke pathology status and the presence of visible bTB-like lesions. There is strong support for an increased risk of having bTB-like lesions in Fulani cattle compared to the mixed breed group. In Cameroon, Fulani cattle have been reported to have a higher prevalence of bTB than other breeds (31) but this study was from the Northwest Region. One explanation may be to do with differing responses in different cattle/host genotypes. It has been reported in the UK that Holstein cattle with the INRA111 genotype appeared to be less likely to develop bTB (46). Similarly, in Ethiopia comparing Holstein cattle and indigenous zebu *B. indicus* cattle, researchers found that Holstein cattle were more susceptible (33).

The presence of fluke pathology in the mixed breed group was associated with an increased risk of visible lesions. In Fulani cattle, which are more likely to have bTB-like lesions, co-infection with fluke was not associated with an increased risk but potentially a reduced probability of having visible bTB-like lesions, although the evidence was weak for this association in Fulani cattle. Our study relied on the butchers classification of breed recorded in the hectic environment of the slaughterhouse. There is the possibility that their classification was incorrect in some cases, however, this is more likely to reduce the chances of observing an association. To improve on this we are currently genotyping the subset of cattle from which we collected lymph nodes for culture (both lesioned and the random sample of non-lesioned lymph nodes). Alternatively, the breed association may be due to confounding by other unobserved variables such as differing management between breeds or differences in exposures interacting with different genotypes of *M. bovis*. However, these are more difficult to untangle.

The IFN-γ assay is particularly useful to detect early (1-4 weeks post infection) *M. bovis* infections as part of control programs often in combination with the SICCT (20, 47). Previous studies have demonstrated that *F. hepatica* co-infection can down-regulate IFN-γ responses to *M. bovis* infection (21, 22). In this African cattle population there was weak evidence of a reduced IFN-γ response (reduced variance in the raw PPD-B - PPD-A value) in fluke infected animals conditional on having been bTB culture positive. This reduction in IFN-γ was also weakly observed in the linear regression analysis (on the continuous test result) and in the logistic regression analysis (on the binary result). However, we did observed a moderate association between increased IFN-γ levels and breed, with Fulani cattle having a higher probability of testing positive. Given the higher rates of infection observed in the lesion data, this is not surprising. This represents relatively weak evidence for a decline in test sensitivity in fluke infected animals and increased risk of leaving potential bTB positive animals in a herd. However, the prevalence of bTB was relatively low in this relatively small study, meaning the power to detect these effects is less than ideal and further larger studies are needed to confirm these findings.

In order to further explore this potential decline in test sensitivity, we looked at the subset of IFN-γ test negative animals (based on the binary cut-off of 0.1 as recommended by the manufacturers). Using visible bTB-like lesions as the comparison test, which we know from culture results from these cattle is reasonably specific (sp=69.7%) and sensitive (95.8%), (with a positive predictive value of 65.1% and negative predictive value of 96.6% calculated from (30) for this sample), we looked at factors associated with false negative results in the IFN-γ negative subset of animals. The risk of false negative results was strongly associated with NTM infections. This is to be expected as we know that lesions are an imperfect predictor of *M. bovis* and that a number of these animals with lesions had NTMs based on culture results (30). Interestingly, having accounted for a major source of the disagreement, there again remained a complex interaction between breed and fluke status. In the mixed breed animals the risk of being a false negative increased from $\sim$ 4% to $\sim$ 10% consistent with suppression of the Th1 response by fluke infections. Also Fulani cattle had higher rates of false positives compared to the mixed breed group but Fulani cattle with fluke pathology had a drop in risk from $\sim$ 16% to $\sim$ 8.5%, although the statistical support for this decline is weak. Again, this may be due to different host genetics, management or exposures and needs further investigation.

More work is needed to understand these interactions between co-infecting pathogens. Variation in immune interaction of the host, with *M. bovis* and *Fasciola gigantica*, at different stages in the pathogenesis of one pathogen may affect the pathogenesis of the other (48, 49). One possible explanation for this complexity may be that we have not accounted for other confounding co-infections. Conducting studies to look at all possible co-infections can become extremely complex, expensive and logistically challenging (50). There have been a number of co-infection studies including nematode infections in buffalo (42) and fluke in cattle (19) with evidence of associations with bTB-like lesions or interference with IFN-γ test results, however, a recent paper failed to find a statistical association with fluke or bovine viral infections (51) in European cattle, although they did find an association with paratuberculosis co-infections, which were associated with an increased probability of observing visible bTB-like lesions.

It is clear that co-infections can have complex impacts on test diagnostics and pathogen invasion and this may have important implications for control programmes. As one bacillus is sufficient to establish *M. bovis* infection within a host (52), leaving any infected animals behind in a control programme has the potential to maintain transmission and there was evidence here that in the mixed breed group at least, fluke infections were associated with an increased risk of a false negative IFN-γ result. Certainly, test and slaughter programs are likely to continue to play their part in bTB control in high income settings and certain wildlife control settings. Mitigating against *Fasciola spp.* co-infections (or other co-infections such as paratuberculosis) or being able to incorporate the likely impact on test performance may improve ante-mortem diagnostic test sensitivity within cattle or wildlife populations.

In conclusion, this study explored the association between co-infection with the tropical liver fluke *F. gigantica* on the pathology and detection of *M. bovis* infections in a natural ecological setting in African cattle. We have shown a complex association between the presence of visible bTB-like lesions in carcasses and the presence of concurrent *Fasciola* infections which appears to be also affected by breed. Furthermore, we have shown that the IFN-γ response may be slightly dampened down in *F. gigantica* infected cattle although further data are needed to confirm this. However, it does appear to be sufficient to increase the false negative risk in the mixed breed group at lest in this population. The reduction in sensitivity of the IFN-γ assay by *Fasciola spp.* co-infection could have profound effects on bTB control and eradication programs as *Fasciola spp.* are present worldwide (53). Given the complexity of determining whether animals are truly *M. bovis* infected, there is a need to develop more subtle and sophisticated algorithms for interpretation of individual animal bTB diagnostic test results that use the raw test readings as well as other animal and related herd variables.

ETHICS STATEMENT

This study did not involve the experimental use of any live vertebrate but reports the results of a pre-slaughter blood sampling and post mortem examinations of cattle for bovine tuberculosis carried out by the local veterinary inspectors in a commercial abattoir in Cameroon. Samples for culture were collected from carcases in accordance with best practice guidelines to minimize contamination. Local approval was given by the Head of Epidemiology at the Ministry of Livestock, Fisheries and Animal Industries responsible for supervision of activities at commercial slaughterhouses in each administrative Division. This project and the protocols had ethical approval

from the University of Edinburgh Ethical Review Committee (Animal (Scientific Procedures) Act, 1986) (ERC No: OS02-13).

AUTHOR CONTRIBUTIONS

BB, RK, LN, VT, KM, RN, MS, and NE: conceived and designed the study; RK, SM, NV, NE, and AM: performed the field work; RK, BB, IH, SM, and RC: analyzed the data; RK, BB, IH, SM, MS, and DW: contributed reagents, expertise, materials, analysis tools; RK and BB: wrote the first draft paper; All authors read and contributed to the final draft of the paper.

ACKNOWLEDGEMENTS

The authors thank the Wellcome Trust (WT094945) for funding this research project. BB also thanks the BBSRC for their support through the Institute Strategic Programme (BB/J004235/1).The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Importantly we would like to thank all the cattle keepers, the butchers and their staff in the abattoirs, the MINEPIA veterinary center staff and delegates without whom this study would not have been possible.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 04 March 2019
doi: 10.3389/fvets.2019.00061

Validation of a Real-Time PCR for the Detection of *Mycobacterium tuberculosis* Complex Members in Bovine Tissue Samples

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 29 June 2018
Accepted: 12 February 2019
Published: 04 March 2019

Citation:
Lorente-Leal V, Liandris E, Castellanos E, Bezos J, Domínguez L, de Juan L and Romero B (2019) Validation of a Real-Time PCR for the Detection of Mycobacterium tuberculosis Complex Members in Bovine Tissue Samples. Front. Vet. Sci. 6:61. doi: 10.3389/fvets.2019.00061

Although the post-mortem diagnosis of bovine tuberculosis is mainly achieved through microbiological culture, the development of other techniques to detect *Mycobacterium tuberculosis* complex (MTBC) members directly from tissue samples has been pursued. The present study describes the development, optimization and validation of a Real-Time PCR based on the *mpb70* gene to detect MTBC members in clinical tissue samples from cattle. Specific primers and a hybridization probe were used to amplify MTBC-specific sequences in order to avoid cross-reaction with non-MTBC species. An Internal Amplification Control (IAC) was included in order to assess the presence of PCR inhibitors in the samples. The PCR was optimized to achieve maximum efficiency, and the limit of detection, limit of quantification and dynamic range of the reaction were determined. The specificity of the reaction was tested against 34 mycobacterial and non-mycobacterial species. The diagnostic sensitivity, specificity and positive and negative predictive values (PPV and NPV) of the method were assessed on 200 bovine tissue samples in relation to bacteriological culture. The dynamic range of the reaction spanned from 5 ng/reaction (10^6 genome equivalents) to 50 fg/reaction (10 genome equivalents). The efficiency of the reaction was 102.6% and the achieved R^2 was 0.999. The limit of detection with 95% confidence was 10 genome equivalents/reaction. No cross-reactions with non-MTBC species were observed. The diagnostic sensitivity and specificity values of the *mpb70* specific Real-Time PCR respect to culture were 94.59% (95% CI: 86.73–98.51%) and 96.03% (95% CI: 90.98–98.70%), respectively, with a PPV of 93.33% (95% CI: 85.55–97.07%) and a NPV of 96.80% (95% CI: 92.10–98.74%). The concordance of the Real-Time PCR based on *mpb70* is comparable to that of culture (K = 0.904) showing a great potential for the detection of members of the MTBC in animal tissues.

Keywords: real-time PCR, *Mycobacterium tuberculosis* complex, tuberculosis, detection, bovine tissue

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic infectious disease caused by members of the *Mycobacterium tuberculosis* complex (MTBC), which affects certain species of mammals including cattle (1). Within this group of bacteria, *M. bovis* followed by *M. caprae* are the most frequent species in bovines. Due to its zoonotic potential and to the economic importance of cattle in the EU, this disease is subject to well-established national eradication campaigns in Member States. According to the legislation in force, i.e., 64/432/ECC, the intradermal tuberculin test is the official test in order to classify TB free herds, areas or countries, and microbiological culture is the method of confirmation of MTBC infections in bovine tissues.

The reported recovery rates for culture in general oscillate between 30 and 95% (2–5) while in a recent study using a Bayesian approach, the diagnostic sensitivity and specificity of culture was 78.1 and 99.1%, respectively (6). This variation between studies can be explained by different factors associated with the technique and the samples, which can affect the performance of the method. Firstly, the choice of tissue samples at the abattoir is a key for culture. Abnormal lymph nodes and parenchymatous organs with bTB-compatible lesions must always be included when present. If pathological lesions are not detected then, specific lymph nodes (retropharyngeal, bronchial, mediastinal, supramammary, mandibular, and mesenteric) should be taken for examination and culture. Secondly, the preservation of samples until culture by refrigeration or freezing, together with the step of chemical decontamination, is mandatory in order to decrease the risk of contamination with other microorganisms. Inadequate storage and sample treatment influence the viability of MTBC and can promote the growth of contaminating microorganisms (2). Thirdly, the type of culture media chosen to grow mycobacteria may influence the recovery rate of microbiological culture. MTBC growth can be detected either by colony formation in agar and egg-based solid media (such as Middlebrook 7H10/7H11, Stonebrink or Löwenstein-Jensen with sodium pyruvate), or fluorescence or pressure differences in liquid media (BACTEC 460 TB and MGIT 960 and VersaTREK system). In studies comparing both culture systems, the recovery rates for liquid media are higher than those reported for solid media with values of 80 to 95% and 65 to 82%, respectively (3, 5). The highest recovery rates within liquid systems are recorded for the BACTEC 460 TB system, which is no longer commercially available. In addition, there is a suspected decrease in selectivity of the MGIT 960, which in turn makes liquid media more prone to overgrowth by rapidly growing microorganisms (4, 5). Members of the MTBC are grouped within the slow growing mycobacteria due to their slow replication cycle. As a result, culture detection of MTBC is extremely slow; around 28 days for liquid media and 43 days for solid media for a positive result (2, 3).

In order to overcome the problems associated with the recovery of MTBC by culture, detection of mycobacterial DNA from animal tissue samples using PCR is being considered as an alternative or complementary test to microbiological culture. Since the early 90's, many conventional PCRs have been developed and used for the direct detection of members of the MTBC in bovine samples (7). In those studies including fresh bovine tissue samples from animals with visible and non-visible lesions (VL and NVL), the reported sensitivity and specificity values of PCR with respect to culture showed great variability, ranging from 63 to 97%, and 50 to 97%, respectively (8–10). After the introduction of Real-Time PCR for the detection of MTBC species, sensitivity values increased with respect to conventional PCR. In those studies implementing Real-Time PCR in which bovine tissue samples with VL and NVL were analyzed, diagnostic sensitivity and specificity by Real-Time PCR ranged between 74 to 100% and 97 to 100%, respectively (6, 11–14). The variability in the values between studies depends not only on the type of PCR (conventional, nested or Real-Time PCR), but also on the PCR target (single- or multiple copy) and reagents, the type and number of samples included in the studies, and the DNA isolation methods. The largest study to date assessing the diagnostic performance of Real-Time PCR for the detection of MTBC using a Bayesian approach reported a diagnostic sensitivity of 87.7% and a specificity of 97% (6).

In this study, we describe the development and validation of a Real-Time PCR based on the *mpb70* gene, which encodes for a major antigenic protein conserved in all MTBC species. In addition, we assess its diagnostic performance in fresh bovine tissue samples obtained within the Spanish national eradication campaign.

MATERIALS AND METHODS

Real-Time PCR

PCR Design and Optimization

The *mpb70* gene was the target of this PCR since it encodes for a majorly expressed antigenic protein in *M. bovis*, which is conserved in all members of the MTBC. An *in silico* specificity analysis was carried out, in order to rule out any sequence homologies between other bacterial species, with the Basic Local Alignment Tool (BLAST) from the NCBI, using the *mpb70* CDS from *M. bovis* AF2122/97 (NC_002945.4). The *mpb70* sequence was then used to obtain *mpb70* homologs from the available MTBC genomic sequences deposited in the genbank (NCBI): *M. tuberculosis* H37Rv (NC_000962.3), *M. africanum* strain 25 (CP010334.1), *M. caprae* Allgeau (CP016401.1), *M. microti* strain 12 (CP010333.1), *M. mungi* strain BM22813 (LXTB01000090.1), *M. orygis* strain 112400015 (APKD01000057.1) and *M. canetti* CIPT 140010059 (NC_015848.1).

Oligonucleotides targeting the *mpb70* gene, specific for members of the MTBC, were designed to target a 133bp conserved amplicon with Oligo primer analysis software 6.0 (Molecular Insights, West Cascade, CO, USA): *mpb70*-forward: 5′-CTCAATCCGCAAGTAAACC-3′, *mpb70*-reverse: 5′-TCAGCAGTGACGAATTGG-3′ (15), and *mpb70*-probe: 5′- FAM-CTCAACAGCGGTCAGTACACGGT-BHQ1-3′. The amplicon sequences were obtained and aligned against the available MTBC sequences, as well as with the closest similarities obtained in the *in silico* specificity analysis (e.g., *M. kansasii, M. indicus pranii,* or *M. marinum*).

The Real-Time PCRs were carried out using the QuantiFast® Pathogen PCR + IC Kit (QIAGEN, Hilden, Germany). This kit includes an Internal Amplification Control (IAC), as well as specific reagents and primers/probes required for its amplification. It employs MAX™NHS Ester as a reporter dye. Different primer/probe concentrations and extension temperatures were tested in order to achieve maximum replication efficiency.

M. tuberculosis H37Rv DNA was used for the generation of the standard curve and positive controls. Ultra-pure distilled water was used as negative controls. This strain was grown in Löwenstein-Jensen slants in the BSL3 facilities at VISAVET Health Surveillance Center. A loop full of colonies was collected and heat inactivated (100°C) in 200 µl of ultra-pure distilled water during 15 min.

The efficiency and dynamic range of the reaction were assessed in triplicates using a standard curve prepared from a stock of 10 ng/µl of *M. tuberculosis* H37Rv genomic DNA, 10-fold serially diluted to a range of 1 ng/µl to 0.1 fg/µl. DNA concentration and quality of the DNA solution were measured in ten replicates using a nano-drop spectrophotometer (ThermoFisher, Waltham, MA, USA). The dynamic range of the reaction was established as the range of standard curve concentrations at which the coefficient of linearity was >0.997 and the cycle separation between the 10-fold dilutions was close or equal to 3.32 cycles. The limit of quantification was established as the lowest concentration point of the dynamic range of the reaction.

The optimized setup with a final 25 µl volume per reaction, including the Internal Amplification Control (IAC) was: 5 µl of 5x Quantifast Pathogen Master Mix, 2.5 µl of 10x IAC assay, 2.5 µl of 10x Internal Control DNA, 2 µl of 10 pmol/µl *mpb70*-Forward primer, 2 µl of 10 pmol/µl *mpb70*-Reverse primer, 0.75 µl of 10 pmol/µl *mpb70*-probe, 5.25 µl of ultrapure sterile distilled water (Sigma-Aldrich, St. Louis, MO, USA), and 5 µl of DNA sample. Primers and probe were obtained from Eurofins Genomics (Ebersberg, Germany).

All PCR reactions were carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) according to the following optimized cycling conditions; 95°C for 5 min followed by 45 2-step cycles of 95°C for 15 s and 60°C for 30 s, with data acquisition at this step.

Analytical Specificity and Sensitivity

The inclusivity of the PCR was tested against seven species of the *M. tuberculosis* complex: *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. caprae*, *M. microti*, and *M. pinnipedii*. Selectivity was assessed using a panel of 69 strains from 24 Non-Tuberculous Mycobacteria (NTM) species and 10 non-mycobacterial species (OM: Other Microorganisms); *M. avium* subsp. *hominissuis* ($n = 7$), *M. avium* subsp. *avium* ($n = 3$), *M. avium* group X ($n = 10$), *M. chitae, M. colombiense, M. europeum, M. flavescens, M. fortuitum* ($n = 3$), *M. gordonae, M. hibernae, M. intracellulare, M. kansasii* ($n = 9$), *M. marinum* ($n = 2$), *M. neoaurum, M. nonchromogenicum* ($n = 4$), *M. parascrofulaceum, M. peregrinum* ($n = 2$), *M. phlei* ($n = 2$), *M. scrofulaceum, M. seoulense, M. shimodei, M. smegmatis* ($n = 2$), *M. terrae, M. thermoresistible, M. vaccae, Brucella mellitensis, Brucella abortus, Salmonella enterica* Sv. Typhimurium, *Serratia maucencens, Rhodococcus equi, Enterococcus hirae, Lysteria monocytogenes, Nocardia* sp., *Streptomyces* sp., and *Corynebacterium pseudotuberculosis*. DNA for these bacteria was obtained from reference strains and clinical isolates from the VISAVET Health Surveillance Center (Complutense University of Madrid).

The analytical sensitivity or limit of detection (LOD) and intra-assay repeatability were estimated using a new standard curve that was prepared from a 10-fold serially diluted stock of *M. tuberculosis* H37Rv genomic DNA ranging from 1 ng/µl to 10 fg/µl, and one 1:5 dilution thereof to a concentration of 2 fg/µl (10 fg/reaction or 2 genomic equivalents). The reaction was carried out using 20 replicates per concentration and the LOD was established as the concentration in which at least 95% of the replicates were positive. Inter-assay repeatability was assessed in 20 replicates of 10 fg/µl *M. tuberculosis* DNA in a period of 6 months.

M. tuberculosis H37Rv genomic equivalents were obtained from the amount of DNA used for each point of the standard curve using the equation previously described (16): [ng of DNA $\times$ 6.023 $\times$ 10^{23} molecules/mol]/[bp length of genome $\times$ 10^9 ng/g $\times$ 660 g/mol]. The genome size recorded at the NCBI Genome entry of *M. tuberculosis* H37Rv (NC_000962.3) was used as a reference (i.e., 4.41 Mb). Genomic equivalents for each sample were obtained by extrapolating the Ct values with the quantities from the standard curve.

Selection, Preparation and Culture of Clinical Samples

Two-hundred fresh tissue samples from cattle were randomly selected from samples processed as part of the Spanish national eradication campaign against bTB during the period 2013–2017, based on the Royal Decree 727/2011. Processing took place within the BSL3 facilities of VISAVET Health Surveillance Center. Simple randomization was carried out by assigning a random value to each sample and by sorting them by increasing order. The first 200 samples of this list were included in the study. The selected tissue samples originated from 11 out of the 17 autonomic regions of Spain. Almost half ($n = 99$) of the samples were obtained from Madrid, followed by Castile-La Mancha ($n = 34$), Aragon ($n = 18$), Extremadura ($n = 13$), Valencia ($n = 12$), Murcia ($n = 8$), La Rioja ($n = 6$), Andalusia ($n = 3$), Canary Islands ($n = 3$), Balearic Islands ($n = 3$), and Castile and Leon ($n = 1$).

From the total amount of samples, 118 came from cattle that were positive to the single intradermal tuberculin (SIT) test (bovine PPD $\geq$ 4 mm), whereas 63 were from SIT-negative animals (bovine PPD $\leq$ 2 mm) and 4 had inconclusive results (2 mm $>$ bovine PPD $<$ 4 mm) according to the Royal Decree 727/2011. Following the regulation in force, in regions with high prevalence of bTB, SIT inconclusive results were considered as positive. Fifteen animals showed bTB-compatible lesions during routine abattoir inspection of carcasses and were also sent for sample collection and processing. Lymph nodes (retropharyngeal, mandibular, mediastinal, bronchial,

prescapular, mesenteric, hepatic, and/or supramammary) and/or organs were then collected for processing, culture and direct PCR.

Once in the laboratory, all tissue samples were visually inspected for lesions and sliced. A total of 78 samples had VL, whereas 122 had NVL. Approximately 2–2.5 g of tissue sample from the same animal were pooled and homogenized in 12 ml sterile distilled water in a Masticator (IUL, Barcelona, Spain) at max speed for up to 5 min. One ml of the homogenized sample was collected for DNA isolation, whereas the remainder of the homogenate was decontaminated with an equal volume of 0.75% (w/v) hexadecyl pyridinium chloride solution in agitation during 30 min (17). Samples were centrifuged during 30 min at 1,300–1,500 g. Pellets were collected with swabs and cultured in Löwenstein-Jensen with sodium pyruvate and Coletsos media (Difco, Spain) at 37°C for a maximum of 3 months. Culture was considered positive when isolates were identified as MTBC by conventional PCR (18) and /or DVR-spoligotyping (19).

Tissue DNA Extraction

DNA from tissues was obtained using the DNeasy Blood & Tissue Kit (QIAGEN) with a few modifications. Briefly, one ml of the homogenized tissue sample was added in a tube containing 100 mg of 0.5 mm and 50 mg of 0.1 mm glass beads and centrifuged for 5 min at 9,000 g. The supernatant was removed from the samples and 200 µl of sterile distilled water and 180 µl of ATL Buffer were added. Samples were then lysed in a Fastprep® FP120 homogenizer (MP Biomedicals, Santa Ana, CA, USA) using 3 cycles of 40 s at a speed of 6.5 m/sec. After an overnight chemical treatment with 20 µl of proteinase K at 56°C, the mechanical lysis step was repeated. Samples were then centrifuged briefly at maximum speed and 300 µl of supernatant were transferred to a new 1.5 ml Eppendorf tube and mixed with 400 µl of a mixture of AL buffer and 96% ethanol (equal volumes). The lysate was transferred to a spin column and was processed according to the manufacturer's instructions. DNA elution was carried out using 200 µl of AE buffer.

Diagnostic Performance

The diagnostic performance of the Real-Time PCR targeting the *mpb70* gene was assessed on 200 randomly selected tissue-extracted DNA samples. The exogenous heterologous IAC supplied with the kit was used to assess the presence or absence of inhibition phenomena. According to the manufacturer, the IAC should show Ct values of 30 ± 3. As a result, complete inhibition was defined when no IAC was amplified and partial inhibition was defined as a $Ct > 33$ for the IAC. If inhibition was detected, samples were diluted 5-fold and PCR was repeated. Results were compared against microbiological culture, and diagnostic sensitivity and specificity, Positive and Negative Predictive Values (PPV/NPVs) as well as Positive and Negative Likelihood Ratios (PLR and NLRs) were calculated using MedCalc 18.2.1 (MedCalc, Ostend, Belgium). Agreement between culture and Real-Time PCR results was assessed using Cohen's Unweighted Kappa in WinEpi 2.0 (20).

Samples with culture-negative and PCR-positive results were further analyzed by DVR-spoligotyping (detection of spacers) and sequencing of the 16S rRNA gene. Sequencing of a 1,030 bp fragment of the 16S rRNA gene (18) was carried out externally by STABvida (Lisbon, Portugal). The obtained sequences were analyzed using the Bioedit software version 7.1.3.0 (21). Samples that gave a positive result to either of the above two techniques were considered as true positives and were used to re-calculate the diagnostic performance of the Real-Time PCR. On the other hand, for samples with a culture-positive and PCR-negative results DNA extraction and PCR were repeated.

RESULTS

In silico Analysis

Sequence similarity between *mpb70* homologs in members of the MTBC is 99.7–99.8% (data not shown). Even though some non-MTBC species -such as *M. kansasii, M. marinum* or *M. gilvum*- have homologous *mpt70/mpb70* sequences (22), sequence similarity with these species is limited (data not shown). Alignments of the *mpb70* amplicons with MTBC species showed 100% identity, with exception of *M. canetti* that had a T/C substitution at position 360 (*M. bovis* AF2122/97 numbering from *mpb70* CDS start). Although this substitution falls within the length of the reverse primer, it did not affect the ability of the primer to anneal to its target in *M. canetti. M. indicus pranii, M. kansasii* and *M. marinum,* had a considerably lower identity, indicating that specificity issues would be unlikely (data not shown).

Optimization and Analytical Sensitivity and Specificity

For optimization of the PCR reaction and repeatability studies, two 10-fold diluted standard curves were prepared from a 10ng/µl stock of *M. tuberculosis* H37Rv (DNA stock concentrations with Standard Deviations or SDs of 0.35 and 0.97, respectively).

The lowest concentration of DNA detected in the standard curve by the Real-Time PCR was 10 fg/µl (50 fg/reaction or ∼ 10 genomic equivalents) with all three replicates showing an amplification curve. The dynamic range of the reaction spanned from 1 ng/µl (5 ng/reaction or approx. 10^6 genome equivalents) to 10 fg/µl (50 fg/reaction or ∼ 10 genome equivalents), with an R^2 of 0.999. The upper and lower Ct values of the dynamic range were 20.06 and 36.33, respectively. The quantification limit was set to 10 genome equivalents/ reaction. Replication efficiency was 102.60% with a slope of −3.27.

All 20 replicates with a concentration of 50 fg/reaction were positive for this PCR, whereas only 14/20 of the 10 fg/reaction aliquots were positive. The Ct values of both dilutions were, respectively, 37.07 (SD 0.98) and 38.92 (SD 1.28). Therefore, the limit of detection for this Real-Time PCR with a 95% confidence was 10 fg/µl (50 fg/reaction or 10 genomic equivalents) and the cut-off was set to a $Ct < 40$.

The Real-Time PCR reacted positively only against members of the MTBC and no cross-reactions were detected against any of the NTMs or non-mycobacterial species tested.

Diagnostic Performance Compared to Microbiological Culture

Two hundred DNA samples obtained from bovine tissues were analyzed using this PCR and microbiological culture. A total of 69 samples were MTBC positive for culture, whereas 131 were negative (**Table 1**). Ten out of the 131 culture-negative samples showed growth of non-tuberculous mycobacteria ($n = 4$) or other microorganisms ($n = 6$) (NTM/OM). The Real-Time PCR detected 71 positive samples, with a minimum and maximum Ct values of 24.39 and 39.35, respectively and a median Ct value of 33.48. Sixty-one out of 69 positive culture samples were also positive for the Real-Time PCR targeting *mpb70,* resulting in a sensitivity relative to culture of 88.41% (95% CI: 74.3 to 94.86%). Ten of the 131 culture-negative samples were positive for the Real-Time PCR, and the specificity value was 92.37% (95% CI: 86.41 to 96.28%). Of the 10 cultures showing growth of NTM/OM, one reacted positively to the direct Real-Time PCR.

The exogenous heterologous IAC used in this PCR detected complete inhibition in only 4 out of 200 samples (2%) and partial inhibition (IAC Ct $>$ 33) in 15 out of 200 samples (7.5%). After dilution, all these samples were PCR negative. One of the completely inhibited samples and 3 of the partially inhibited samples were positive to culture.

PPVs and NPVs were 85.92% (95% CI: 76.97 to 91.76%) and 93.80% (95% CI: 88.72 to 96.67%), respectively. The positive and negative likelihood ratios were, respectively, 11.58 (95% CI: 6.34–21.14) and 0.13 (95% CI: 0.07–0.24). There was a very good correlation between culture and PCR results (Cohen's Unweighted Kappa = 0.802).

Samples with discording results between the two methods used were further analyzed. DNA isolation was repeated for the 8 culture-positive PCR-negative samples. Of these, half ($n = 4$) gave a positive result. For samples with culture-negative and PCR-positive results ($n = 10$), spoligotyping and 16S RNA sequencing were applied and the presence of MTBC DNA was confirmed in 5 of them. Of these, one presented growth by an actinomycete and 4 were negative to culture. These samples, in addition to all culture-positives, were considered to be true positives. As a result, the corrected relative sensitivity and specificity of PCR was calculated to be 94.59% (95% CI: 86.73% to 98.51%) and 96.03% (95% CI: 90.98–98.70%), respectively (**Table 1**). PPVs and NPVs were, then, 93.33% (95% CI: 85.55–97.07%) and 96.80 % (95% CI: 92.10–98.74%). PLRs and NLRs increased to 23.84 (95% CI: 10.08–56.37) and 0.06 (95% CI: 0.02–0.15), respectively. Correlation between culture and PCR increased to 0.904.

Among samples with VL ($n = 78$), 65 and 61 were positive to PCR and culture, respectively (**Table 2**). Three out of 7 culture-negative and PCR-positive samples were shown to contain MTBC DNA by sequencing or spoligotyping. Although 3 culture-positive samples were negative for this PCR, they became positive after the extraction protocol was repeated. Regarding NVL samples (n=122), a total of 6 samples were positive to PCR whereas 116 were found to be negative. In contrast, 8 NVL samples were culture-positive and 114 samples were culture-negative. Of these culture-negative samples, 3 were positive for the Real-Time PCR, of which 2 were confirmed as true positives by sequencing or spoligotyping. On the other hand, 5 culture-positive samples were negative for the *mpb70*-specific PCR. However, one of them was positive after the repetition of the extraction protocol. After confirmation of the true positives, Cohen's Unweighted Kappa between culture and PCR for VL and NVL samples was, respectively, 0.804 and 0.685.

Intra and Inter-Assay Variation

The intra-assay repeatability at a concentration of 10 fg/μl showed an average Ct value of 37.07 with a standard deviation of 0.98 and a coefficient of variation of 2.63%. Inter-assay repeatability using 20 replicates from a stock of 10 fg/μl in a 6 month period showed an average Ct value of 36.70 with a SD of 1.40 and a CV of 3.82%.

DISCUSSION

The purpose of this study was the design, optimization, and validation of the *mpb70* Real-Time PCR for the detection of members of the *M. tuberculosis* complex directly from animal tissue samples. In addition, this study compared the diagnostic performance of this PCR and bacteriological culture using a large number of bovine tissue samples ($n = 200$) collected in the framework of the Spanish bTB eradication program.

The Real-Time PCR targeting the *mpb70* gene showed 100% of inclusivity and selectivity. Moreover, it shows good replication efficiency (102.6%), and an analytical sensitivity of at least 10 genome equivalents with 95% confidence. Furthermore, very little variation was seen at the LOD both within and between assays (CV=2.63 and 3.82%, respectively). In addition, the linear range of the reaction spans from 5 ng/reaction (approximately 10^6 genomic equivalents) to 50 fg/reaction ($\sim$ 10 genomic equivalents). Although this PCR was developed for the detection of MTBC, the single-copy nature of the target and the wide linear range of the reaction make this PCR a suitable candidate for absolute quantification studies of MTBC in tissues. In fact, 59 out of the 71 *mpb70* PCR-positive samples showed a Ct value within the dynamic range of the reaction (data not shown). Although the quantification was not possible due to the absence of the standard curve in all runs, the range of concentrations was estimated to be between 2.29×10^5 and 63 genomic equivalents, with an average Ct value of 33.33 ($\sim$ 415 genome equivalents).

Overall, there was a good correlation between microbiological culture and PCR results in this study. Furthermore, diagnostic sensitivity and specificity values were very good when compared to microbiological culture (88.41 and 92.37%, respectively). Eight samples were negative to the direct Real-Time PCR but positive to microbiological culture. After repetition of the DNA extraction protocol, half of them became positive to the PCR. This implies that the DNA extraction protocol is very important and directly affects the sensitivity of the PCR. Several factors influence the DNA yield and quality obtained through DNA extraction protocols.

TABLE 1 | Comparison of results obtained by analyzing 200 randomly selected cattle samples by microbiological culture and Real-Time PCR.

		Culture/True positives*				Diagnostic performance	
		Result	**+**	**–**	**Total**	**Sensitivity**	**Specificity**
Raw results	PCR	+	61	10	71	88.41% [95% CI: 78.43–94.86%]	92.37% [95% CI: 86.41–96.28%]
		–	8	121	129		
		Total	69	131	200		
Corrected results	PCR	+	70	5	75	94.59% [95% CI: 86.73% to 98.51%]	96.03% [95% CI: 90.98–98.70%]
		–	4	121	125		
		Total	74	126	200		

**Corrected results consider as true positives: (1) those samples that were culture positive, (2) samples that were culture-negative but PCR-positive, and for which MTBC presence was demonstrated by 16S sequencing and/or spoligotyping, and (3) culture-positive and PCR-negative samples that became positive after the DNA extraction was repeated.*

TABLE 2 | Comparison of results obtained by analyzing 200 randomly selected veterinary samples by microbiological culture and Real-Time PCR, according to the presence or absence of anatomic lesions.

		Culture (True positives)		
		+	**–**	**Total**
VL	PCR +	58 (64)	7 (4)	65 (68)
	PCR –	3 (0)	10	10
	Total	61 (64)	17 (14)	78
NVL	PCR +	3 (6)	3 (1)	6 (7)
	PCR –	5 (4)	111	116 (115)
	Total	8 (10)	114 (112)	122

Culture negative and PCR-positive samples were considered true positives (in brackets) after the confirmation of the presence of MTBC DNA by 16S rRNA gene sequencing and/or spoligotyping.

Firstly, the amount and type of processed tissue could determine the bacterial load in the sample. The extraction protocol used in this study uses a volume of sample that is 1/10 the amount of sample used for microbiological culture, which could produce a loss of sensitivity due to the decreasing amount of bacteria available for extraction. In addition, the presence or absence of lesions can affect the amount of bacteria in the sample which in turn could determine the quantity of available DNA. In this study, the four remaining culture-positive and PCR-negative samples had NVLs, of which 3 were positive to the SIT test. This suggests that the animal may have been at early stages of infection and, therefore, have low bacterial loads. On the other hand, the recovered samples after the second extraction ($n = 4$) had mostly VLs ($n = 3$).

Secondly, the type of disruption technique used can have an important effect in the DNA extraction process. Even though the protocol in this study has been optimized to obtain a high amount of DNA through two mechanical and one overnight chemical lysis steps, improvements in the extraction protocol may reduce the number of discording results. Park et al. showed that increasing the incubation time before mechanical lysis with ATL buffer up to 3 h increased the DNA yield in *M. avium* subsp. *paratuberculosis* when compared to no pre-treatment (23). On the other hand, an 8-h pre-treatment was detrimental to the amount of extracted DNA, achieving the same amount of DNA as the no pretreatment controls. The effect of reduction in the pre-treatment incubation time should be assessed in the future. Another improvement could include the use of a homogenizer instead of a masticator in the tissue homogenization step, which could release a larger amount of bacteria from tissue samples for extraction.

Furthermore, several factors associated with the extraction protocol may introduce inhibitors in the sample, such as organic compounds or excess host DNA. In order to detect the inhibition of the PCR, the reaction mix includes an exogenous heterologous IAC, with a randomly generated DNA supplied by the manufacturer. By using the IAC, 4 and 15 samples were found to be completely or partially inhibited, respectively. Of these, 1 inhibited and 3 partially inhibited samples were culture-positive, and they remained PCR-negative after a 1:5 dilution. After repeating the extraction protocol on these samples, the inhibited and one partially-inhibited sample became positive, indicating that their dilution may have caused the further dilution of the target DNA and, therefore, may have resulted in the loss of sensitivity in the PCR. Furthermore, this could imply that the inhibitor was not present in the sample and was introduced as a result of the DNA extraction procedure, or that the extraction protocol failed to remove it in the first place. Other reported PCRs also include IACs, but only a few include information regarding the presence or absence of inhibition (11–13). Although no cases of inhibition were detected in these publications, they used endogenous or exogenous homologous IACs, which may present some disadvantages with respect to exogenous heterologous IACs. For instance, the amount of endogenous IAC template (i.e., bovine β-actin gene) varies depending on the type of sample or extraction method used, which means that readouts vary between samples and there is no indication of the level of inhibition present in the sample. In addition, they can overcome inhibitory effects in the sample as they are usually in higher concentrations than the target. Exogenous homologous IAC (i.e., *M. bovis* DNA), on the other

hand, are recognized by the target's primers and can, therefore, give rise to competition events that can hinder the amplification of the target DNA in low-concentrated samples, such as those close to the LOD. Exogenous heterologous IACs, such as the ones used in this study, use a consistent amount of control template, different to the target of interest, with a set amplification cycle. As a result, it allows the detection of complete or partial inhibition phenomena and minimizes competition, since the primers and the control target sequence are completely different to those of the target of interest.

Ten samples were negative to culture but positive to the Real-Time PCR. The use of spoligotyping and/or 16S rRNA sequencing on these discording samples showed the presence of MTBC DNA in 5 of them. The inability of culture to detect MTBC in these samples may be due to sample processing issues in which bacterial integrity is hampered and growth is impeded. In addition, very advanced granulomatous lesions may contain lower numbers of viable bacteria than early granulomas (24). Nevertheless, MTBC DNA can still be present in non-viable bacteria in enough quantity to be detected by PCR after purification. Although no histopathological evaluation was done on these samples, 7 of the culture-negative and PCR-positive samples were obtained from animals with VLs whereas 3 were obtained from animals with NVLs. Finally, growth of NTM/OM could be another reason for these discrepancies. In fact, 1 of the 10 tissue samples that showed growth of NTM/OM during culture was positive to this PCR, indicating that the growth of MTBC in culture could have been hampered by the growth of other NTM/OM. The detection of MTBC DNA in this sample by 16S rRNA sequencing and spoligotyping supported the analytical specificity of the *mpb70* oligonucleotides, indicating that the presence of other microorganisms in the sample will not interfere with this PCR.

When the presence of MTBC DNA was confirmed in the discording samples, these were considered as true positives. Therefore, 70 positive samples were correctly identified by PCR, increasing the diagnostic sensitivity and specificity values with respect to culture (from 88.41 to 94.59% and from 92.37 to 96.03%, respectively). PPVs and NPVs were 93.33 and 96.80%, respectively. Furthermore, the PLR was 23.84 indicating a high probability of correctly identifying a bTB-positive tissue sample. In addition, the NLR was very low (0.06), indicating a low probability of a negative result being positive.

The most commonly used genetic target for PCR detection of MTBC species is the IS*6110* transposon (25). Other targets used in the detection of MTBC members in veterinary samples through PCR include the 16S-23S rRNA Internally Transcribed Spacer or ITS (14), *hupB* (26), TbD1 (11), *rv2807* (12), and *devR* (16). The high sequence similarity between the different MTBC species and the single-copy nature of the *mpb70* gene make it also a suitable target for both detection and quantification through Real-Time PCR. Since the early 1990's, the *mpb70* gene has been used extensively for the detection of MTBC species through conventional PCR (18, 27–29). Additionally, it has been used as a target for Real-Time PCR quantification of MTBC members in infected cell culture extracts (15). However, in this study hybridization probes were added to increase specificity.

Although the diagnostic specificity of this PCR was similar (96.03 vs. 97%) to that seen for the Real-Time PCR used by Courcoul et al. targeting the IS*6110* element (6), diagnostic sensitivity was higher in this study (94.59% vs. 87.7%). When compared against a Real-Time PCR detecting the IS*6110* element based on melting curve analysis and hybridization probes (30), the *mpb70*-targeting PCR showed a better correlation with culture results and increased diagnostic sensitivity. A semi-nested Real-Time PCR targeting the IS*6110* showed very similar diagnostic sensitivity, specificity and predictive values to those obtained in this study; 100% diagnostic sensitivity, 97.7% diagnostic specificity, 96.3% PPV and 100% NPV (13). Even though the LOD is lower for this semi-nested Real-Time PCR (1.5fg vs. 50fg), the requirement of two PCR steps increases the risk of cross-contamination. In addition, a Real-Time PCR targeting the 16S-23S ITS showed a moderate diagnostic sensitivity of 73.87% (14).

It is important to consider that the diagnostic performance of this PCR in this study does not give information about the infection status of all animals included in this study, as it only compares culture and PCR on tissue samples. Based on the results of this study and previous publications, direct PCR has some advantages compared to culture for the detection of MTBC species in animal tissue samples. In the first place, PCR takes a few hours to complete in comparison to the weeks required for microbiological culture. Secondly, analytical specificity can be extremely high if the appropriate oligonucleotides are designed, limiting cross-reaction with contaminating microorganisms. This removes the requirement for a decontamination step, decreasing the hazardous conditions applied to the sample. Furthermore, it would reduce the risk of exposure to mycobacteria as it decreases the processing time of tissues with suspected MTBC infections before inactivation, the amount of time spent at BSL3 facilities and the bacterial load to which the user is exposed to. In addition, the *mpb70* PCR showed a comparable limit of detection and diagnostic sensitivity to that seen in IS*6110* PCRs. One disadvantage of the IS*6110* target over the *mpb70* is the risk of horizontal transfer of mobile elements between mycobacterial species, as has been recorded for IS*1245* and *M. kansasii* (31). Moreover, the IS*6110* is present in a variable number of copies within the genome of certain MTBC species, which limits its use in quantitative studies, unlike the *mpb70* gene, which is a single-copy gene.

The results obtained in this study open the possibility of using the direct Real-Time PCR as an alternative to microbiological culture in the short term. Although microbiological culture is still needed for bacterial isolation and molecular characterization with epidemiological purposes, PCR could decrease considerably the time needed until results are obtained, improving the decision making capacity during the eradication campaigns.

In conclusion, the Real-Time targeting the *mpb70* gene is a time-effective and efficient method for the detection of MTBC members in veterinary tissue samples, which shows improved diagnostic performance with respect to culture. In addition, it has a low detection limit of 10 genomic equivalents/reaction of MTBC species. Furthermore, being a single copy gene and

having a dynamic range of 10^6-10 genomic equivalents/reaction, it could be used for quantification studies of as little as 10 genomic equivalents.

AUTHOR CONTRIBUTIONS

VL-L and EL performed all experiments in this study and the *in silico* specificity analysis. EC participated in the design of the *mpb70* specific oligonucleotides used in this study. BR, EL, and LdJ designed the study. LD and LdJ are responsible for the obtaining of samples. VL-L wrote the manuscript with the invaluable insights of EC, JB, EL, LD, BR, and LdJ. BR directed and supervised the complete study.

FUNDING

VL-L was funded with a predoctoral grant from the Complutense University of Madrid and Banco Santander 2017–2018.

ACKNOWLEDGMENTS

We would like to thank the excellent work performed by laboratory technicians F. Lozano, T. Alende, A. Gutiérrez, N. Moya, C. Viñolo, D. de la Cruz, and L. Jimenez, in culturing and spoligotyping. We would also like to thank the work carried out by Susana Gómez during the design of the primers and probe, and Pilar Pozo with sequencing. In addition, we would like to appreciate valuable statistical insights given by María Luisa de la Cruz and Julio Álvarez. This work was supported by the Área de Ganadería de la Comunidad de Madrid, the Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente, and the Programa de Tecnologías Avanzadas en Vigilancia Sanitaria (TAVS) de la Comunidad de Madrid (S2013/ABI2747). This work was supported by the Programa de Tecnologías Avanzadas en Vigilancia Sanitaria (TAVS) from the Comunidad de Madrid (ref. S2013/ABI-2747).

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Conflict of Interest Statement: EC is a full-time employee of Exome Diagnostics Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 26 June 2018
doi: 10.3389/fvets.2018.00141

Development of a Multidimensional Proteomic Approach to Detect Circulating Immune Complexes in Cattle Experimentally Infected With *Mycobacterium bovis*

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Objective: To evaluate a high-resolution method to identify pathogen-specific biomarkers in serum of calves infected with *Mycobacterium bovis*.

Methods: Serum samples from four calves infected with *M. bovis* were collected before and after infection at weeks 9, 14, 15, 31, and 36. Immune-complex-associated mycobacterial antigens in the serum were enriched using an immunochromatography method termed, dual path platform (DPP). All regions of antigen capture zones, that consisted of monospecific rabbit polyclonal antibodies raised against *M. tuberculosis* lysates, on DPP strips were excised and analyzed by multidimensional proteomics. The resulting proteins were then passed through 4 rigorous peptide quality filters-false-hits, decoys, non-*M. tuberculosis* complex proteins were all removed followed by individual quality check of those remaining. Peptides were then checked on NCBI's BLASTp for *M. tuberculosis* complex specificity.

Results: Proteins in 2 of the animals passed the multipronged-highly stringent peptide quality analysis. Animal#54 had 7 unique *M. tuberculosis* complex proteins at week 14 post-infection, while animal#56 had 4 at week 36 post-infection along with 1 immunoglobulin.

Conclusion: *M. tuberculosis* complex -specific peptides identified in this study were identified in 2 animals and at 2 separate time points post infection. Further studies with better enrichment protocols and using larger sample sizes and replications are required to develop a TB-specific diagnostic tool for bovine tuberculosis.

Keywords: bovine tuberculosis, dual path platform, immune-complexes, mass-spectrometry, *Mycobacterium bovis*, mycobacteria, biomarkers, diagnostics

OPEN ACCESS

Edited by:
Adrian Allen,
Agri Food and Biosciences Institute, United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *14 February 2018*
Accepted: *06 June 2018*
Published: *26 June 2018*

Citation:
Hadi SA, Waters WR, Palmer M, Lyashchenko KP and Sreevatsan S (2018) Development of a Multidimensional Proteomic Approach to Detect Circulating Immune Complexes in Cattle Experimentally Infected With Mycobacterium bovis. Front. Vet. Sci. 5:141. doi: 10.3389/fvets.2018.00141

INTRODUCTION

Mycobacterium bovis causes tuberculosis primarily in cattle but it is also zoonotic. Transmission to humans occurs through close contact with infected animals or via consumption of contaminated animal products (e.g., unpasteurized milk or dairy products) (1–3). The primary screening test used in the field is tuberculin-based skin test which is time-consuming, labor intensive and associated

with low sensitivity and variable specificity. Variability in specificity is caused by species differences and technique being used (4, 5). Ultimately a false-positive can lead to a considerable financial burden on farmers deterring control measures. Thus, there is a need for highly specific rapid field tests that are cost effective.

Immune complexes are formed by the non-covalent binding of antigens with antibody molecules circulating real-time (6). Lyashchenko et al. (7), reported the presence of *Mycobacterium* specific immune complexes in cattle experimentally infected with *M. bovis* detectable by the dual-path platform (DPP) assay that utilizes polyclonal antibodies against *M. tuberculosis* whole-cell antigens. This provided an unprecedented opportunity to interrogate *M. tuberculosis* complex-specific antigens enriched by polyclonal tuberculosis-specific antibodies using high resolution technique of liquid chromatography followed by dual mass-spectrometry (LC-MS/MS). LC-MS/MS can detect proteins at abundances as low as 10^{-15} moles, thereby enabling discovery of circulating in infected animals. In the present study, high-resolution multidimensional mass spectrometry analysis of the DPP-captured immune complexes was evaluated for its ability to identify the captured *M. bovis*-specific peptides that may aid in the development of a highly accurate tuberculosis diagnostics for animals and humans.

MATERIALS AND METHODS

Seven Holstein calves obtained from a TB-free herd in IA and housed in a biosafety level 3 (BSL-3) facility at the National Animal Disease Center, Ames, IA were infected at 11 months of age with 8×10^3 CFU of virulent *M. bovis* (95-1315; USDA Animal Plant and Health Inspection Service [APHIS] designation) by aerosol. This strain of *M. bovis* was isolated from a white-tailed deer in Michigan, USA. Animals were sampled for serum at multiple time points pre- and post-infection over the next 11.5 months at which point they were euthanized (7, 8). Necropsy of all the calves revealed presence of gross lesions in multiple organs specific to bovine tuberculosis and bacterial culturing from infected tissues confirmed the presence of *M. bovis* in all 7 animals infected.

In this pilot study we focused on 4 out of the 7 calves present in the original study (7), since they had the highest levels of circulating immune complexes to increase the probability of biomarker discovery. Pre- and post-inoculation samples collected at weeks 9, 14, 15, 31, and 36 were used to identify mycobacterial specific peptides. To characterize the circulating immune complexes-associated with *M. tuberculosis* complex, a rapid DPP-Ag assay was performed (**Figure 1**). The DPP antigen capture zone (test line) was coated with rabbit polyclonal antibodies raised against *M. tuberculosis* whole-cell lysate to enable capture of mycobacterial antigen-antibody complexes (7, 8). Pre-infection (baseline) sera from these four animals served as negative controls. Triplicates of each time points from every animal were made pre and post-infection (which summed up to 27 DPP-Ag assay strips for analysis) for each week 0, 9, 14, 31 and 36. A 50 μL aliquot of serum sample was placed on three independent DPP-Ag strips for each time-point, to allow for antigen enrichment of molecules on the capture zone, which were then processed as one single sample to allow for maximum enrichment, enhanced sensitivity, efficient use of the LC-MS/MS and improved proteomics profile generation.

The immune-complex capture zone of 2 mm width was excised and analyzed by LC-MS/MS analysis performed at University of Minnesota's Center for Mass Spectrometry and Proteomics (CMSP). Triplicates of DPP-assays for every animal were pooled for analysis. A region 2 mm upstream of the antigen capture zone (or the DPP test line) was also analyzed by LC-MS/MS. The enormous volume of peptide data generated by LC-MS/MS was passed through a series of stringent filters before the final candidates were considered.

First, PEAKS (Bioinformatics Solutions Inc.) software was used to query peptides generated in each triplicate-pooled-sample through LC/MS-MS against a database that included all documented peptides from *M. tuberculosis* Complex, cattle and rabbit proteins. These results were then analyzed by Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) to validate all MS/MS based peptide identifications and to allow combined visualization of all sample results. All identified peptides were compared against a decoy database (generated in Scaffold_4.7.5), consisting of randomized peptide sequences, to remove any spurious hits. Second, any protein that matched against the decoy database, was removed from further analysis. We focused only on the *M. tuberculosis* complex proteins because they offer highest possible specificity for bovine tuberculosis diagnostics. The third filter was based on an individual quality check of the proteins with in Scaffold. Peptide identifications were accepted in Scaffold if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (9) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Peptides and proteins that were selected in the third filter had percent probabilities varying from 74 to 100%. The fourth and the last filter was the identification of *M. tuberculosis* complex specificity using the National Center for Biotechnology Information (NCBI)'s non-redundant database BLASTp (basic local alignment search tool for proteins) analysis where two aspects were investigated: (1) *E*-value ($<1e^{-10}$) and (2) the species match of the peptides. If the proteins matched with any other bacteria other than *M. tuberculosis* complex, they were excluded from further consideration. Additionally, if any peptides had an *E*-value higher than $1e^{-10}$, which suggested that the species match was likely non-random, they were also removed from further consideration. This last filter was excessively stringent as it eliminated most of peptide hits discovered after decoy database search. Some of the peptides eliminated may still be useful in a future validation study.

The same pipeline was followed for identifying cattle specific immunoglobulins, where immunoglobulins were passed through all the filters described for *M. tuberculosis* complex proteins. Additionally, the proteins that overlapped between pre-infection and post-infection test-lines were excluded as it suggested that they were not associated with the *M. bovis* infection, rather existed in the background.

RESULTS AND DISCUSSION

The peptides generated from LC-MS/MS analysis resulted in identification of 26,945 proteins. Forty-nine percent of these were eliminated after the decoy database search. Of these, 3.73% were identified with the *M. tuberculosis* complex repertoire, 26.02% proteins were of host (bovine) origin and 21.35% were of leporine origin. DPP strips of all post-infection samples, except at week 31, had *M. bovis* proteins. After analysis, 11 *M. tuberculosis* complex-specific proteins were identified in two *M. bovis*-infected animals (**Table 1**). At week 14 (post-infection) serum from animal #54 showed 7 proteins that corresponded to peptides in *M. tuberculosis* complex with a BLAST *E*-value lower than $1e^{-10}$ (**Table 2**). At week 36 post-infection, serum of animal #56 had 4 proteins that corresponded to *M. tuberculosis* complex with an E-value lower than $1e^{-10}$(**Table 2**).

At week 14 post-infection in animal #54 polyketide synthase was detected, which plays a role in the growth of the bacteria and is considered a potential virulence factor (10). The detection of this protein at such an early stage in *M. bovis* infection agrees with other studies (11, 12) where polyketide synthase was detected through different techniques but at similar time points. Lamont et al. (11), showed that polyketide synthase can be used as a useful marker for detecting *M. bovis* infection in a multi-cut off fashion, based on the prevalence of the disease.

Killer cell immunoglobulin-like receptor ($1e^{-18}$) in animal #56 at week 36 corresponded to cattle (*Bos taurus*) specific immunoglobulin, alone passed all analysis filters. Even though pre-infection DPP-assays from all 4 animals were pooled together to enhance the probability of capturing all mycobacterial circulating immune complexes at base-line to compare them with proteins detected post-infection, no immune-complexes were detected at base-line.

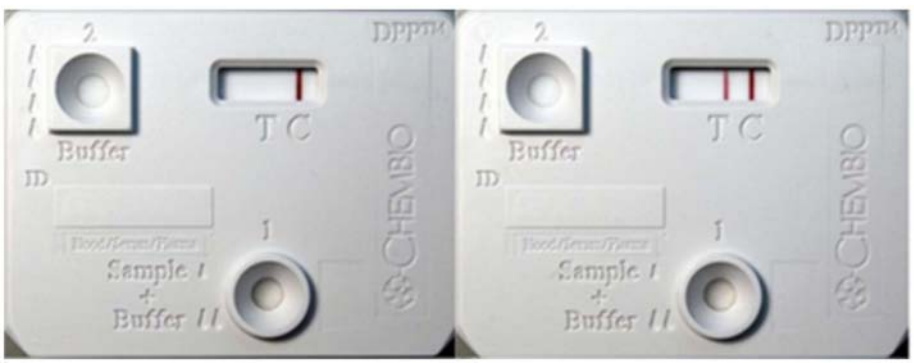

FIGURE 1 | Dual-path platform assay kit showing positive and negative controls. Dual-path platform assay was used to detect circulating antigen-antibody complexes in calves infected with *Mycobacterium bovis*. The rabbit polyclonal antibodies immobilized on the test line(T) acted as the capture reagent for the circulating immune-complexes in the infected animal's serum as well as signal detector when coated onto nano-gold-particles. DPP strip case: **Left**: Negative Control (serum from uninfected animals), **Right**: Positive control.

TABLE 1 | Enumeration of pathogen-derived proteins detected by mass spectrometry from DPP-Ag assay strips processed with serum samples from cattle experimentally infected with *Mycobacterium bovis*.

Animal ID	Pre-infection			*Mycobacterium bovis* infection			
	DPPAg result[a])	*Mycobacterium bovis* proteins identified[b]	Cattle specific immunoglobulins	Week post-inoculation	DPP-Ag result[a]	*Mycobacterium bovis* proteins identified	Cattle specific immunoglobulins
51	0	0	0	9	124	0	0
54	0	0	0	14	788	7	0
				15	772	0	0
56	0	0	0	36	485	4	1
57	0	0	0	31	447	0	0

[a] *DPP reader data (reflectance) in relative light units obtained as described (7).*
[b] *Pooled DPP-Ag strips processed with pre-infection sera from four calves.*

TABLE 2 | List of Mycobacterium tuberculosis complex-specific high confidence proteins at week 14 and week 36 and cattle immunoglobulin at week 36 that passed exclusion criteria.

***Mycobacterium tuberculosis* complex proteins at wk14**	**Peptide sequences used for BLASTp analysis**
Acyltransferase	QDGSASYDAAVR-MLKAGELVGVYPEATISR
Esterase	VFGAADPR-FACVVRAFASMFPGR
LLM class F420-dependent oxidoreductase	QKDYDEYGYR-FGTAGSRLDDLAAPLPR
Transposase, partial	MDPTEDQARALAR-VTGIGTVKPSLRVLR
Transcriptional regulatory protein embr2	FGILGPLEISAGFRSLPLGTPK-SPLGRLPLR
Hypothetical protein Mb3478	GASPATAAR-LPPALNPDDADALPTTDRLTTR
Polyketide synthase	DGDRVLAIVR-LVDAPLPSWTHRTLMLSR-MFNSLGIQYGPAFSGLVAVHTAR-LFVVTRSAASVLPSDLANLEQAGMR
***Mycobacterium tuberculosis* complex proteins at wk36**	**Peptide sequences used for BLASTp analysis**
Helicase helz	VYAHHGGARLHGEALRDHLER-RGNVLAAMAKLK-IDEMIEEKKALADLVVTDGEGWLTELST
Hypothetical protein Mb1791	FGVTINDVVVALCAGALRR-VPSQISDPAQR
Hypothetical protein Mb2390c	HGHGRDVAAHR-TGGHRQASSRIK-HQKPGDVPRDPRC
Chromosome partition protein Smc	LDTMAANLARLTDLTTELR-LAVRTAEER
Cattle Immunoglobulin at week 36	**Peptide sequences used for BLASTp analysis**
PREDICTED: killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2 isoform X2	GEMLTSGHAPADFVIGPMTLASAGTYR

The proteins that did not comply with exclusion criteria were removed such as non-Mycobacterium tuberculosis complex proteins, decoy proteins, low quality proteins and proteins that did not identify with Mycobacterium tuberculosis complex when NCBI's non-redundant database BLASTp (basic local alignment search tool for proteins) analysis was used. The same pipeline was used to identify cattle immunoglobulins. The hyphen represents the space given in NCBI's BLASTp search that allowed to account for the presence of other amino acids in between the peptides that formed the proteins.

The panel of mycobacterial proteins and cattle specific immunoglobulin reported in the present study may be specific to the infection stage at which they were detected, as the proteins seen at week 14 did not overlap with those detected at week 36 post infection. Alternatively, since these distinct proteins sets were found in two different animals, they could be a result of animal-to-animal variation in host response to the infection.

A major limitation of our study was sample size. Since the LC/MS-MS analysis itself was expensive and limited amounts of infected animal sera were available, multiple replications on the same animals were not possible. Additionally, multiple logistical and financial issues precluded us working with larger sample sizes: (1) working with agricultural animals for experimental infection with a BSL-3 pathogen and (2) Expenses associated with a BSL-3 cost, animal costs as well as personnel. Thus, to compensate for this limitation triplication of every animal's sample was performed.

Furthermore, the use of an antiserum derived against *M. tuberculosis* may have compromised specificity of our approach to detect *M. bovis* specific antigens, although these organisms are genetically very closely related. Future analysis though should include multiple replications of experimental infections followed by DPP assay and LC-MS/MS to discover *M. bovis* specific peptides in a reproducible and accurate fashion. Furthermore, a field validation on multiple exposure levels in outbreaks would be necessary for this technology to be applicable in routine bovine TB diagnostics.

In conclusion, the panel of 11 proteins reported in this study are specific to *M. bovis*. Further studies with more robust enrichment methods and larger sample sizes would be required to confirm these findings. Further validation of the identified circulating immune-complexes in naturally infected cattle would enable us to effectively and broadly apply the DPP technology in field.

ETHICS STATEMENT

All studies were approved by the National Animal Disease Center Animal Care and Use and Institutional Biosafety committees and performed under appropriate project licenses within the conditions of the Animal Welfare Act.

AUTHOR CONTRIBUTION

WW and MP conducted the animal infection and testing studies. KL developed the DPP lateral flow devices. SS and SH developed protocols for extraction of total proteins from DPP devices, performed proteomics and data analysis. WW, SH, KL, and SS wrote the paper.

FUNDING

The project was supported by Grand Challenges – UMN (Interspecies transmission of tuberculosis in Uganda) and USDA-NIFA grants funded to SS and to KL (Award No. 2016-33610-25688).

ACKNOWLEDGMENTS

We would like to thank University of Minnesota's Center of Mass Spectrometry and Proteomics, especially Mr. Todd W. Markowski for his input and guidance.

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Conflict of Interest Statement: KL is employed by Chembio Diagnostic Systems, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 13 August 2018
doi: 10.3389/fvets.2018.00189

Development and Evaluation of a Serological Assay for the Diagnosis of Tuberculosis in Alpacas and Llamas

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 07 May 2018
Accepted: 24 July 2018
Published: 13 August 2018

Citation:
Infantes-Lorenzo JA, Whitehead CE, Moreno I, Bezos J, Roy A, Domínguez L, Domínguez M and Salguero FJ (2018) Development and Evaluation of a Serological Assay for the Diagnosis of Tuberculosis in Alpacas and Llamas. Front. Vet. Sci. 5:189. doi: 10.3389/fvets.2018.00189

South American camelids are susceptible to tuberculosis, caused mainly by *Mycobacterium bovis* and *M. microti*. Despite the tuberculin skin test being the official test for tuberculosis, it has a very low sensitivity in these species (14–20%). Serological tests present the advantages of being rapid, easy to perform and facilitate analysis of large numbers of samples in a short period of time. Novel antigen discovery and evaluation would provide enhanced detection of specific antibodies against members of *M. tuberculosis* complex. Here, we describe the development and evaluation of an ELISA-type immunoassays to use in the diagnosis of tuberculosis in llamas and alpacas based on P22, a multiprotein complex obtained by affinity chromatography from bovine Purified Protein Derivative (bPPD), that showed high sensitivity and specificity in mice, cattle and goats. This work was performed in two stages. First, a preliminary panel of samples collected from tuberculosis-free ($n = 396$) and *M. bovis*-infected herds ($n = 56$) was assayed, obtaining high specificity (100%) and sensitivity ranging from 63 to 96%. Subsequently, the use of the serological assay was tested using samples from two herds suffering from clinical *M. bovis* ($n = 88$) and *M. microti* ($n = 25$) infection to evaluate the ability of the ELISA to detect infected animals. 11 out of 88 alpacas were positive to the ELISA in a *M. bovis* outbreak and 7 out of 25 in a *M. microti* outbreak. The P22 ELISA potentially provides a sensitive and specific platform for improved tuberculosis surveillance in camelids.

Keywords: South American camelids, diagnosis, ELISA, P22, tuberculosis

INTRODUCTION

To date, tuberculosis (TB) is one of the most important diseases globally, both in animals and humans (1, 2). Animal TB has a broad range of domestic and wild mammal species hosts, including South American Camelids (SAC) that have become increasingly popular as production animals in recent years. Although llamas and alpacas are gaining more importance in fiber production (3, 4),

these animals also have companion animal value and may have regular contact with humans and other susceptible animal species. SACs are a potential source of different pathogens that might be transmitted to humans and could pose a risk to human health (5). Among these diseases, alpacas and llamas are very susceptible to TB, caused by bacteria from the *Mycobacterium tuberculosis* complex (MTC), mainly by *M. bovis* and *M. microti* (6, 7).

The diagnosis of tuberculosis in SAC has been mainly based on the tuberculin skin test, both single and comparative intradermal tuberculin test (SIT and SCIT, respectively), but these show poor performance in general in these species (8–10) and low sensitivity between 14 and 20%. A sensitivity of only 14% was found in one llama herd outbreak for animals that presented with visible lesions at post-mortem examination within 3 months of the SCIT test (11). In another report, only one llama tested positive out of five that were subsequently found to have visible lesions from which *M. bovis* was cultured (12). The interferon gamma (IFN-γ) test, based on the stimulation of blood cells with Purified Protein Derivatives (PPDs) and subsequent detection of the IFN-γ released, has been also developed for the diagnosis of TB, but it has been difficult to standardize, is labor-intensive, and in SAC yields a low sensitivity and specificity (63.6 and 89.1%, respectively) (13). In addition, in-house and commercial serological assays for the detection of specific antibodies have been previously investigated with a wide range of results (8, 11–14), but have been tested in a low number of animals.

Serological tests have been able to detect infected animals before the onset of clinical disease (8). In addition, the booster effect on the antibody response caused after injection of tuberculin has been reported as a strategic option to increase the sensitivity of serological assays in some species (15, 16). In general terms, the specificity of the serological assays are moderate to high, ranged from 84.6 to 98%, depending on the study and serological test employed (13, 17, 18). However, they showed low to moderate sensitivity, ranging from 43 to 75%, even using sera samples collected after intradermal PPD injection (7, 13, 17, 18). More details of the serological test evaluated in SAC are provided in **Table 1**. For these reasons, it is necessary to develop and evaluate new assays in order to provide more sensitive and specific options for the serological diagnosis of TB in SACs.

The aim of the present study was to develop and evaluate a novel ELISA type assay for the detection of specific antibodies of MTC in alpacas and llamas based on P22 multiprotein complex (20), which is affinity-purified from the PPD of *M. bovis,* and has been shown to provide greater sensitivity in other host species (15, 16). The P22-based ELISA was tested in serum samples from alpacas naturally infected with *M. bovis* and *M. microti* from Spain and England and uninfected llamas and alpacas from Peru and England.

MATERIALS AND METHODS

This work was performed in two stages: the first one included a preliminary panel of samples collected in TB-free and naturally *M. bovis*-infected herds to set the optimal cut-off point and calculate specificity and sensitivity of the ELISA; in a second stage, two farms suffering from clinical TB infection under different epidemiological situations were used to validate the test. Handling of the animals, testing and sampling were performed by accredited veterinarians. These were residual samples collected as part of routine surveillance or during breakdown sampling. All samples used in this study were serum samples. The animals used in this study were not experimental animals. All handling and sampling procedures were performed by veterinarians in accordance with the local legislation (Real Decreto 53/2013 in Spain, Ley de Protección y Bienestar animal N° 30407 in Peru, and the The Veterinary Surgeon Act 1966 in England).

Assessment of Specificity and Sensitivity

The specificity of the serological tests was evaluated in two different TB-free herds of alpacas and llamas located in different regions in Peru (19). The first alpaca herd was located at 4,000 m of altitude in La Libertad (northwest) and the second llama herd was located at approximately 4,200 m of altitude in Puno (southeast). 120 alpacas (104 male and 16 female) and 40 llamas (all female) were tested. Both herds were considered TB-free (based on long history of TB-free infection, absence of compatible lesions and epidemiological investigations). No lesions consistent with TB were observed in any animal in the 5 years prior to the study during slaughterhouse surveillance and no TB outbreak was reported on farms near the herds of the study. In addition, one TB-free herd from southern England was also included. 236 samples were available from adult alpacas at this herd including 93 males and 143 females. The regulatory program for TB surveillance in SAC in England can be found in the Bovine TB Eradication Programme for England (http://apha.defra.gov.uk).

The sensitivity was evaluated using serum samples from animals ($n = 56$) from a herd located in central Spain where an *M. bovis* outbreak was detected. The herd was a mix of alpacas of Suri and Huacaya breed. No previous history of TBs was reported before this outbreak. In December 2011, field veterinarians detected clinical signs (anorexia, cachexia, respiratory distress) and/or sudden deaths in three alpacas. Compatible TB-like lesions were observed in the post-mortem examination of one of these alpacas and *M. bovis* infection was subsequently confirmed by bacterial culture (18). A total of 67 animals were slaughtered and subjected to post-mortem examination within 4 weeks after the ante-mortem tests. Animals with positive *M. bovis* cultures and/or presence of visible TB-like lesions compatible with TB ($n = 56$) were included in the study to assess sensitivity. Serum samples for detection of specific antibodies were collected prior to PPD inoculation and 15 days after.

Testing the ELISA Under Field Conditions in Two TB Outbreaks

The analysis was carried out in two herds with natural *M. bovis* or *M. microti* infection confirmed by the presence of lesions compatible with TB and/or microbiological culture. Herd A consisted of 88 animals of Huacaya breed in England. This farm was selected due to a TB outbreak commencing in November

TABLE 1 | Details of different serodiagnostic tests in llamas and alpacas.

Assay test	Specie	Number of animals ($n_{Se} + n_{Sp}$)	Antigens	Sensitivity (%)	Specificity (%)	References
Enzyme linked immunosorbent assay (ELISA)	Alpaca	65	MPB83	43.1	–	(18)
	Llama and alpaca	160	MPB83	–	96.3	(19)
	Alpacas	52 + 306	MPB70 and MPB83	69.2	97.4	(13)
	Alpacas	52 + 257	*M. bovis* antigens[a]	66.7	96.9	(13)
VetTB STAT-PAK	Llama	14	MPB83, ESAT-6 and CFP-10	64.3	–	(11)
	Llama and alpaca	8 + 79	MPB83, ESAT-6 and CFP-10	62.5	89.9	(8)
	Llama and alpaca	52 + 279	MPB83, ESAT-6 and CFP-10	73.1	94.6	(17)
	Alpacas	52 + 306	MPB83, ESAT-6 and CFP-10	67.3	97.4	(13)
Dual-path platform (DPP)	Llama and alpaca	52 + 279	MPB70 and MPB83	75	97.5	(17)
	Alpacas	52 + 306	MPB70 and MPB83	57.7	96.7	(13)
Multiantigen print immunoassay (MAPIA)	Llama	14	*M. bovis* antigens[b]	100	–	(11)
	Llama and alpaca	8 + 79	*M. bovis* antigens[c]	87.5	97.5	(8)

nSe, number of TB positive animals used for evaluation of Se; nSp, number of negative animals used for evaluation of Sp.

[a] bPPD, ESAT6, CFP10, Rv3616c, MPB83, MPB70, and an MPB70 peptide.

[b] ESAT-6, CFP-10, MPB64, MPB59, MPB70, MPB83, the 16-kDa protein, the 38-kDa protein, two fusion proteins comprising CFP10/ESAT-6 and the 16-kDa protein/MPB83, and two native antigens, bPPD and M. bovis culture filtrate.

[c] Purified recombinant proteins (ESAT-6, CFP10, MPB70, MPB83, Mtb8, Mtb48, Acr1, and the 38 kDa protein), two native antigens, MPB83 protein and M. bovis culture filtrate (MBCF), and four protein fusions (CFP10/ESAT-6, Acr1/MPB83, F10, and F6).

2016. Two initial clinical cases were disclosed at necropsy with compatible TB lesions and *M. bovis* was isolated. Subsequently, a whole herd SCIT was performed and one alpaca was culled on the basis of a positive test. This alpaca was found to have lesions at necropsy. Serological testing took place 14 days later using Enferplex and cervid-DPP tests: two animals tested positive on the Enferplex test, were culled but found to have no visible lesions. All animals were skin-tested again 3 months later (using bovine tuberculin only) and also bled for further serological analysis (Enferplex only) 10 days following the skin test. Three animals were found positive on serology and were culled. At necropsy examination, two of these animals had no visible lesions while the third alpaca was found to have atypical lesions, comprising multiple small caseous lesions in a prescapular lymph node.

The herd B outbreak of TB was detected a herd of approximately 80 animals located in England in July 2017. The owner had performed surveillance serological testing (Enferplex) in May and identified a single animal that tested positive. At a retest 1 month later, the animal remained positive and was culled voluntarily on the basis of suspicion of disease. He was found to have lesions in the liver as well as bronchial and hepatic lymph nodes but no lesions in the lungs. At whole herd skin testing (SCIT), three further animals were disclosed and culled, although no visible lesions were found. At serological testing performed after the skin test, six animals were identified as positive on Enferplex and culled. Five of these animals had atypical lesions identified at post-mortem examination while the sixth had typical lesions in the lungs. A seventh alpaca was culled as a dangerous contact and also displayed atypical lesions at necropsy. 25 samples were available for analysis from 22 Suri alpacas (3 males and 19 females), one Huacaya male alpaca and two male llamas. *M microti* was never successfully cultured from these cases although PCR testing of lesion material was positive for *M microti*.

Development of an Indirect and a Competitive ELISA

An in-house indirect ELISA that detects antibodies against a protein complex named P22, purified by affinity chromatography from bovine PPD [CZ Veterinaria (Porriño, Spain)] was developed. The indirect ELISA was performed as described previously with minor modifications (15). Briefly, plates were coated with P22 (10 μg/ml) and then blocked with 5% skimmed milk powder solution in phosphate buffered saline (PBS). After

three washes with PBS plus 0.05%Tween 20 (PBST), sera were added in duplicate at 1:100 dilutions in skimmed milk and incubated for 60 min at 37°C. The optimal dilution of test serum was determined before by evaluating the reactivity of serum diluted from 1:10 to 1:640. 100 µl of detection antibody (Anti-llama IgG-HRP conjugate at 1:4,000 were added and the plates were incubated for 30 min at room temperature (RT). As before, the secondary antibody was titrated from 1:1,000 to 1:8,000 to choose the optimal dilution. The reaction was developed by adding 100 µl of o-phenylenediamine dihydrochloride substrate (FAST OPD, Sigma–Aldrich, St Louise, USA) incubated for 15 min in darkness and RT conditions. After that, the reaction was stopped with 50 µl of H_2SO_4 (3 N). The optical density (OD) was measured at 492 nm with an ELISA reader.

Negative control serum was obtained from TB-free llama previously described as *M. bovis* culture negative from TB-free areas and was included in every plate in quadruplicate. Positive controls were obtained from llamas previously described as *M. bovis*-infected confirmed by the presence of TB compatible lesions and *M. bovis* positive culture.

In order to reduce the cross-reactivity with non-tuberculous mycobacteria (NTM), a competitive ELISA was included. In this case the serum samples were diluted in skimmed milk supplemented with avian PPD [CZ Veterinaria (Porriño, Spain)] at 150 µg/ml. Only samples that yielded positive results to the indirect ELISA were analyzed by the competitive ELISA.

Data Treatment

Sample results were expressed as an ELISA percentage (E%), calculated by the following formula: [sample E% = (mean sample OD/(2 × mean of negative control OD)) × 100]. Specificity was calculated in the TB-free population using the formula [Sp = true negatives/(true negatives + false positives) × 100]. Sensitivity was calculated in the TB-infected population by the formula [Se = true positives/(true positives + false negatives) × 100]. The cut-off value was calculated using a ROC analysis and was defined as the value at which the highest sum of Se plus Sp was obtained (21). Confidence intervals for Se and Sp were calculated using the 95% Wilson's confident interval (Epitools, Ausvet Pty Ltd., Canberra, Australia).

RESULTS

The ROC analysis evidenced the diagnostic value the P22 ELISA in SAC (**Figure 1**). The cut-off value was defined as the ratio of the mean sample OD to the double of the mean OD of the negative control. The P22 ELISA with a cut-off value set at 100 E% showed the best balance between sensitivity and specificity. Modifying the cut-off value (>100E%<) resulted in either a decreased specificity or a constant sensitivity and a cut-off value of 100 was, therefore, chosen for the P22 ELISA.

The data including sensitivity, specificity, positive predictive value, negative predictive value and area under the curve (AUC), using confidence intervals of 95% (95% CI) for the ELISA with a chosen cut-off value of 100, are summarized in **Table 2**. Once the optimal cut-off was calculated, the specificity and sensitivity was studied in greater depth.

Determination of Test Specificity

Specificity of the P22 ELISA in llama and alpaca herds is shown in **Table 3**. The 396 animals from TB-free herds were negative to the indirect ELISA. Thus, overall the specificity of P22 indirect

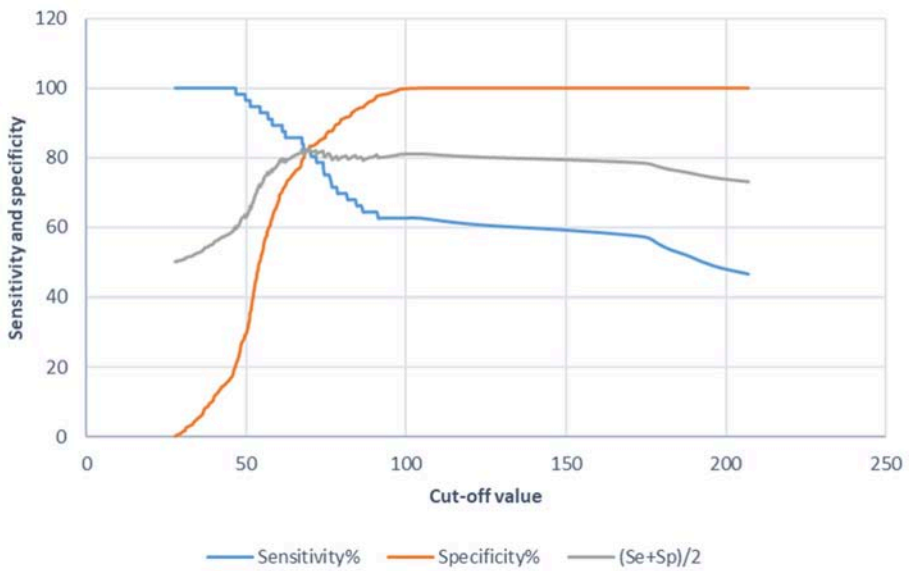

FIGURE 1 | Diagnostic value graphics for the tuberculosis indirect ELISA in SACs when using the P22 as an antigen. Sensitivity (Se), specificity (Sp) and their semi-sum are the percentages on the Y-axis and the cut-off value on the X-axis.

TABLE 2 | Sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and area under the curve (AUC) with 95% confidence intervals (CI_{95}) in the chosen cut-off value of 100 for P22 indirect ELISA in llamas and alpacas.

Se		Sp		PPV		NPV		AUC
%	CI_{95}	%	CI_{95}	%	CI_{95}	%	CI_{95}	
62.5	49.4–74	100	99–100	100	90.1–100	95	92.4–96.7	0.91

TABLE 3 | Specificity and 95% Wilson's confident interval of the ELISA using serum samples from llama and alpacas taken before and 5 days after the SCIT test.

			Pre-SCIT		Post-SCIT	
Country	**Specie**	**Total**	***N*[a]**	**Sp[b]**	***N*[a]**	**Sp[b]**
Peru	Alpaca	120	0	100 (96.9–100)	0	100 (96.9–100)
Peru	Llama	40	0	100 (91.2–100)	0	100 (91.2–100)
UK	Alpaca	236	0	100 (98.4–100)	–	–
	Total	396	0	100 (99–100)	0	100 (97.7–100)

[a] *Number of positive animals.*
[b] *95% Confidence interval for specificity.*

ELISA was 100% (95% CI 99–100) in llamas and alpacas. In the absence of any positive animal, the competitive ELISA was not carried out.

Determination of Test Sensitivity

The sensitivity achieved with P22 indirect ELISA in the samples from Spain was 62.5% (35/56) (95% CI 49.4–74) before PPD inoculation, and 96.4% (54/56) (95% CI 87.9–99) 15 days after PPD inoculation (**Table 4**). The competitive ELISA showed similar sensitivity.

Study of Two TB Outbreaks in England

M. bovis Outbreak

In herd A, of the 88 animal analyzed, 11 were positive to indirect ELISA (**Figure 2**). However, three animals had E% over 150 and the remaining eight animals had values between 100 and 150%. The competitive ELISA showed similar results. The same three animals had over E% 150 again and only seven were between 100 and 150%, one less than using the indirect ELISA. This animal was negative in competitive ELISA and positive in indirect ELISA maybe due to cross reaction by NTM. Considering that four animals had visible lesions at necropsy, three were positive to both the indirect and competitive ELISA.

M. microti Outbreak

In herd B, 25 serum samples were analyzed. Seven animals were positive in both indirect and competitive ELISA. Only one animal showed an E% value close to the cut-off point. The other six animals that had an E% over 300 (**Figure 2**), had visible lesions at post-mortem examination. Two animals with visible lesions at necropsy were negative to the ELISAs.

TABLE 4 | Sensitivity and 95% Wilson's confident interval of indirect (Ei) and competitive ELISA (Ec) in TB-infected animals based on post-mortem examination (culture positive and/or presence of visible TB lesions).

***N* of animals**	**Pre-SCIT**		**15 days after SCIT**	
	Ei	**Ec**	**Ei**	**Ec**
56	62.5 (49.4–74)	60.7 (47.6–72.4)	96.4 (87.9–99)	96.4 (87.9–99)

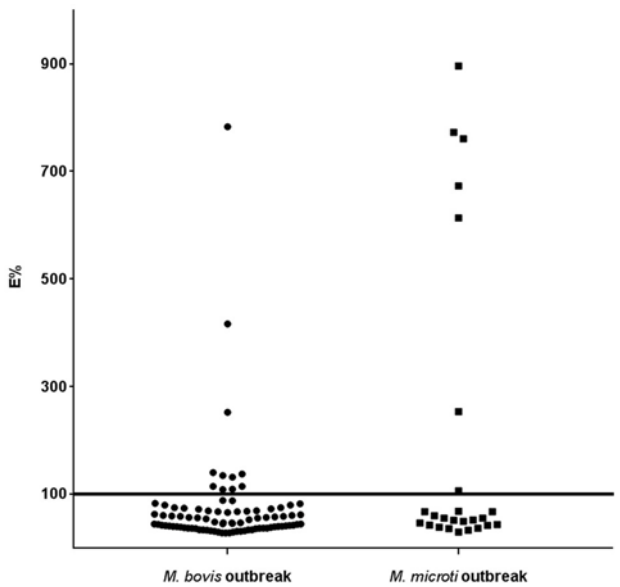

FIGURE 2 | Histogram of the ELISA E% value of individual llama or alpaca tested by indirect ELISA using P22 as antigen in *M. bovis* and *M. microti* outbreaks. The horizontal line represents the chosen cut-off value.

DISCUSSION

Since alpacas became an important animal in Europe and more tools for the diagnosis of TB in alpacas are needed, our results suggest that the indirect P22 ELISAs described here can provide better sensitivity and specificity than other TB antibody detection tests currently used in alpacas and could be used to detect both *M. bovis* and *M. microti* infection in SAC. As the indirect ELISA showed a specificity of 100%, and the purpose for the competitive ELISA was to remove antibodies against proteins shared between *M. tuberculosis complex* and non-tuberculous mycobacteria, the competitive ELISA is not useful in this case to improve the specificity of the diagnosis. Therefore, we focused on the indirect ELISA and propose this ELISA as a new tool for the diagnosis of TB in SACs.

Several serological tests for detection of antibodies against TB described previously showed specificity range from 84 to 98% (13, 17, 19). The P22 ELISA achieved an excellent specificity of 100%, higher than all serological test described up to date for diagnosis of TB in camelids. In addition, no effect of the injection of PPD was observed. The number of animal included in this study was large enough to have a reliable specificity data,

including with samples 5 days after PPD injection. However, further studies with samples taken 15 days after the skin test are necessary to confirm this finding because 5 days post-PPD may be insufficient to observe optimal antibody boost.

Regarding sensitivity, our ELISA yielded a moderate average sensitivity of 62.5%, similar to those reported by other serological assays in SACs, which are between 43 and 74% (13, 17, 18). These results are similar to those obtained for TB in bovine using a P22-based ELISA test (15). Using samples obtained 15 days after skin test, the sensitivity of P22 ELISA increased to 96%. This result was higher than reported by all previous serological assays using samples 15 days post-skin test, which sensitivity was between 77 and 89% (18). This boosting effect has been reported in TB in goats, bovines and alpacas (16, 18, 22). Casal et al. (22) demonstrated that sensitivity was significantly higher in cattle using samples collected 15 days post-skin test (ranging from 66.7 to 85.2%). Our results obtained using samples 15 days after injection of PPD were promising and suggested that the P22 ELISA could be a useful TB diagnostic tool in SACs. Taking a blood sample at 15 days post-PPD injection would require an additional veterinary visit, with an associate cost. For this reason, it may not be suitable as a routine method. Despite the costly strategy, the increase of the sensitivity to almost 100% could justify its use in certain situations. The booster effect, including the P22 ELISA, has also been described as a useful approach in cases of explosive TB outbreaks in other species as goats (16).

Humoral response occurs primarily in advances stage of infection and its detection has been considered less effective in early stages of TB infection (23, 24). However, although the skin test is the official diagnostic test for TB in alpacas, SIT test showed poor performances in terms of sensitivity and our results showed a higher sensitivity than SIT. Similar results were obtained previously (11, 13, 18). The combination of the skin test and a serological assay could be an approach to maximize the detection of infected animal instead of IFN-γ because of low sensitivity and difficulties to perform (18). Therefore, implementation of serology in parallel with the skin test could reach sensitivity of 100% (18). Since serology represents a rapid and inexpensive assay, a previous study recommended testing the same samples using several serological assays for a better diagnosis of infected animals (13). In this sense, our P22 ELISA may serve as a preferred technique for the diagnosis of TB, together with other serological assays or skin test. In addition, previous published batches of P22 showed similar qualitative and quantitative composition (20) and, consequently make P22 a stable and reliable product.

TB in SACs is mainly caused by *M. bovis* and *M. microti*, and has been reported in several European countries including Spain, the Netherlands, Switzerland, Ireland and the UK (7, 9, 14, 25). The present study has demonstrated the potential of the ELISA in serodiagnosis of TB due to *M. bovis* and also *M. microti*. The high OD observed in six *M. microti* and three *M. bovis* infected animals suggest a new promising sensitive serological test. Moreover, out of four animals in *M. bovis* outbreak and eight animals in *M. microti* outbreak with visible lesions, three and six animals, respectively were positive to the ELISA, showing a good ability to detect animals in advance stages of diseases, which are considered to be the major excretors of bacterias (26, 27). In addition, the low rates of positive results found in the herd A also confirm the high specificity of the assays. Eight and one animals in herds A and B, respectively had an E% close to the cut-off. However, the specificity of the ELISA was 100% and, for this reason, the cross-reaction with other proteins in P22 shared with environmental mycobacteria was discarded. The level of antibodies in these animals was low and consequently the OD in the ELISA was also low.

In conclusion, the new multiprotein complex named P22 could be an alternative antigen for the detection of specific *M. tuberculosis* complex antibodies in SAC. Moreover, the P22-based indirect ELISA can be used as a cost effective, rapid and reliable tool for the large-scale screening and therefore, support the detection and management of tuberculosis in llamas and alpacas.

AUTHOR CONTRIBUTIONS

CW, JB, AR, and LD obtained the serum samples from the animals. JI-L, CW, IM, MD, and FS performed the laboratory techniques. JI-L, CW, MD, and FS wrote the manuscript that was edited, discussed and reviewed and accepted by all authors.

FUNDING

JI-L was supported by an FPU contract-fellowship (Formación de Profesorado Universitario) from the Ministerio de Educación, Cultura y Deporte of the Spanish Government (FPU2013/6000). AR is the recipient of an Industrial Doctorate contract (DI-15-08110) funded by the Spanish Ministry of Economy, Industry and Competitiveness (MINECO) and the European Social Fund. This work was funded by the University of Surrey Innovation Voucher Scheme 2017-18.

ACKNOWLEDGMENTS

Authors would like to thank Ana Belén Martinez and Soledad Crespo for their technical assistance and María Luisa de la Cruz for her support in statistical analysis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 24 July 2018
doi: 10.3389/fvets.2018.00169

Risk Perceptions and Protective Behaviors Toward Bovine Tuberculosis Among Abattoir and Butcher Workers in Ethiopia

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Bovine Tuberculosis (BTB) is a serious cause of economic losses and public health threat, especially in developing countries. Humans acquire BTB through consumption of raw or undercooked meat, inhalation of aerosol and occupational exposure. A cross-disciplinary approach to study diseases connecting society and biology helps to understand the ways in which social, cultural, behavioral, and economic circumstances influence a healthy life. The objective of this study was to assess the risk perceptions and protective behaviors toward BTB among abattoir and butcher workers in central Ethiopia. A health belief model was used to generate the desired data following health belief model constructs. A total of 300 meat handlers working in local abattoirs, export abattoirs and butcher houses in Bishoftu, Modjo, Dukem, and Akaki towns of central Ethiopia were selected using a systematic random sampling method. Univariate and multivariable logistic regression analysis were used to assess factors associated with risk of exposure to BTB through the consumption of raw meat. The results showed that among the study participants, 95% heard about BTB and 93% knew that eating raw meat could be a source of BTB for humans. More than 62.7% of the respondents in the high risk group strongly agreed that contracting BTB would prevent them from coming to work, keep them in bed for an extended period of time and cause death. The majority of the respondents believed that free provision of personal protective clothing, compensation with test and slaughter campaigns, television and radio advertisements, educational programs and government-imposed penalties would help in prevention of BTB. Despite the high perceived severity and risk perception, the multivarable logistic regression model showed low-risk protective behavior among male (OR: 2.3, 95% CI: 1.2–4.3) and older age (>30) individuals (OR: 14.4 95% CI: 2.1–125.8). The study also noted the importance of media for health education as means for prevention of BTB. The authors strongly recommended the need of promotion of behavioral change toward the consumption of raw meat wich would have potential implications for the public health impacts of zoonotic tuberculosis and ultimately help national and global efforts toward prevention and control of tuberculosis.

Keywords: bovine tuberculosis, health belief model, protective behavior, raw meat, risk perception, Ethiopia

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *28 March 2018*
Accepted: *02 July 2018*
Published: *24 July 2018*

Citation:
Fekadu F, Beyene TJ, Beyi AF, Edao BM, Tufa TB, Woldemariyam FT and Gutema FD (2018) Risk Perceptions and Protective Behaviors Toward Bovine Tuberculosis Among Abattoir and Butcher Workers in Ethiopia. Front. Vet. Sci. 5:169. doi: 10.3389/fvets.2018.00169

INTRODUCTION

Bovine Tuberculosis (BTB) is a zoonotic Tuberculosis disease (TB) caused by *Mycobacterium bovis (M. bovis)* with cattle being serving as a primary host. In 2016, an estimated 147,000 new human cases of zoonotic TB and 12,500 deaths due to the disease occurred globally. The African region carries the heaviest burden, followed by the South-East Asian region (1–3). In Africa, zoonotic TB due to *M. bovis* is transmitted through inhalation of aerosols, leading to pulmonary TB, and through ingestion of contaminated animal products such as milk and meat, leading primarily to extrapulmonary TB (4). In most developed countries, it was eliminated or controlled in the domestic animal population through strict control and eradication measures including test-and-slaughter strategies and compulsory pasteurization of milk. As a result, human infection is reduced, even though the potential risk remains in place (5, 6).

In Ethiopia, the average prevalence of bovine tuberculosis based on studies done between 2000 and 2016 showed to be 6% in cattle. The prevalence also varied based on the breeds of cattle and the production systems (7). The fact that *M. bovis* is frequently isolated from various animal organs/tissues such as lesions in the lungs and lymph nodes at slaughterhouses gestures that the disease can spread through both direct and indirect modes to human (8). Out of all human TB cases, the contribution of *M. bovis* was estimated to be 17.0% (9). This is of great importance, especially for livestock traders, farmers and animal product handlers.

Occupational exposures to *M. bovis* have been reported in many countries including Australia (10). In Nigeria, 10% prevalence of TB was diagnosed among livestock traders; and about one-quarter of the identified TB cases were caused by *M. bovis* strains. This study indicated that several factors including poor living conditions contributed to exposure of the people to *M. bovis* infections (11). In addition to the health effects, the economic loss in livestock caused by TB is enormous. Direct economic losses due to the infection become evident by 10 to 18% and 15% reduction in milk and meat production, respectively (12).

Collecting data on the status of BTB can enhance the understanding of the effects and patterns of transmission of the diseases and the associated determinant factors in population (13). To communicate the potential risks and protective measures effectively, health authorities need to understand the determinants of a particular behavior such as the role of beliefs, the perception of risk, benefits, and barriers to change to protect oneself (14, 15).

The Health Belief Model (HBM), a theory that is used to incorporate each of these factors, allows researchers to assess what might constitute one's protective behavior which is influenced by constructs of knowledge, perceived benefits, perceived susceptibility and severity, perceived barrier, self-efficacy, and cue to action (16). Addressing the occupational risks related to such infectious diseases is necessary by exploring the risk perception and protective behavior against the disease. According to this model, meat handlers at abattoirs and butcher shops are likely to overlook health-related precautionary measures including avoiding eating raw meat and refrain from contacting contaminated meat, if the meat handlers consider BTB to be a threat to their health and believe to be susceptible to the disease BTB. In other words, a meat handler and trader are less likely to eat the visibly infected parts of the meat when they feel they are at a heightened risk of BTB owing to their general work conditions such as working in the abattoirs and habits of processing raw meat with inadequate protective wear and not washing their hands before and after processing meat. A meat handler is also likely to read messages related to health if they believe that the benefits of the measures taken as a precaution to avert BTB outweigh the costs and if factors have synergistic rather than hindering contributions. The meat handlers will also need to feel that they are capable of undertaking the required actions to avoid risky behaviors which are called here self-efficacy. The cues or readiness to action component of the model is the least systematically studied or understood of all constructs (16).

However, such information is limited in Ethiopia. To this end, addressing the occupational risks related to such infectious diseases is needed by exploring the risk perception and protective behaviors against the disease. Therefore, the objective of this study was to assess the occupational exposure risk perceptions and protective behaviors toward BTB among abattoir and butcher house workers in four selected towns of central Ethiopia.

MATERIALS AND METHODS

Study Site and Population

The study was conducted in four selected towns in central Ethiopia (Bishoftu, Modjo, Dukem, and Akaki towns) on randomly selected people working in local and export abattoirs as well as butcher houses (**Figure 1**). The study population consists of people living in the towns working in abattoirs and butcher houses. The eligibility criteria were meat handlers who were 15 years old or above, working in local or export abattoirs and butcher houses.

Study Design and Theoretical Framework

The study employed a cross-sectional study design following the HBM. The model is commonly used to explain a wide variety of health behaviors and can be successfully used to guide public health interventions (16). It emphasizes the subjective perceptions of the individuals in understanding behaviors. Perceived susceptibility and severity of a health hazard as well as perceived benefits and barriers of preventive health behaviors are key components of the HBM. They are theorized to underline the cognitive processes involved in health-related decision making (17). The current study followed similar protocol used by Hambolu et al. (18) in adopting the HBM.

The main study outcome was whether respondents did or did not eat raw meat. Those who ate raw meat were classified as "high risk" and otherwise "low risk." The independent variables were related to socio-demographic variables, knowledge

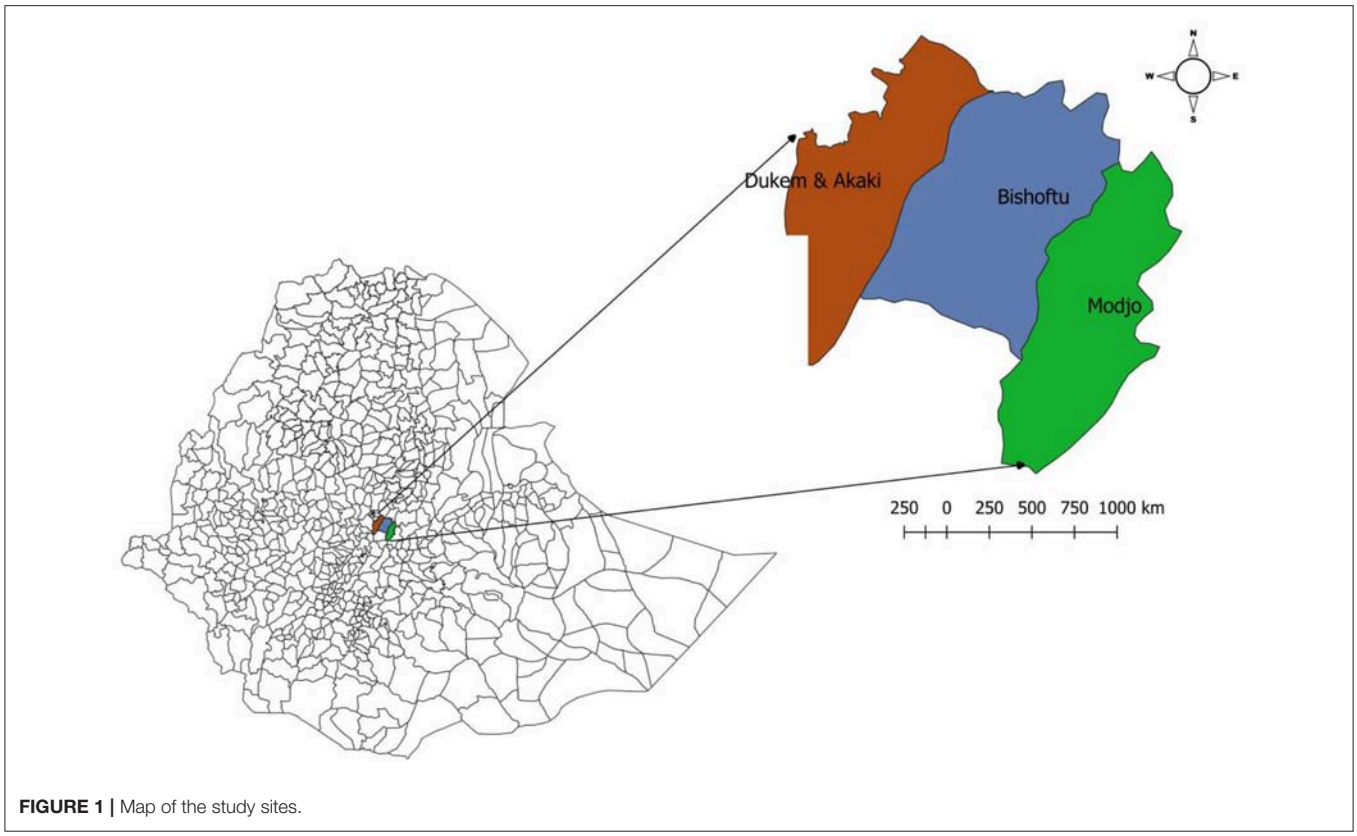

FIGURE 1 | Map of the study sites.

indicators related to TB and BTB, other risky behaviors related to BTB, participants' perceived susceptibility, perceived severity, perceived barriers, self-efficacy, and cues to action to BTB.

Sample Size and Sampling

The sample size was calculated by considering 74.4% expected prevalence of raw meat eating habit (16), 95% confidence interval and 5% required precision. Accordingly, the minimum target sample size was 289 and we collected data from 300 study people working the abattoirs in the study area.

Data Collection Tool and Eligibility

Data were collected through face-to-face interview using a pre-tested and structured questionnaire. The questionnaire format consisted of four sections. The first part included questions about participants' socio-demographic characteristics such as age, gender, level of education, monthly income, and religion. The second part comprised of questions examining the knowledge on BTB, with response options of "yes," "no" or "I don't know." The third part had items asking about risk-taking behavior, including whether participants eating raw meat with response options of either "yes" or "no." The final part consists of questions relating to each of the health belief model constructs: perceived susceptibility, severity, barriers, self-efficacy, and readiness or cues to action. For the items in the health belief model constructs, participants were asked to indicate their level of agreement to the given statements eliciting their own views on a five-point Likert scale:(1) Strongly disagree, (2) Disagree, (3) Neither agree nor disagree, (4) Agree, and (5) Strongly Agree.

Statistical Analysis

The collected data were entered into excel spreadsheet and analyzed using SPSS version 20. As all the variables were categorical, the values in each category were presented together with their corresponding percentages. Univariate and multivariate logistic regression analyses were conducted to examine the effects of the independent variables on the dependent variable (eating raw meat). Correlation between responses to items within a construct was tested using Cronbach alpha. If the correlation was high (> 0.7), then the average of the Likert scale was considered. Candidate variables having a *P*-value less than 0.05 during the univariate analysis were further included in the multivariate logistic regression model to see their association with risk of consuming raw meat. The significance level was set at $\alpha < 0.05$ (19).

RESULTS

Socio-Demographic Characteristics

A total of 300 people were interviewed and all responded to the questionnaires. Of these, 75% (225) of the respondents had eaten/consumed raw meat and categorized as having high-risk behavior whereas the remaining who did not consume raw meat (25%) were referred as "the low-risk group."

Eighty percent (241/300) of the respondents were male, and more than half of them (165/300) were in the age category of 21–30 years. The univariate analysis showed that among the socio-demographic variables, only the gender variable was shown to have a significant difference across proportions of categories of a variable as compared between low and high risk groups ($P < 0.01$). Irrespective of the risk category, people in the categories of male gender, 21–30 age range, Orthodox religion followers, those who work in abattoirs, and those with income range of 1–5 USD per day where found to make 80.3, 55, 83.3, 69.3, and 97% of the participants having the habit of consuming raw meat, respectively (**Table 1**).

Knowledge

Among the respondents, about 95.3% of the respondents had awareness about TB. In spite of this fact, 97.3% of them were found to consume raw meat becoming a high-risk group. Ninety-three percent of all the interviewed people knew about the transmission mode of TB from animals to humans. More than 70% of them were aware about that the healthy-looking meat could be contaminated. On the other hand, about 90% of respondents knew that consumption of contaminated meat could be a source of BTB in humans (**Table 2**). Based on the univariate analysis, all the variables related to knowledge about BTB: heard of TB, spread of TB from animals to humans, healthy-looking meat contains TB causing pathogen and consumption of contaminated meat can be a source of infection in humans were found statistically associated with the high-risk behavior of the habit of consuming raw meat ($P < 0.05$).

Perceived Susceptibility

The univarate logistic regression analysis showed statistically significant association of all the considered evidence for the respondents' perceived susceptibility with the high risk behavior for contracting BTB ($P < 0.05$). Most of respondents perceived that they had a probability of increased chance of contracting BTB because of their work, when they use bare hands, when they would eat in the slaughterhouses and perceived that contaminated (unwashed) hands and eating raw meat (**Table 3**).

Perceived Barriers to Prevention

Contrary to the perceived susceptibility all the attributes of the perceived barriers to prevention of BTB were not statistically associated with the high-risk behavior ($P > 0.05$) (**Table 4**).

Perceived Severity

More than 62.7% of the respondents in the high risk group strongly agreed that contracting BTB would prevent them from coming to work, keep them in bed for an extended period of time and cause death. These were statistically significant ($p < 0.05$). There were no significant difference between high risk and low-risk groups based on contacting BTB is scaring and treatable or not ($P > 0.05$) (**Table 5**).

TABLE 1 | Univariate analysis of demographic characteristics and their association with raw meat eating habit of abattoir and butcher house workers.

Variables	Number (%) $n = 300$	High risk (%) $n = 225$	Low risk (%) $n = 75$	*p*-value
GENDER				
Male	241(80.3)	153 (68.0)	63(84.4)	<0.01
Female	59 (19.7)	72(32.0)	12(15.6)	
AGE				
15–20	18 (6.0)	21(9.3)	4(4.9)	0.06
21–30	165 (55.0)	135(60.0)	40(53.3)	
31–40	68 (22.7)	42(18.7)	18(24.1)	
>40	49 (14.3)	27(12.0)	13(17.7)	
LEVEL OF EDUCATION				
Illiterate	11 (3.7)	12(5.4)	4(4.9)	0.66
At school	274 (91.3)	200(89.3)	69(92.0)	
Graduate	15 (5.0)	12(5.3)	2(3.1)	
RELIGION				
Orthodox	250 (83.3)	198(88.0)	61(81.8)	0.46
Muslim	18 (6.0)	12(5.3)	5(6.2)	
Protestant	27 (9.0)	15(6.7)	7(9.8)	
Traditional	5 (1.7)	0(0.0)	2(2.2)	
OCCUPATION				
Abattoir worker	208 (69.3)	171 (76.0)	50 (67.1)	0.19
Butcher man	92 (30.7)	54 (24.0)	25 (32.9)	
INCOME PER DAY				
<1 USD	6 (2.0)	3 (1.3)	2 (2.2)	0.85
1–5 USD	291 (97.0)	219 (97.3)	73 (96.9)	
>6 USD	3 (1.0)	3 (1.3)	1 (0.9)	

TABLE 2 | Univariate analysis of knowledge about bovine tuberculosis (BTB) by risk categories among workers of abattoirs and butcher shops.

Knowledge related variables	Number (%)	High risk (%) $N = 225$	Low risk (%) $N = 75$	*p*-value
HAVE YOU HEARD OF TB?				
Yes	286 (95.3)	201 (89.3)	73 (97.3)	0.02
No	2 (0.7)	3 (1.3)	3 (0.4)	
Don't Know	12 (4.0)	33 (14.7)	33 (3.6)	
CAN TB SPREAD FROM ANIMALS TO HUMANS?				
Yes	279 (93.0)	192 (85.3)	72 (95.6)	<0.01
No	2 (3.3)	0 (0)	1 (0.9)	
Don't know	19 (4.0)	33 (14.7)	3 (3.6)	
CAN HEALTHY LOOKING MEAT CONTAIN TB CAUSING PATHOGENS?				
Yes	221 (73.7)	138 (61.3)	58 (77.8)	0.02
No	25 (8.3)	24 (10.7)	6 (7.6)	
Don't know	54 (18.0)	63 (28)	11 (14.7)	
IS CONSUMPTION OF CONTAMINATED MEAT A SOURCE OF BTB INFECTION IN HUMANS?				
Yes	267 (89.0)	186 (82.7)	68 (91.1)	<0.01
No	6 (2.0)	0 (0)	2 (2.7)	
Don't know	27 (9.00)	39 (17.3)	5 (6.2)	

TABLE 3 | Univariate analysis of perceived susceptibility to bovine tuberculosis (BTB) by risk groups (Percent sum up to 100 for each risk group across the levels of Likert scales.

Questions	Strongly disagree (%)	Disagree (%)	Neither agree nor disagree (%)	Agree (%)	Strongly agree (%)	*p*-value
DO YOU THINK THAT YOU HAVE AN INCREASED CHANCE OF CONTRACTING BTB BECAUSE OF YOUR WORK?						
Low risk	26.7	2.7	6.7	30.7	33.3	0.02
High risk	19.1	2.2	2.2	22.2	53.3	
DO YOU THINK THAT YOU ARE AT INCREASED RISK OF CONTRACTING BTB WHEN YOU USE A BARE HAND?						
Low risk	26.7	1.3	6.7	36.0	29.3	<0.01
High risk	17.3	1.3	2.2	27.1	52.0	
DO YOU THINK THAT YOU ARE AT INCREASED RISK OF CONTRACTING BTB WHEN YOU EAT IN THE SLAUGHTER SLAB?						
Low risk	24.0	1.3	6.7	41.3	26.7	<0.01
High risk	10.7	0.4	8.5	27.2	52.7	
DO YOU THINK THAT YOU ARE AT INCREASED RISK OF CONTRACTING BTB WHEN YOU DON'T WASH YOUR HANDS AFTER HANDLING CARCASSES?						
Low risk	24.3	1.4	4.1	36.5	33.8	0.01
High risk	14.2	0.4	2.7	25.2	57.1	
DO YOU THINK THAT YOU ARE AT INCREASED RISK OF CONTRACTING BTB WHEN YOU EAT RAW MEAT?						
Low risk	69.3	1.3	4.0	14.7	10.7	<0.01
High risk	15.5	0.9	2.7	45.1	35.4	

TABLE 4 | Univariate analysis of perceived barriers to prevent bovine tuberculosis (BTB) among workers of abattoirs and butcher shops.

Questions	Strongly disagree (%)	Disagree (%)	Neither agree nor disagree (%)	Agree (%)	Strongly agree (%)	*p*-value
DO YOU NEED TO TASTE MEAT BEFORE SELLING TO SHOW THAT IT IS SAFE?						
Low risk	80.0	0.0	0.0	1.3	18.7	0.36
High risk	72.0	0.0	0.0	0.9	26.8	
CANNOT WEAR PROTECTIVE CLOTHING BECAUSE THEY ARE NOT CONDUCIVE TO WORK?						
Low risk	94.7	0.0	0.0	4.0	1.3	0.11
High risk	98.2	0.9	0.9	0.4	0.9	
CANNOT WEAR PROTECTIVE CLOTHING BECAUSE THEY ARE EXPENSIVE?						
Low risk	98.7	0.0	0.0	1.3	0.0	0.30
High risk	99.6	0.4	0.0	0.0	0.4	
DO NOT WEAR PROTECTIVE CLOTHING BECAUSE MY COLLEAGUES DO NOT?						
Low risk	98.7	0.0	0.0	1.3	0.0	0.26
High risk	99.1	0.4	0.0	0.0	0.9	

Self-Efficacy

Only 25% of the respondents in the high risk group agreed or strongly agreed that they were able to tell if carcasses were infected with TB or not ($P < 0.05$). There were no significant difference between high risk and low-risk groups based on the capacity to buy protective wear and wearing of protective wear when their colleagues are not wearing $P > 0.05$ (**Table 6**).

Cue to Action

Over 65% of respondents in the high risk group agreed or strongly agreed that free provision of protective clothing and compensation with test and slaughter campaigns would help to protect BTB ($P < 0.05$). Even though, not statically significant ($P > 0.05$), the majority of the respondents agreed or strongly agreed that both television and radio advertisements, educational programs, and government-imposed penalties would help to protect BTB (**Table 7**).

Evaluation of the way the public protection could be prompted shows that the majority of respondents agreed or strongly agreed that radio advertisements and adequate compensation would help (**Figure 2**). In addition, about 65% of respondents felt that they would need educational programs and free provision of protective clothing in order to comply with the procedures and 60% felt that government-imposed penalties for those who do not practice safe measures would work.

All the potential predictors of the high-risk behavior for contacting BTB under each construct of HBM based the univariate analysis having statistically significant association at a *p*-value less than 0.05 and high correlation (>0.7) between significant variables with in each construct were further analyzed using multivariate logistic regression to determine the predictors of the high risk behavior. The analyses were done using the

TABLE 5 | Univariate analysis of perceived severity to prevent bovine tuberculosis (BTB) among workers of abattoirs and butcher shops.

Questions	Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree	*P*-value
DO YOU THINK THAT CONTRACTING BTB WILL PREVENT YOU COMING TO WORK?						
Low risk	6.7	0.0	5.3	29.3	57.3	<0.01
High risk	2.7	0.0	1.3	17.3	78.7	
DO YOU THINK THAT CONTRACTING BTB WILL KEEP YOU IN BED FOR AN EXTENDED PERIOD OF TIME?						
Low risk	10.7	0.0	4.0	28.0	56.0	0.02
High risk	4.4	0.0	1.3	20.0	74.2	
DO YOU THINK THAT CONTRACTING BTB SCARES YOU?						
Low risk	29.3	4.0	4.0	14.7	46.7	0.12
High risk	21.7	1.8	1.3	12.4	62.7	
DO YOU THINK THAT BTB CAN CAUSE DEATH						
Low risk	5.3	2.7	2.7	22.7	65.3	0.01
High risk	2.7	0.4	1.8	10.7	84.4	
DO YOU THINK THAT TB IS TREATABLE?						
Low risk	5.33	2.7	4.0	12.0	74.7	0.53
High risk	3.6	0.9	2.2	10.2	83.1	

TABLE 6 | Univariate analysis of self- efficacy to prevent bovine tuberculosis (BTB) among workers of abattoirs and butcher shops in Central Ethiopia.

Questions	Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree	*P*-value
CAN YOU BUY PROTECTIVE WEAR?						
Low risk	85.3	2.7	1.3	1.3	9.3	0.25
High risk	78.7	0.9	0.4	4.4	15.6	
CAN YOU WEAR PROTECTIVE WEAR EVEN IF YOUR COLLEAGUES ARE NOT?						
Low risk	1.3	0.0	1.3	1.3	96.0	0.39
High risk	1.3	0.0	0.0	1.3	97.3	
ARE YOU ABLE TO TELL IF CARCASSES ARE INFECTED WITH TB OR NOT?						
Low risk	76.0	6.7	2.7	2.7	12.0	<0.01
High risk	63.1	2.2	0.9	25.3	8.4	

TABLE 7 | Univariate analysis of Cues to action to prevent bovine tuberculosis (BTB) among workers of abattoirs and butcher shops in Central Ethiopia.

Questions	Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree	*P*-value
DO YOU THINK THAT EDUCATIONAL PROGRAMS WOULD HELP TO PROTECT BTB?						
Low risk	5.3		5.3	32.0	56.0	0.29
High risk	4.4		2.2	26.2	67.1	
DO YOU THINK THAT SUPPLY OF FREE CLOTHING WOULD HELP TO PROTECT BTB?						
Low risk	4.0	0.0	9.3	34.6	52.0	0.01
High risk	1.3	0.4	2.2	27.1	68.8	
DO YOU THINK THAT ADEQUATE COMPENSATION FOR COOPERATING WITH TEST AND SLAUGHTER CAMPAIGNS WOULD HELP TO PROTECT BTB?						
Low risk	5.3		13.3	34.6	44.0	<0.01
High risk	1.7		4. %	28.4	65.3	
DO YOU THINK THAT GOVERNMENT IMPOSED PENALTIES WOULD HELP TO PROTECT BTB?						
Low risk	2.6	2.6	6.6	22.6	64.0	0.06
High risk	2.2	0.0	2.6	25.3	69.7	
DO YOU THINK THAT TELEVISION ADVERTISEMENT WOULD HELP TO PROTECT BTB?						
Low risk	1.3		5.3	20.0	72.0	0.66
High risk	0.8		2.6	18.6	77.7	
DO YOU THINK THAT RADIO ADVERTISEMENT WOULD HELP TO PROTECT BTB?						
Low risk	1.3		5.3	18.6	73.3	0.39
High risk	0.8		1.7	18.6	78.6	

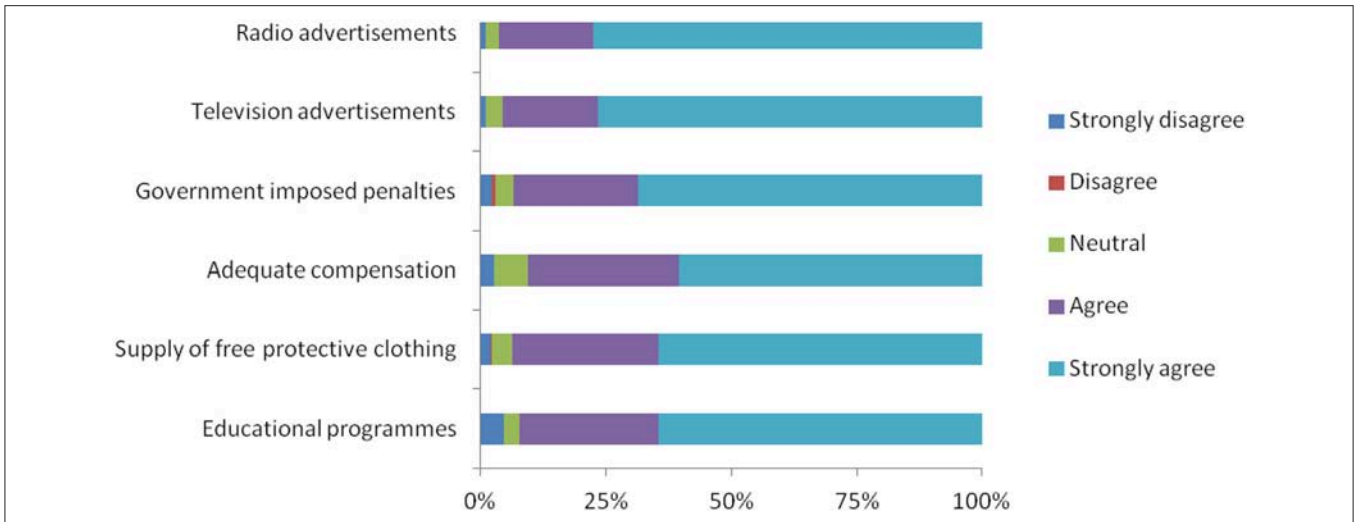

FIGURE 2 | Major interventions for facilitating the adoption of protective behaviors and practices.

TABLE 8 | Multivariate logistic regression analysis of HBM constructs to prevent bovine tuberculosis (BTB) among workers of abattoirs and butcher shops.

Risk factors identified	Adjusted odds ratio (95% CI)	*P*-value
PERCEIVED SUSCEPTIBILITY		
High susceptibility	1	
Low susceptibility	1.6 (1.2–2.1)	<0.01
GENDER		
Female	1	
Male	2.3 (1.2–4.3)	0.01
AGE CATEGORY		
<15	1	
15–20	4.6 (0.6–44.2)	0.15
21–30	6.4 (0.9–51.5)	0.15
31–40	14.4 (2.1–125.8)	<0.01
>40	9.6 (1.3–89.9)	0.03

significant variables and averaged Likert scale of the significant variables under each construct. Accordingly, only the male gender, those who claim to be older and those who perceive that are not susceptible to BTB were associated with the risk of high risk behavior of consuming raw meat (**Table 8**).

DISCUSSION

This study attempted to assess the risk perceptions and protective behaviors on BTB and identify the determinants of the high-risk behavior, eating raw meat, among workers of abattoirs and butcher shops in central Ethiopia using the health belief model. The present study showed the high prevalence of the risky behavior of eating raw meat for BTB (75%, 225/300), which was in agreement with the findings of Biru et al. (20) in which 79.3% people were found to consume raw meat in and around Sululta, central Ethiopia. About 95% of the respondents were aware of TB, and 93% of them were aware that TB can spread from animals to humans and the was relatively higher as compared to a previous study (21), which reported that about 82% of the respondents in western highland regions of Cameroon aware of TB. The high-risk group was found to exhibit better knowledge (95.3%) about TB, despite that, they were found to consume raw meat becoming a high-risk group. This is not in line with theories of the health belief model as well as other health behavior models, which might be due to the longtime and deep entrenched cultural habit of eating raw meat in Ethiopia, particularly eating "*kurt*" (raw beef) and "*kitfo*" (raw or undercooked minced beef mixed with blend of several spices) in many social groups including educated people such as animal and human health professionals in the country (22).

The health belief model recognizes the importance of raising awareness in the populations for the promotion of health and disease protective life strategy. Our finding was in contrary to other findings that concluded as "patchy awareness" and lack of knowledge of zoonosis combined with raw meat eating habits and poor livestock keeping systems are likely to expose respondents to an increased risk of contracting zoonosis (18, 23).

Out of the demographic factors male gender and age (above30 years) were found to associate with the high-risk behavior, consumption of raw meat. This might be due to the fact that most of the workers in the abattoir were male individuals (80.3%). The finding of risky behavior related to the age was not in agreement with another finding, this might be due to the raw meat eating culture of adult people as compared to young ones in Ethiopia (22). In this study, the male respondents were found to be more in a high-risk group compared to the female counterpart. This finding is in agreement with the reports of Hambolu et al. (18) who reported 78.2% of males were in the high group in Nigeria. As the matter of the fact and the high probability of the exposure, older groups and male individuals working in abattoirs in Ethiopia will be at greater

risk of contracting BTB. Behavioral sciences explain that the observed predominance of risk-bearing behavior among males as inherently linked to the social construction of masculinity in many African countries. Given that, further in depth studies might be required to get insight into the Ethiopian context (23).

In our study, even though they were not statistically significant, free provision of personal protective clothing, compensation with test and slaughter campaigns wherever economic benefits allow, television and radio advertisements, educational programs and government-imposed penalties found to help to protect BTB. These findings are comparable with a study conducted in Nigeria (18).

The respondents perceived susceptibility to contracting BTB showed that there was increased chance of contracting BTB because of handling meat using a bare and contaminated hand, their work, eating at slaughter slab and eating raw meat. In terms of perceived barrier namely the perception that one cannot wear personal protective clothing because they are not conducive for work, and the perception that one cannot sell meat without tasting were not found to be predictors of the high-risk behavior. However, according to Janz et al. (24) the perceived barriers were the most important predictors of behavior while perceived susceptibility was the most important amongst predictors of preventative behavior.

The main limitation of this study was the use of cross-sectional study design, which is unable to verify causal relationships between the dependent and independent variables. It is documented that other methods such as longitudinal designs have a clear superiority in studies of belief-behavior relationships (25). The face to face semi-structured interviews which were used in this study might have increased the likelihood of respondents' inclination to give socially acceptable answers as also hypothesized by Hambolu et al. (18). Despite the limitation, there was a high response rate (100%), making the results likely to be the beliefs of the study population.

In conclusion, the study revealed low-risk protective behavior among male and older age (>30) individuals despite the high-risk perception and the importance of media for health education as means for prevention of BTB. We believe that the findings of the study would help and serve as a baseline data for policy and decision makers to take appropriate actions aimed at mitigating the risk of tuberculosis transmission to humans from animals following consumption of raw meat. Avoiding eating raw meat, avoiding handling of meat using bare and contaminated hands, creation of awareness for workers in abattoirs and butcher houses in particular and the general population in general about zoonotic importance of BTB using radio and television streaming and a national level study to assess the public perception regarding zoonotic importance of BTB were recommended.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This research was approved by the Academic Commission of College of Veterinary Medicine and Agriculture, Addis Ababa University. As the research was not involving invasive procedures and collection of biological samples, separate ethical clearance was not solicited. The study purpose was explained to participants and verbal agreement was obtained before proceeding to the study. Due to the low literacy rate and lack of interest of the participants to put their signature on consent form, the researchers opt to collect the desired data merely based on oral consent.

AUTHOR CONTRIBUTIONS

FF collected field data. TB, FG, AB, FW conceived the idea of the study, participated in the study and questionnaire design, organized the data collection, wrote the draft of the manuscript, and completed the final version for submission. TB analyzed the data and interpreted the results. TT participated in developing the research idea and study design. BE participated in the development of the idea, supervised the work, and edited the final version of the manuscript before submission.

ACKNOWLEDGMENTS

The authors would like to thank all study participants and Addis Ababa University, College of Veterinary Medicine and Agriculture for supporting this study. The authors acknowledge Dr. Samson Leta for developing the map of the study sites.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

PERSPECTIVE
published: 27 November 2018
doi: 10.3389/fvets.2018.00298

TB Control in Humans and Animals in South Africa: A Perspective on Problems and Successes

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Mycobacterium tuberculosis (*M. tb*) remains one of the most globally serious infectious agents for human morbidity and mortality, but with significant differences in prevalence across the globe. In many countries, the incidence is now low and declining, but control and eradication remain a distant view. Similarly, the prevalence of bovine TB caused by *Mycobacterium bovis (M. bovis),* varies significantly across regions, although unlike for *M. tuberculosis*, data are sparse. The reduction in incidence and prevalence and control of both human and bovine TB is difficult and costly, yet some countries have managed to do this with some success. This perspective will consider some of the critical control steps we now know to be important for the control of TB from *M. tuberculosis* in humans living in South Africa, where the incidence of TB is the highest currently experienced. Despite the high incidence of human TB, South Africa has been able to reduce this incidence remarkably in the past few years, despite limited resources and high HIV prevalence. We draw from our experience to ascertain whether we may learn useful lessons from control efforts for both diseases in order to suggest effective control measures for bovine TB.

Keywords: tuberculosis, *Mycobacterium bovis*, bovine TB, infectious diseases, zoonotic TB

OPEN ACCESS

Edited by:
Andrew William Byrne, Agri-Food and Biosciences Institute (AFBI), United Kingdom

Reviewed by:
Mitchell Palmer, National Animal Disease Center (USDA ARS), United States
Francisco Olea-Popelka, Colorado State University, United States

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *26 June 2018*
Accepted: *06 November 2018*
Published: *27 November 2018*

Citation:
Meiring C, van Helden PD and Goosen WJ (2018) TB Control in Humans and Animals in South Africa: A Perspective on Problems and Successes. Front. Vet. Sci. 5:298. doi: 10.3389/fvets.2018.00298

INTRODUCTION

Mycobacterium bovis (M. bovis), the causative agent of bovine tuberculosis (BTB), has perhaps the broadest host range of the pathogenic mycobacteria (1). Although the most commonly affected species are members of the Bovidae, even humans can be affected.

Considerably more attention is devoted to control of *Mycobacterium tuberculosis* in humans, than *M. bovis* in its multiple hosts (2). Although there are some similarities between TB control in humans and animals, such as the need for diagnosis, there are also very different disease management options, such as antibiotic therapy for humans, in comparison to test and slaughter for domestic cattle. Disease control measures include the need to find and deal with cases and prevent transmission. Although this seems self-evident, achieving these goals is not simple and require critical activities such as those shown below and discussed later.

Steps to TB control:

1. Awareness
2. Risk factor reduction
3. Access
4. Diagnosis
5. Retention
6. Treatment
7. Adherence
8. Follow up

Actions attributable to these steps allowed South Africa to steadily reduce human TB incidence from a peak of 977/100,000 per annum in 2007 to 781 in 2016. This observed reduction in incidence is perhaps remarkable because the reduction alone exceeds by far the incidence rate seen in most countries (3).

The reported occurrence of bovine TB in South African domestic bovine herds is far lower (**Table 1**), although since full testing coverage is not done the actual numbers are likely to be higher. TB and BTB control activities will be discussed below.

AWARENESS AND STIGMA

Ignorance of TB is rife. For this reason, many organizations tasked with human health care such as WHO (World Health Organization), The Union (International Union Against Tuberculosis and Lung Disease), and MSF (Médecins Sans Frontières), start their campaigns with generating awareness. Such campaigns leverage media, to create interest and awareness.

TABLE 1 | *Mycobacterium bovis* cases reported in South Africa from 2000 to 2018 (Department of Agriculture, Forestry and Fisheries: http://www.daff.gov.za/daffweb3/Branches/Agricultural-Production-Health-Food-Safety/Animal-Health/Epidemiology).

Year	Outbreaks	Cases	Dead/Culled
2000	10	174	181
2001	1	33	1
2002	4	123	32
2003	17	394	370
2004	11	1,525	737
2005	14	747	856
2006	4	42	37
2007	6	102	50
2008	4	50	37
2009	18	36	1,236
2010	8	18	7
2011	7	34	29
2012	3	90	0
2013	2	8	29
2014	8	102	66
2015	8	32	28
2016	3	247	0
2017	1	8	0
2018	3	4	3

Estimated cattle herd size 13.5 million in 2003.

Our own academic department has reached out to schools and communities in multiple activities in 2018 alone. Using past and cured patients to propagate the message through their own experiences can be quite effective at community level. Such public activities have the benefit of addressing and reducing stigma that might be attached to TB. There is now improved awareness amongst the South African public concerning human TB. However, there is little awareness of bovine TB. In general, there has not been much media attention, there is no large or even small-scale campaign, no rallying cry, no catch phrases, and essentially it is left to private and state veterinarians and technicians to work with farmers as they see fit. To date, one awareness day has been organized in only one location, and the limitations of this hardly need to be discussed.

RISK FACTOR REDUCTION

Humans and animals share some common risk factors for TB, such as nutrition or malnutrition, age, crowding, and extent of exposure (4). There are many others which are likely to be restricted to humans or animals only, such as substance abuse in humans and environmental contamination in animals. Many risk factors in humans relate to poverty and are very difficult to address. Risk factors for cattle include historical TB on a farm, movement of animals, TB on neighboring property or in wildlife in contact with domestic stock, prevalence of TB in a herd or area and herd size, multiple premises, poor housing, and nutrition (5). It is often possible to mitigate against these risks for livestock.

A cornerstone of bovine TB control is movement restriction of animals. This is a vital activity, which is not generally possible with humans and therefore presents veterinarians with an enormous advantage to prevent ongoing disease transmission. Most countries have a test and slaughter policy in place for bovine TB in domestic stock (6, 7). However, having a policy and program does not necessarily mean that full coverage is achieved and appropriate action is followed. For example, many resource-poor countries such as South Africa do not have the resources for rigorous testing and there is a lack of compensation to affected livestock owners. Movement restriction requires proper monitoring, which is extremely difficult even under optimal circumstances. Although TB does not have a vector, we can argue that a contaminated environment (soil, water) and multiple hosts may act as reservoirs for infection and therefore also need active management.

ACCESS

In order to capitalize on awareness campaigns, it is vital that access to appropriate facilities and experts are available to persons who are ill. In South Africa, there is a large network of state-funded public health clinics (8) and private practitioners which addresses health problems including TB. In the veterinary field, there is a network of state veterinary services as well as private veterinarians who can deal with bovine TB. However, the veterinary service is far smaller than the human health service component and overall they must deal with far larger numbers

of potential hosts on a per capita basis than clinicians for the human population. Testing for bovine TB is voluntary, except for dairy herds. However, there are inadequate numbers of state veterinarians to do regular TB testing, including for dairy herds where compulsory testing every 2 years is required. Therefore, private veterinarians have to be hired at considerable cost to the owners. On occasion, state veterinary services will provide TB testing for impecunious owners or commonage herds. Since there is no compensation paid to owners for culled positive animals or herds that need to be slaughtered, there is little or no incentive for testing to be done, in fact, there can be active resistance to testing.

One of the key elements envisaged for successful TB control remains the goal of a point-of-care (PoC) diagnostic test, the value of which is illustrated by scenarios (**Figure 1**): we highlight firstly the South African human TB diagnostic program prior to 2011, which required three sputum samples from a client on different days over a week. This resulted in a loss to follow up of 17–25% (9, 10). Let us also assume that we use the test still used in many resource-poor settings, i.e., acid-fast staining with diagnostic sensitivity of 50–60%. The implication (**Figure 1** scenario A) is that only a small percentage of patients initiated proper therapy, which allowed ongoing disease and transmission events (8). In a different hypothetical scenario (scenario B), using a test of the same sensitivity but PoC based, with immediate initiation of therapy, the proportion of TB cases that could initiate therapy almost doubles. Scenario B will also imply a reduction in infectiousness time and fewer transmission events. In a third hypothetical scenario (scenario C), an Xpert® MTB/RIF test is conducted (PoC) where indicated, therapy can be initiated immediately. Given the test sensitivity of 82–89% (11, 12), it implies that over 80% of TB cases could initiate therapy. Ignoring specificity discussion to illustrate this point, a high sensitivity PoC diagnostic test results in less loss or default.

By far the majority of the human TB diagnostic tests based on GeneXpert, are done at no cost to clients utilizing public clinics, since laboratory-based tests are done by the National Health Laboratory Service (NHLS, funded by the National Department of Health) which has many laboratories scattered in a network across the country (8). In contrast, the Department of Agriculture, Forestry and Fisheries (DAFF) subsidizes laboratory diagnostics at only one laboratory for BTB in suspect animal cases, but does not pay costs in full. Tests require that samples be taken at necropsy, or that fresh blood samples for immunological tests arrive within hours under ideal conditions, the latter being

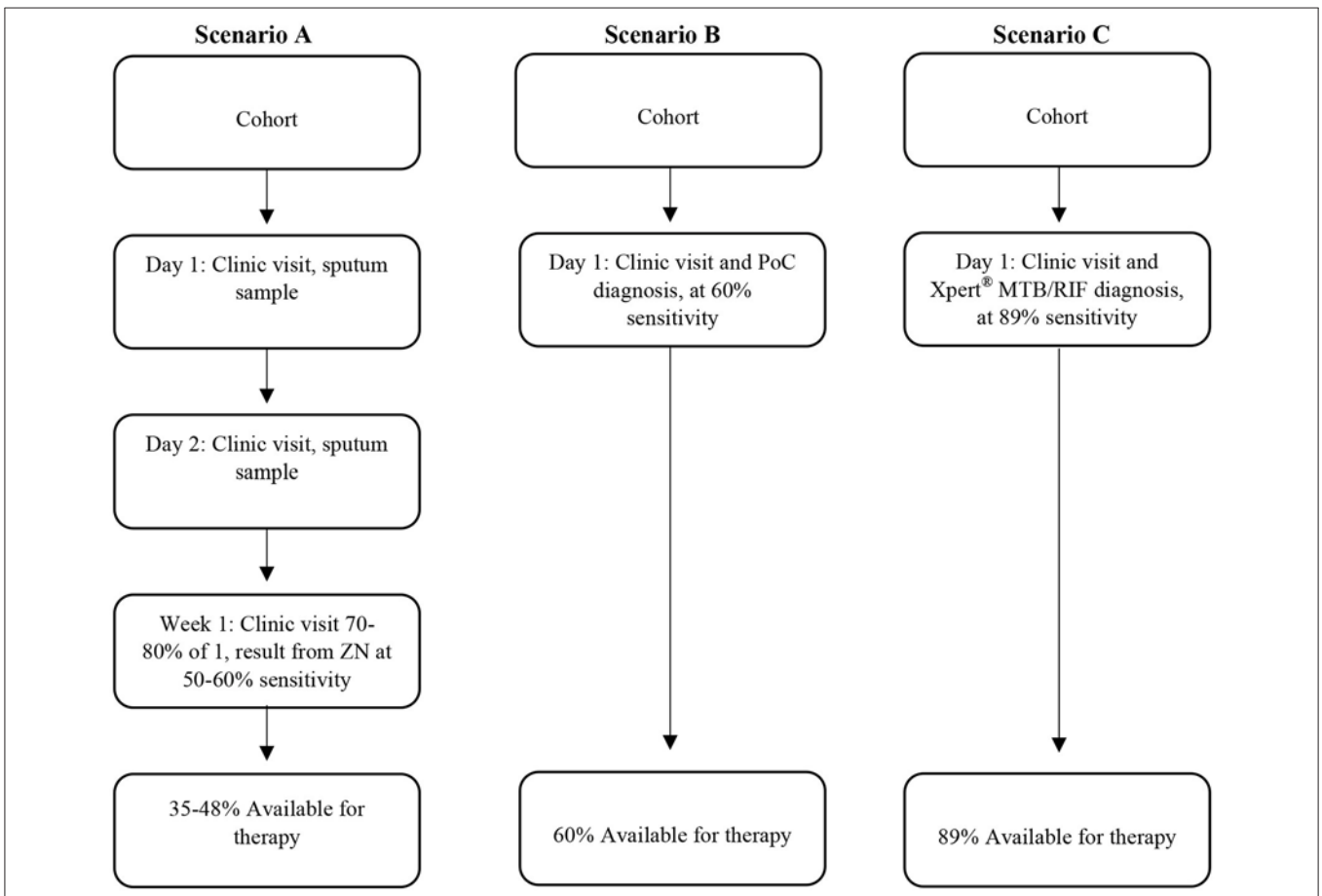

FIGURE 1 | Different scenarios representing different human TB diagnostic approaches which include the sensitivity of the diagnostic tests and corresponding availability of therapy for individuals. Scenario 1 is a previous TB program now obsolete, scenario 2 is hypothetical, illustrating the advantage of point of care test, and scenario 3 is what could be achieved using the GeneXpert system if used for same day diagnosis in the clinic.

largely impossible in a large country with distant rural farms. Owners are not compensated for their animals which will result in a reluctance to test animals. Samples from necropsy are set up for mycobacterial culture followed by speciation (13). Unlike the NHLS, there is only one state lab, Onderstepoort Veterinary Institute (OVI), accredited for testing for bovine TB, largely because there is no financial incentive for other laboratories to be accredited. Such a monopoly is unlikely to be the best way forward.

Clearly, surveillance or suspicion of bovine TB should not lead immediately to slaughter and necropsy. Therefore, non-lethal diagnostics for animals are needed. Only once such diagnostics strongly suggest bovine TB, necropsy, culture, and speciation is done to confirm bovine TB. Bovine TB has been tested for in Bovidae by skin testing and more recently by *in vitro* blood-based interferon gamma (IFN-γ) release assays (IGRA) or other biomarkers (14–19). These tests although useful, are limited owing to the need for blood transport to accredited laboratories under time and temperature constraints, as well as the need for a reasonably well-equipped laboratory. In order to circumvent this logistics problem, serum-based diagnostics are being researched. Serum-based biomarker research in humans shows promise for a diagnostic, but as yet, although sensitivity is high (94%), specificity (73%) is inadequate for implementation (20). However, it may be that such biomarkers discovered for human TB diagnosis, may be applicable to bovine TB.

Bovine TB can also infect many species other than the Bovidae. Therefore, particularly in the case of wildlife, species-specific diagnostic tests may be required. This is necessary to prevent the disease from being maintained in an ecosystem outside of monitored hosts, e.g., cattle or buffaloes and where there may be concerns for endangered species, such as rhinoceroses. Failure to diagnose and treat a TB case has significant downstream cost implications, not least of which is ongoing transmission and disease propagation. Thus, a considerable and ongoing investment in the best diagnostics and control programs to implement these is justified.

RETENTION

Many TB control programs suffer client losses along the care cascade. Such work shows the importance and advantages of the "Holy Grail" of TB researchers, the PoC diagnostic (21). The consequence of losses on the cascade is that successful completion of treatment for TB was estimated to be only 53% of cases (8). In the case of livestock or wildlife, the difficulties involved in accessing animals for repeat testing or dealing with positive responders are familiar to state veterinarians. No similar quantitative care cascade loss studies have been done in veterinary medicine in South Africa and thus information is anecdotal. However, the future cost of missed cases, as for humans, cannot be overemphasized.

TREATMENT

There is perhaps little that can be learnt from current therapeutic management of human TB and extrapolated to animals. The standard treatment for TB in humans is antibiotic therapy (22), which with the exception of animals in captivity is not feasible in animals. Sometimes physical isolation is also practiced, i.e., the TB case is placed in a treatment facility to isolate them from the general populace. For TB in animals, the same basic principle applies: remove the bacterial threat by removing the animal (i.e., physical isolation), usually by slaughter.

ADHERENCE

The basic clinical principle applies: complete the course of treatment. This must apply, whether it is antibiotic treatment in humans, movement control or removal of animals with TB, usually by slaughter. Failure to do so will result in ongoing disease and transmission, and failure to eradicate the problem (22).

FOLLOW-UP

This is an important step and often not done in human TB management in higher incidence areas owing to sheer volume of work and resource limitations. The reason for this activity is that even under ideal conditions and with proper adherence, some individuals will experience recurrent disease. Furthermore, prior to becoming bacillus negative, TB cases can transmit the disease. Ideally, therefore, treated and cured individuals need follow up for at least 2 years (23) and their contacts should be investigated. In the case of free-living humans, particularly in a high incidence society, investigating all contacts is impossible. Likewise for free-ranging wildlife. However, these principles are part of bovine TB control practice in South Africa, i.e., test and remove and subsequent follow up testing and retesting until disease is cleared according to protocol. This practice should always be followed. It is encouraging that even culling of limited infected animals in a free-ranging wildlife system can reduce prevalence rate (7).

Although the steps discussed above are arguably critical for TB control, there are many other factors that are important and will impact on any control measures undertaken. Some of these are discussed further below.

TRANSMISSION

Arguably the most important step in combatting TB is to stop transmission. Close contact is important, but not definitive for transmission. For example, a study in a very high incidence area showed that only a small proportion of human TB cases result from household contact (24, 25). Furthermore, the passive detection of TB cases in high prevalence communities is insufficient to limit disease transmission (8, 26). We still have an inadequate understanding of TB transmission, although we know that aerosol transmission is one of the main sources for humans, and most likely also bovis. In the case of some other animals, it may be ingestion of contaminated meat or biting. Clearly, adequate distance must be maintained to avoid

ongoing transmission. Therefore, attention should be given to the potential for a contaminated environment, and there should be space and free airflow such that transmission may be minimized.

INFECTION, LATENCY, AND DISEASE

It is generally stated that (in the absence of immunosuppression), only 10% of infected humans will develop active TB (4, 27). Traditionally and commonly stated: approximately half of those who will develop active disease will do so within 2 years after infection and the other half sometime after that, owing to reactivation of latent infection (LTBI) (28). Controversy characterizes opinions concerning whether a positive diagnostic assay, such as those that are host-based, really prove disease or are indicative of infection but do not necessarily represent disease or the presence of live bacilli. We previously considered four possible states: (1) not exposed, (2) exposed and infected, no response detectable, no sign of disease, (3) infected, bacilli present, no active disease (latent TB), (4) infected, active disease. In clinical medicine, distinguishing between these four states is not necessarily clear. A recent comprehensive review (23), suggests that the burden of disease from latent TB in humans has been vastly overestimated, suggests only three states and that TB has a shorter incubation period than previously thought. If this is correct, it has major implications for public health. Unfortunately, there is little clear-cut data on whether three or four states apply to the multiple animal hosts of *M. bovis,* nor clear-data regarding progression between states.

Therefore, the interpretation of immunological tests for human as well as bovine TB is complex. Possible outcomes of exposure from cattle to *M. bovis* are believed to be in line with that of humans. Briefly, following exposure to bacilli, the innate immune response can either clear the infection or fail to do so. This failure then leads to the need for intervention by the host's adaptive immune response. A successful response leads to the clearance of the infection with no delayed-type hypersensitivity responses (skin test and whole blood gamma interferon release assay negativity), or failure leads to active disease (skin test and IFN-γ release assay positivity) (29). In cattle, failure to detect visible lesions at *post-mortem* examinations does not indicate absence of infection (30). A systematic review of many studies has previously shown that 50% of reactor animals had no visible lesions (31), which was seen in a separate study where only 43% of reactors had visible lesions at slaughter (32). This suggests that as for humans (23) active disease may be significantly underestimated in studies where culture is the gold standard.

Recent modeling suggests that the WHO's (human) TB elimination target cannot be achieved by 2050 using LTBI screening as the sole control strategy (33, 34). The assumptions used include maximum coverage, no imported infections due to travel and migration, and application of an additional 4% annual decrease. This model suggests that a TB incidence of $<$1/100,000 will only be achieved about 50 years after implementation of LTBI screening and prophylactic treatment (33). These findings are optimistic assumptions, but illustrate the difficulties involved in eliminating TB when LTBI exists. Furthermore, they emphasize that continued surveillance and follow up will be essential. However, if latent TB is far less important than previously assumed, then eradication or good control far sooner than this is possible. Therefore, in veterinary medicine, the approach taken thus far has been wise, i.e., if any test is positive, take action. This should arguably continue to be the case and is probably the reason for the low prevalence of bovine TB in domestic stock in South Africa.

BOVINE TB IN WILDLIFE

Although bovine TB in livestock appears to be of low prevalence in South Africa (**Table 1**), this is not the case in at least three of our large national park systems (35, 36). Thus, far no effective plan has been made to combat it in an open system in South Africa, although some limited culling has been done in one park (7). Such areas pose a risk for spread beyond the park boundaries, but is limited as far as possible by testing, animal movement control, and breeding of disease-free animals, such as TB free herds of African buffalo (37). Insufficient research has been done to show whether or not this disease will impact species to affect the ecosystem and which species are maintenance or end-stage hosts.

ECONOMICS

Stable systems require a healthy society, a healthy economy and a healthy environment. TB, whether in animal or human form impacts on all three of these pillars. The problem with giving inadequate attention to current TB using as the excuse "we can't afford it," will leave us with the situation we currently have. The latest estimates (2014) from WHO are that 1.7 billion humans were latently infected by *M. tuberculosis* (28). We have no idea how many animals are infected by *M. bovis*, as a comparison, but an estimated 147,000 human (zoonotic) cases of bovine TB alone per annum occur (38). This implies many animal cases and neglect now will mean high future costs.

WAY FORWARD

The nature of TB, whether human or animal form, makes eradication in the short term impossible. However, it is clear that transmission must be stopped in order to eradicate the disease. The essential lessons from this are many: one cannot be complacent, one cannot relax vigilance, and care for this disease (39). Active and latent cases must be dealt with before eradication can be considered.

Countries or regions should take the threat of bovine TB seriously. If this is not the case, then perhaps we can learn from one initiative started in South Africa recently to try to improve TB control. A TB Think Tank was established (40) bringing together researchers in the basic sciences, clinical sciences, epidemiology, social sciences, public

health, and Health systems experts, and government staff. This body has promoted evidence-based decision-making, and in addition, lobbied successfully for increased funding for TB management (human) in South Africa. By involving national TB control staff and other experts, it is believed that significant impact on TB can be achieved. Similar think tank initiatives could be developed for other settings including bovine TB control to support evidence-based policy development and disease control and lobby for the finances to support such efforts.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

The authors work was supported by the South African Medical Research Council and National Research Foundation of South Africa.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 26 October 2018
doi: 10.3389/fvets.2018.00270

A Retrospective Study on Bovine Tuberculosis in Cattle on Fiji: Study Findings and Stakeholder Responses

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Bovine tuberculosis (bTB) is globally significant due to its impacts on cattle production. A Brucellosis and Tuberculosis Eradication and Control (BTEC) program commenced in Fiji during the 1980's and has since been sustained by government funding and industry cooperation. A retrospective study of bTB data obtained during the Fiji BTEC program from 1999 to 2014 was undertaken at the University of Sydney with support from the Government of Fiji. It confirmed that bTB is well-established in dairy cattle farms in Naitasiri and Tailevu provinces of Central Division on the main island of Viti Levu, and suggested that the disease is present among cattle on farms in all or most provinces across three (Central, Northern, Western) of the four divisions in the country. It was evident that despite sustained efforts, disease reduction and containment was not being achieved. Reasons contributing to this situation included the appropriateness of the protocol for conduct of the single intradermal test (SID) in cattle, absence of regular quality assurance training of BTEC field staff, lack of standard procedures for bTB data collation and evaluation, unregulated cattle movements and the presence of stray cattle. The Fiji Ministry of Agriculture responded proactively to these findings by implementing revision to the use of the SID in cattle and refresher training for staff along with the Biosecurity Authority of Fiji who implemented cattle movement restriction. A subsequent apparent outbreak of bTB in some farms due to increased detection by the new test protocol raised concerns for the local dairy industry. To clarify the status and extent of bTB infection and the challenges faced by the industry, a stakeholder forum was held in May 2017, and a new BTEC strategy was formulated and endorsed by stakeholders. bTB remains a focus for cattle disease control by the government of Fiji. This case study highlights the challenges for bTB control in Fiji and underlines the importance of technical and social considerations to achieve success in disease control.

Keywords: bovine tuberculosis, disease control, surveillance, BTEC, Fiji

OPEN ACCESS

Edited by:
Andrew William Byrne, Agri-Food and Biosciences Institute (AFBI), United Kingdom

Reviewed by:
Maria Laura Boschiroli, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), France
Margaret Good, Private Consultant, Dun Laoghaire, Ireland

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *29 June 2018*
Accepted: *08 October 2018*
Published: *26 October 2018*

Citation:
Borja E, Borja LF, Prasad R, Tunabuna T and Toribio J-ALML (2018) A Retrospective Study on Bovine Tuberculosis in Cattle on Fiji: Study Findings and Stakeholder Responses. Front. Vet. Sci. 5:270. doi: 10.3389/fvets.2018.00270

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic bacterial disease of cattle caused mainly by *Mycobacterium bovis*, although other zoonotic members of the *M. tuberculosis* complex may be the cause, such as *M. caprae*, the common cause of bTB in central Europe (1). bTB results in serious economic losses for the livestock industry worldwide due to animal disposal, carcass confiscation, premature

culling, low production and poor reproductive performance (2). Further infection in people results in disease that is predominantly extra-pulmonary but cannot be clinically distinguished from *M. tuberculosis* infection. Official estimates of human zoonotic TB cases due to *M. bovis* in 2016 stand at 147,000 new cases and over 12,500 deaths, mainly in Africa and South-East Asia (3).

In Fiji, bTB leads to decreased production and opportunity for local trade due to sick animals and early culling of potentially productive stock. The bTB situation in Fiji is becoming an increasing concern for industry stakeholders as the culling of stock further aggravates the low milk production in the country. Data in 2014 from cattle sent to slaughter after a positive skin test on farm showed that one in three reactors (animals with positive single intradermal test) had generalized TB, and 85% had some form of gross TB lesion at post-mortem examination. Successful eradication of bTB is recognized by the Government of Fiji to be of benefit to individual cattle owners and to the country in relation to trade and potentially to human health. Hardest hit is the dairy sector which has suffered the greatest loss of cattle numbers (4).

This paper provides a case study of bTB control in an endemically infected cattle population in the Pacific. It outlines the bTB control program in Fiji, presents the methods and results of a retrospective study of bTB from 1999 to 2014 in Fiji, discusses the actions of the Ministry of Agriculture and other government agencies in response to study findings, and considers the implications of this response for industry, and longer-term, for the eradication program. As bTB remains a high priority for cattle disease control by the Government of Fiji, this case study highlights the challenges for bTB control in Fiji and underlines the importance of technical and social considerations to achieve success in disease control.

bTB CONTROL IN Fiji

It is likely that bTB was introduced in Fiji through cattle brought in by European settlers during the 1830's (5). During the 1970's the deleterious effect of brucellosis and tuberculosis in local cattle farms was recognized and the need to establish a national control program voiced (6). Subsequently the Ministry of Agriculture (MOA) commenced the Fiji Bovine Brucellosis and Tuberculosis eradication and control (BTEC) program in the early 1980's with support from the Australian Government (6, 7) implementing dairy farm registration, cattle movement monitoring, and mandatory bTB testing and ear tagging of tested cattle, and carcass inspection at abattoir with compensation paid for condemnations at slaughter. These activities were based on property identification, animal tagging and surveillance programs of Australia (8) and the United Kingdom (9). However, the requirement for annual cattle farm registration is limited to dairy farms as the basis for legal sale of milk and milk products, with only some beef herds being voluntarily registered. Field testing was conducted annually although inconsistently between farms. Historical documentation on the BTEC program and bTB occurrence in Fiji is sparse with no information available prior to 1999. For example, the Animal Health Survey published in 1999 by the Secretariat of the Pacific Community (10) did not include a report for Fiji. Information about Fiji bTB from 1999 is limited to government reports and record books, and data reported to the World Organization for Animal Health (OIE) since Fiji became a member in 2007.

Cattle farms of all types (dairy and beef farms of individual farmers, school farms, village/settlement, government stations, middlemen) are included in the program and participation is mandatory although some farmers do not comply. There are no specific consequences for non-compliance other than ongoing transmission among cattle in non-compliant infected farms. All cattle aged 6 months and above are tested and have a metal tag with a unique number placed in the right ear to indicate the animal has been tested. The single intradermal test (SID) using purified protein derivative antigen from *M. bovis* (PPD-B) is administered at the caudal fold of the tail (CFT) with the result read 3 days after administration. Up to September 2014 a positive result was determined by the presence of a wheal not <4 mm in diameter. All log books and handwritten data collected from the field were filed by the Fiji Ministry of Agriculture in a government stock room. There was no written protocol for standard data management and analysis, and no systematic analysis of data to evaluate progress of bTB control over time. Quarterly and annual reports were prepared based on manual counts of records. Designated responsibility for the conduct of the bTB program was at the level of the division offices from 1999 to 2010 in an effort to increase surveillance coverage. This was centralized to the national office from 2011 to improve monitoring of the quality of testing.

Abattoir monitoring consists of carcass inspection for tubercle lesions by government meat inspectors at the two main abattoirs of the Fiji Meat Industry Board (FMIB) located in Nasinu, Central Division and Vuda, Western Division, respectively (11). Affected organs or whole carcasses are condemned based on the severity and location of tubercle lesions. Compensation to farmers is paid at slaughter of affected animal at a rate of FJD$1.60 per kg for the condemned part of the carcass and applies to animals detected through on-farm testing (reactors sent to slaughter) and to animals detected via carcass inspection at slaughter. Thus, this compensation is available to both farmers that comply with on-farm testing and those that do not. During 2015 the compensation rate was improved to equal the market price at the time of culling (12).

Farms with positive animals determined by on-farm testing or abattoir monitoring are classified as "Infected." BTEC requires an infected farm to be free from bTB for 3 consecutive SID tests held at a minimum of 3-months intervals to obtain "Restricted," "Provisionally clear," and "Clear" statuses, respectively. It requires a minimum of 9 months from the time of detection for a farm to complete three consecutive clear tests and obtain "Clear" (bTB-free) status.

The Fiji BTEC program, a long-term activity sustained by annual government funding and industry cooperation, demonstrates collective commitment to address bTB in the cattle population. To underpin a review of the BTEC program, a retrospective study of bTB surveillance data from 1999 to 2014

was conducted over 12 months during 2014–2015. The aim of the retrospective study was to document the progress of the BTEC program and to provide recommendations to strengthen it. The final results of the study were formally presented to Ministry of Agriculture in September 2016 and to the industry stakeholders during the BTEC Forum held in March 2017.

RETROSPECTIVE STUDY

Materials and Methods

Data Sources

This study was conducted using data collected by the BTEC program from 1999 to 2014. The Fiji Ministry of Agriculture granted approval for use of the Fiji BTEC data to conduct this study in March 2014. Hard copies of batch books, reactor books, field sheets, annual reports, memorandum and other documents related to bTB in Fiji were used to collate and cross-check data from 1999 to 2014. The dataset compiled by year included farm identification number, location, farm type, date of testing, total number of cattle tested, total number of cattle test positive and farm TB status. For 2011 to 2014, the dataset for each year listed tests conducted by individual animal tag number. The few bTB test results from species other than cattle (horse, pig) were excluded, as were farm record data on the number of cattle younger than 6 months. It was assumed that all TB test results were read 3 days after the date of tuberculin administration.

Records of carcass inspection at slaughter from 2011 to 2014 were obtained for FMIB abattoirs at Nasinu and Vuda. Individual cattle records for slaughtered bTB positive animals were identified, including reactors identified during on-farm testing and subsequently sent for slaughter, and other animals identified at slaughter via detection of lesions during carcass inspection. The dataset compiled included farm identification number, animal identification number, date of slaughter and type of lesion detected. Complete records were only available for the Nasinu abattoir.

Due to the absence of a formal national registration system for all cattle farms, no absolute total cattle number were available for use as a denominator to calculate the population coverage of testing or infection prevalence in this study. In place of this, cattle population estimates for 2011 to 2014 published in the World Animal Health Information Database (WAHIS Interface) (13) were used. However, no reliable cattle population numbers at the national and division level were available prior to 2011.

Data Transcription and Sorting

For each year from 1999 to 2014, data were transcribed from hard copy sources into a purpose-built spreadsheet in Microsoft Excel version 2003. *Farm ID spreadsheet* included farm registration number, farm name, farm location (division, province, district, village/settlement), farm type (dairy, beef, other), date of test, number of cattle on farm by age group, number of animals tested, number of animals tested positive, number of animals tested negative and TB status of farm. *Animal ID spreadsheet* included farm registration number, farm name, farm location (division, province, district, village/settlement), date of test, TB tag number, age-gender description (heifer, dry cow, lactating cow, bull, steer), and TB test result.

Data transcription was performed by BTEC personnel from May 2014 to May 2015. Data sorting and validation conducted by the first author produced a comprehensive inventory of cattle farms and farmers from 1999 to 2014. This was verified for dairy farms by matching farm registration number and farm name to the MOA dairy farm registration list, and for beef farms based on familiarity of BTEC staff with farmers and farm operations as there is no formal registration system for beef farms. The list identified 2,141 cattle holding facilities (dairy and beef farms of individual farmers, school farms, village/settlement, government stations, middlemen) including subsistence or irregular cattle farm operations. When needed, missing values for farm location were entered based on recall. This list was sent to MOA Economic Planning and Statistics Division (EP&S) to validate location details recorded for each farm. To ensure that all testing data were for bTB SID tests, a cross-check against records for bovine brucellosis testing was performed.

Data Analysis

Farm ID data from 1999 to 2014 and *Animal ID* data from 2011 to 2014 were available for analysis. Descriptive statistics for the number of positive farms and animals were calculated, and the number of positive animals detected through on-farm surveillance and abattoir monitoring were tabulated separately by division and by province per year.

Farm ID data were also analyzed to determine the number of tests conducted on infected farms each year and the status of each infected farm by year end. Within a calendar year, farms that had undergone one test with at least one reactor (SID test positive animal) were designated as "Infected." Farms that had undergone one test round with at least one positive result and undergone one follow up test within the same calendar year with no positive result were designated as "Restricted." Farms that had undergone two and three consecutive test rounds without any positive results were designated as "Provisionally clear" and "Cleared" farms, respectively.

For 2011 to 2014, the status of Infected farms from one calendar year to the next was investigated to identify farms that had positive cattle detected over consecutive years and did not attain cleared status within a period of two or more consecutive calendar years.

Results

From 1999 to 2014 ∼2,141 cattle holding facilities were included in the BTEC program across the 4 divisions of Fiji (**Figure 1**). On average, 25,693 cattle (median: 27,562, range: 7,552–43,516) from 258 farms (median: 272, range: 96–438) were tested per year during these 16 years. The majority of animals tested were located in the Central Division with an average of 21,339 cattle (median: 25,102, range: 4,701–34,955) tested in this division every year from 1999. Less testing was undertaken elsewhere, with number of years testing conducted and total cattle numbers tested per division being for Western Division (16 years; median: 3,155, range: 139–9,064), Eastern Division (9 years; median: 28,

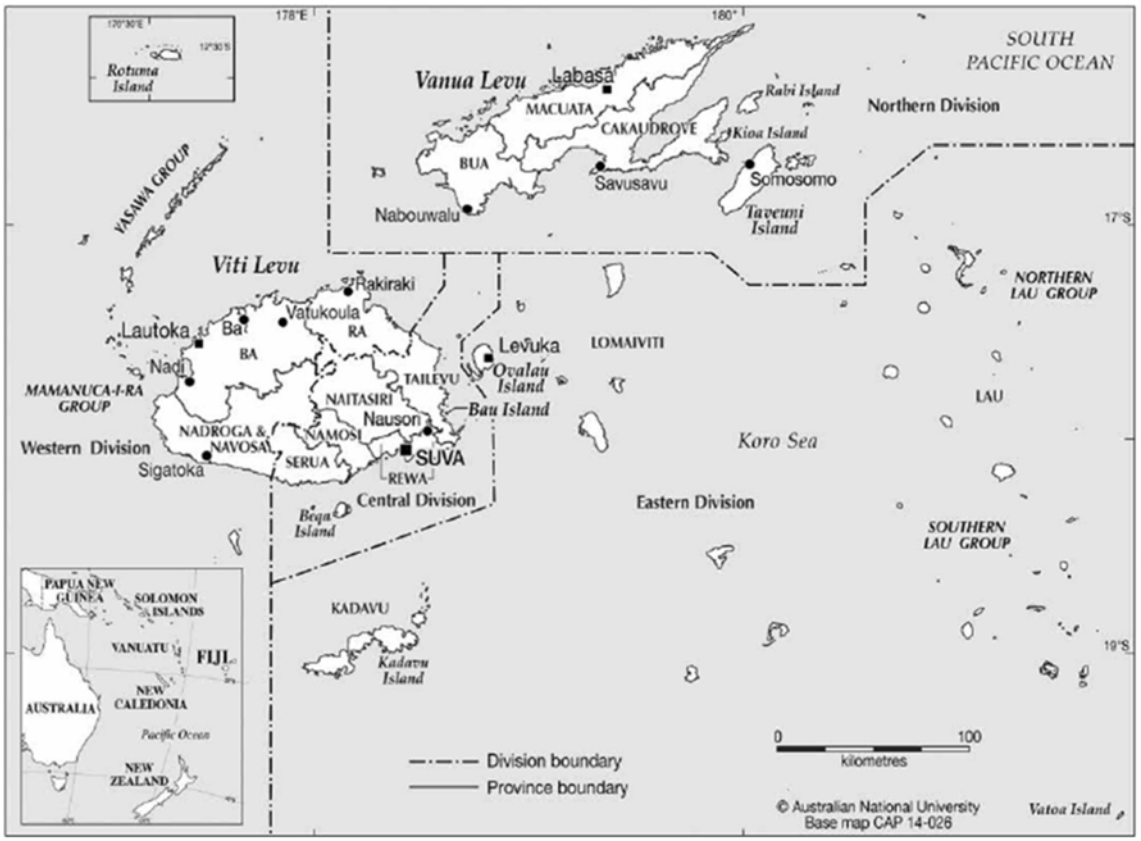

FIGURE 1 | Map of Fiji showing Division boundaries. Source: Maps Online, CartoGIS Services, ANU College of Asia and the Pacific, The Australian National University.

range: 0–397) and Northern Division (6 years; median: 0 range: 0–2,265).

Coverage of Fiji's BTEC Program

Cattle population numbers reported to the FAO and OIE required to estimate population coverage by the BTEC program were deemed unreliable prior to 2011 because the process used to estimate the reported numbers was not documented. For 2011 to 2014, the cattle population reported to the OIE based on data collated by a government veterinarian ranged between 40,008 and 44,388 cattle and the percentage of cattle tested ranged between 33.6 and 74.0%, with variation between years arising mainly from changes in the number of cattle tested. The total tested was markedly lower in 2004, 2006, 2007, 2010, 2011, and 2013, and for 2010 this aligned with a lower budget allocation compared to the previous year (**Table 1**).

bTB Positive Animals and Farms

A total of 2,823 TB positive cattle were identified from 1999 to 2014 with an average number of 176 (median 181.5) reactors per year (**Table 1**). The lowest number of positive cattle in a year was 17 from 7 positive farms in 2010, and the highest 721 reactors from 32 positive farms in 2014.

bTB positive cattle were identified in all four divisions of Fiji although the level of testing and the proportion of positive cattle varied between divisions (**Figure 2**).

For the Northern Division, testing was conducted in 1 of 4 provinces (Cakaudrove) in 6 years with positive cattle (termed reactors) detected in 1999 and 2004 (**Figure 2**). In 1999, 3 out of 9 (33%) farms tested in Cakaudrove province were positive (18 reactors). Testing was conducted only once on each positive farm and no further testing was scheduled in the Northern Division during the same year nor the following year to monitor the infected farms. In 2004, 1 out of 11 (9%) farms tested in the Northern Division was positive (4 reactors). This farm had been identified as infected in 1999 (15 of 18 reactors). No follow-up test was conducted to monitor this infected farm in 2004 or in 2005. No records were available to confirm if any of the reactors from the Northern Division were immediately culled**.**

In Eastern Division testing was conducted in 3 of 5 provinces (Kadavu, Lakeba Lau, Lomaiviti) in 9 years with three positive cattle from the two farms tested in Lomaiviti in 2005, and none in the other years (**Figure 2**). No further testing was conducted to monitor these two farms in 2005 or in 2006. No records were available to confirm if reactors were immediately culled from these farms.

In Western Division testing was conducted each year, and though usually undertaken in at least 2 of the 4 provinces

TABLE 1 | Budget for the Fiji BTEC program[a] and the number of farms and cattle tested for bovine tuberculosis by the program from 1999 to 2014.

Year	BTEC budget (USD)	Number of		Number and percentage with positive result			
		Farms tested	Animals tested	Farms		Animals	
				No.	%	No.	%
1999	36,450	373	38,870	56	15	220	1
2000	36,450	245	29,303	37	15	230	1
2001	72,900	299	26,277	50	17	293	1
2002	72,900	228	30,880	31	14	183	1
2003	72,900	170	27,506	26	15	121	0
2004	72,900	105	19,323	22	21	180	1
2005	72,900	438	41,591	34	8	192	0
2006	72,900	96	7,552	27	28	186	2
2007	114,079	98	9,569	23	23	61	1
2008	85,335	377	43,516	43	11	212	0
2009	718,065	417	32,160	11	3	39	0
2010	96,228	113	14,967	7	6	17	0
2011	437,400	136	14,916	14	10	60	0
2012	370,641	303	27,618	15	5	47	0
2013	364,500	324	17,439	11	3	61	0
2014	729,000	401	29,597	32	8	721	2
TOTAL	3,425,548	4,123	411,084	439		2,823	

[a] *Budget listed is the annual total for bovine brucellosis and bovine tuberculosis activities in the BTEC program.*

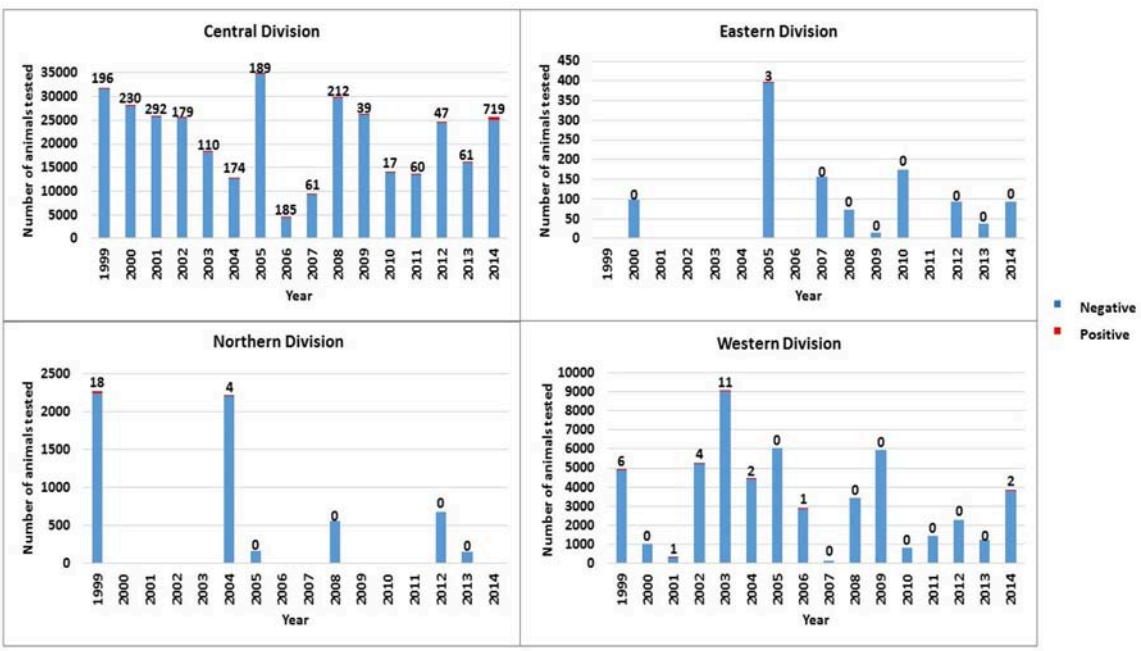

FIGURE 2 | Total number of cattle tested and number of bTB test positive cattle by division per year from 1999 to 2014 in Fiji.

annually for 16 years, most testing was conducted in Ba and Navosa/Nadroga provinces. Positive animals were detected in 7 of 16 years with the number and percentage of positive cattle ranging from 1 to 11 positive cattle or 0.04–2.27% (**Figure 2**). One farm was identified as positive in 2002 (all 4 out of total 4 reactors detected in this division in 2002 were located on this farm), 2003 (10 of total 11 reactors located on this farm), 2004 (2 of total 2 reactors located on this farm) and 2014 (2 of total 2 reactors located on this farm). No records were available from the abattoir at Vuda,

in Western Division for this study to confirm if reactors were culled.

For Central Division, testing was conducted each year in the 5 provinces (Naitasiri, Namosi, Serua, Tailevu, Rewa) with positive cattle detected consistently (**Figure 2**), particularly in Tailevu province that had test positive cattle each of the 16 years with the highest number of positives recorded in 2014 (**Figure 3**). Seven hundred reactors were detected in Tailevu from 23 of the 147 farms tested (15.7%) in the province during 2014.

For the 16 years that testing was conducted in Naitaisiri province, reactors were detected each year except in 2010 and 2011 when lower numbers of cattle were tested (862 in 2010, 1,833 in 2011). For the other years, higher numbers were tested with 7,046 animals (3 reactors) tested in 2009, 5,044 animals (4 reactors) tested in 2012, 4,466 animals (2 reactors) in 2013, and 7,831 animals (6 reactors) tested in 2014.

Serua province had the second highest number of reactors (12 of 721 reactors) in 2014, compared to earlier years when Naitasiri commonly ranked next to Tailevu. Data showed that in 2014, reactors in Serua came from only 1 of 6 farms (17%) tested in the province.

Farm Types

An average of 168 dairy cattle (average: 168, median: 172.5, range: 16–717) were detected positive each year during the last 16 years compared to beef cattle (average: 3, median: 3, range: 0–18). Ninety-nine percent (2,685 of 2,690) of positive dairy animals detected from 1999 to 2014 were from Central Division.

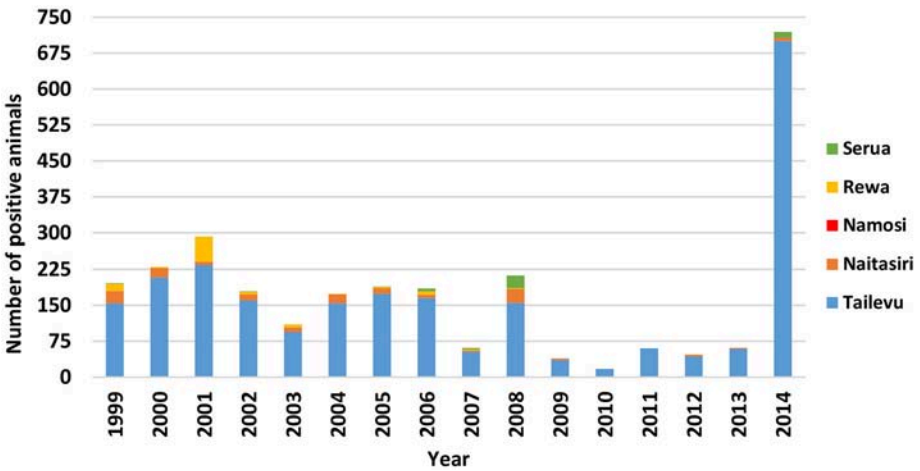

FIGURE 3 | Number of bTB test positive cattle per year in the five provinces of Central Division in Fiji from 1999 to 2014.

TABLE 2 | Number of cattle tested and of bTB test positive cattle by farm type from 1999 to 2014 in Central Division and Western Division Fiji.

Year	Central Division							Western Division						
	% Of positive animals			Number of animals tested				% Of positive animals			Number of animals tested			
	Beef	Dairy	Other[a]	Beef	Dairy	Other[a]	Total	Beef	Dairy	Other[a]	Beef	Dairy	Other[a]	Total
1999	0.2	0.7	0.3	919	23,598	7,195	31,712	0	0.5	0.1	982	396	3,515	4,893
2000	0	0.9	0.5	1,218	23,414	3,525	28,157	0	0	0	0	184	864	1048
2001	0.5	1.3	0.4	366	21,459	4,154	25,979	1.2	0	0	82	216	0	298
2002	0	0.8	0.3	662	23,064	1,935	25,661	0.1	0	0	4,900	0	319	5,219
2003	0	0.7	0.1	347	16,386	1,709	18,442	0.1	0.5	0	8,699	186	179	9,064
2004	0	1.4	0.9	387	12,110	214	12,711	0.2	0	0	1,029	17	3,363	4,409
2005	0.1	0.6	0	1,583	29,990	3,382	34,955	0	0	0	3517	132	2427	6076
2006	1.5	4.2	0	197	4,341	163	4,701	0	0.5	0	2,609	183	59	2,851
2007	0	0.7	0.4	304	7,662	1,308	9,274	0	0	0	0	0	73	73
2008	0.2	0.8	0	1,261	27,038	1,561	29,860	0	0	0	1,006	323	2,130	3,459
2009	0	0.2	0	904	24,559	716	26,179	0	0	0	1,695	4,134	97	5,926
2010	0	0.1	0.2	106	13,356	531	13,993	0	0	0	483	41	275	799
2011	0	0.5	0	453	12,776	263	13,492	0	0	0	1,102	0	322	1,424
2012	0.1	0.2	0.8	1,171	22,987	384	24,542	0	0	0	941	1,030	334	2,305
2013	0.5	0.4	0	560	15,173	286	16,019	0	0	0	122	1,037	68	1,227
2014	0.1	3.1	0.1	1,509	22,856	1,356	25,721	0.1	0	0	2,301	72	1,410	3,783

[a]*Farms with beef and/or dairy cattle that included school farms, villages/settlements, government stations and middlemen.*

For 1999 to 2014 in Central Division, a small proportion of dairy cattle tested positive every year (range 0.1–4.2%), from 0.1 to 1.5% of beef cattle tested positive in 8 of 16 years, and <1% of cattle from other farm types tested positive in 10 of 16 years (**Table 2**). In Western Division, lower proportions of positive cattle were detected in 4 of 14 years that beef cattle were tested, in 3 of the 13 years that dairy cattle were tested, and in 2 of the 15 years that cattle from other farms were tested (**Table 2**).

Among the dairy cattle that tested positive from 2011 to 2014 in Central Division, a high proportion were productive female cattle, for example, in 2011 when all test positive animals were dairy cattle, 61.7% were dairy cows and a further 16.7% were heifers selected to be milkers (**Table 3**).

Classification of bTB Infected Farms

Data show that from 1999 to 2014, no farms were cleared of bTB infection within a calendar year (**Table 4**).

Nine farms with positive cattle detected from 2011 to 2014 through on-farm testing and abattoir monitoring were all dairy farms situated in the localities of Waimaro and Namalata in Tailevu province (**Table 5**). Farms A and B had reactors consistently from 1999 to 2014. Except for Farms C and G, all other farms listed had their highest count of reactors in 2014. These farms are all located along an estimated 9.6 km stretch of the single major road in Tailevu.

Case Detection at Carcass Inspection

Each year from 2011 to 2014, cattle from Central, Western and Northern Divisions slaughtered at the FMIB Nasinu abattoir were found to have tubercle lesions during meat inspection (**Table 6**). The highest number of positive animals were from Tailevu province in Central Division with an average of 67 animals (268 total positives) detected in the abattoir per year. Although there was no reactor detected in Naitasiri during field testing in 2011 (**Figure 3**), three positive animals were detected at this abattoir. Further positive animals from the Northern Division were detected consistently from 2011 to 2014 with no on-farm detections despite testing conducted in 2012 and 2013 (**Figure 2**). Trace back of positive cattle from the Northern Division using the individual TB tag numbers showed that the 10 positive cattle had either read negative during on-farm testing or had never been tested on farm. This may imply that there are positive animals that are non-reactive to SID PPD-B affecting BTEC's proficiency in detection of infected animals in the field.

TABLE 3 | Number of bTB test positive cattle by age-gender group from 2011 to 2014 in Central Division Fiji.

Year	Heifer[a]	Dry cow[b]	Lactating cow[c]	Bull[d]	Steer[e]	No data	Total
2011	10	26	11	9	4	0	60
2012	14	8	17	4	4	0	47
2013	16	12	27	5	1	0	61
2014	134	212	260	76	33	4	719
Total	175	259	315	94	42	4	889

[a] *Heifer,female at least 6 months of age and not yet mated.*
[b] *Dry cow,adult female more than 12 months of age not being milked at time of test.*
[c] *Lactating cow,adult female more than 12 months of age being milked at time of test.*
[d] *Bull,adult uncastrated male.*
[e] *Steer,castrated male at least 6 months of age.*

Situation Analysis and Recommendations

The findings of the retrospective study confirmed that bTB had been endemic in Fiji for more than 16 years. Between 3 and 28% of farms tested per year in the BTEC program included cattle that tested positive to the SID test determined by the presence of a wheal size ≥4 mm until September 2014. This designation for a positive result at the highly specific interpretation of wheal ≥4 mm at the caudal fold was not adequate to identify sufficient positive animals for culling on infected farms to prevent ongoing bTB transmission.

There is clear evidence that bTB is well-established in the dairy cattle farms in Naitasiri and Tailevu provinces of Central Division on the main island of Viti Levu. While the strength of evidence for these provinces arises from a concentration of the BTEC program on-farm testing on the dairy farms in these two provinces, the abattoir monitoring results also support the conclusion of higher infection in these provinces at least for 2011–2014. Identification of SID test positive cattle in Central Division over multiple years also in beef farms (8 of 16 years) and other farm types (10 or 16 years) suggests that bTB infection is established throughout the cattle population.

Further the on-farm testing results and abattoir detections provide evidence that bTB is present among cattle farms in the other three divisions of the country, and in all 4 provinces of Western Division and in 3 of 4 provinces of Northern Division. Given the substantially lower numbers of dairy cattle in these other 3 divisions, this suggests that bTB is established at least among some beef cattle farms in Western Division and Northern Division.

From 1999 to 2014, the consistent positive status of a small number of farms and the fact that no farms were cleared of bTB infection within a calendar year (whilst acknowledging that a minimum of 9 months is required to progress from infected to clear status) is clear evidence that the test and cull plus quarantine procedures as applied for infected farms were inadequate to clear infection from a farm. The example of nine dairy farms located along one road in Tailevu province that were consistently positive for 2011–2014 exemplifies the situation with persisting infection.

The descriptive analysis of the BTEC data from 1999 to 2014 provided disturbing evidence that despite sustained efforts in on-farm testing and carcass inspection at abattoirs, BTB disease reduction and containment was not being achieved. This situation is well-illustrated although limitations of the 1999–2014 BTEC data, such as considerable variation in number of farms and animals tested between years and the positive SID designation based on wheal ≥4 mm, restricted the retrospective study to descriptive analyses.

Factors contributing to this situation and recommendations to strengthen the BTEC program are presented in **Table 7**. Further, given the need to identify bTB-free areas in Fiji that may be sources of replacement stock, surveillance sampling of farms in the provinces of Kadavu and Lakeba Lau in Eastern Division

TABLE 4 | Number of farms tested per division and total number of bTB positive farms from 1999 to 2014 with classification of these farms by the end of the calendar year.

Year	Farms tested	Central	Eastern	Northern	Western	Total Positive	Infected[a]	Restricted[b]	Provisionally clear[c]	Clear[d]
1999	373	49	0	3	4	56	47	9	0	0
2000	245	37	0	0	0	37	24	9	4	0
2001	299	49	0	0	1	50	40	8	2	0
2002	228	30	0	0	1	31	22	8	1	0
2003	170	24	0	0	2	26	24	2	0	0
2004	105	20	0	1	1	22	20	2	0	0
2005	438	32	2	0	0	34	20	9	5	0
2006	96	26	0	0	1	27	26	1	0	0
2007	98	23	0	0	0	23	23	0	0	0
2008	377	43	0	0	0	43	16	23	4	0
2009	417	11	0	0	0	11	8	2	1	0
2010	113	7	0	0	0	7	2	5	0	0
2011	136	14	0	0	0	14	12	2	0	0
2012	303	15	0	0	0	15	11	3	1	0
2013	324	11	0	0	0	11	10	1	0	0
2014	401	31	0	0	1	32	25	4	3	0
Total	4,123	460	2	4	11	439	330	88	21	0

[a] *Infected,farm with bTB positive cattle determined by on-farm testing or abattoir monitoring.*
[b] *Restricted,an infected farm after one negative round of testing.*
[c] *Provisionally free,an infected farm after two negative rounds of testing a minimum of 3 months apart.*
[d] *Clear,an infected farm declared bTB-free after three consecutive negative rounds of testing each a minimum of 3 months apart.*

TABLE 5 | Number of bTB positive cattle per year for the nine dairy farms in Tailevu province that were consistently positive for bovine tuberculosis from 2011 to 2014 detected through on-farm testing and carcass inspection at the abattoir.

Farm	Number of positive animals per year															
	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
A	10	43	34	31	27	48	18	21	7	19	10	5	21	6	36	76
B	2	53	11	27	8	4	29	16	2	28	5	2	7	14	20	199
C	9	9	10	15	5	10	1	12	1	1	0	0	8	3	23	11
D	0	2	4	4	0	5	4	6	2	3	6	0	6	6	5	43
E	11	9	20	5	2	41	31	20	4	39	0	2	17	23	17	53
F	0	6	11	1	0	0	10	7	2	10	0	1	18	4	16	66
G	6	6	10	6	0	8	3	13	0	0	0	0	7	6	1	4
H	23	18	77	6	26	8	7	22	6	15	9	0	31	11	12	76
I	0	0	0	0	0	1	4	0	0	4	0	0	4	3	1	24

should be conducted to confirm if these areas are bTB-free and permit declaration of a bTB-free zone in the country (14).

RESPONSES TO FINDINGS OF THE RETROSPECTIVE STUDY

The Fiji Ministry of Agriculture responded proactively to the findings of the retrospective study along with the Biosecurity Authority of Fiji (BAF) and the Fiji Cooperative Dairy Company Limited (FCDCL). The earliest responses commenced in late 2014 initiated following a preliminary analysis of the bTB records for 2011–2013. The response actions taken from 2014 to 2018 are described in detail below.

SOP for On-Farm Testing

The MOA updated the 2010 BTEC SOP in September 2014 and consequently implemented re-training of staff and calibration of BTEC field testing equipment. The revised protocol identified reactors as all animals that developed any size of wheal or redness in the SID injection site at the caudal fold 3 days after administration of PPD-B, following the OIE recommendation for detection of reactors in known infected farms (1). On the assumption that all cattle in Fiji are potentially bTB infected, this new protocol was applied to all farms, regardless of whether farms

TABLE 6 | Provinces with bTB positive cattle detected by meat inspectors at the FMIB Nasinu abattoir in Central Division, Fiji from 2011 to 2014.

Division	Province	Number of positive animals detected in the abattoir				
		2011	2012	2013	2014	Total
Central	Naitasiri	3	3	2	5	13
	Rewa	2	0	1	1	4
	Serua	1	0	0	0	1
	Tailevu	80	42	84	62	268
Northern	Bua	2	0	1	2	5
	Macuata	0	1	2	2	5
Western	Ba	1	0	1	0	2
	Navosa/Nadroga	0	6	6	2	14
	Ra	1	1	1	0	3
Grand Total		90	53	98	74	315

were previously identified as disease-free or infected (15, 16). This SOP change was implemented to improve the sensitivity of detection of infected animals in the field. A change that was needed for example due to identification at carcass inspection of some cattle with tubercle lesions that had previously tested negative using SID. A subsequent apparent outbreak of bTB in some farms was due to increased detection by the new test protocol, with a total of 721 reactors from 32 farms in 2014 compared to 61 reactors from 11 farms in 2013 (**Table 1**). This event raised concerns for the local dairy industry. The extent of infection in these farms was confirmed by post-mortem inspection of bTB reactors. For reactors at slaughter at FMIB Nasinu abattoir in Central Division, the percentages with generalized TB, gross TB lesions and no visible lesions were 33, 51, 16% in late 2014 ($n = 301$), and 26, 40, 34% in 2015 ($n = 1101$), respectively (17). The dairy sector experienced the greatest loss of cattle due to culling of reactors, and this had a serious economic impact for individual farmers and for the industry leading to a shortage of dairy cattle in the country, a reduction in the volume of milk produced, and an increase in the volume of imported processed milk (4). Dairy farmers with smaller, semi-commercial farms slowly converted to cash crops to supplement their dwindling income. In response to this serious situation, the MOA improved its compensation scheme in August 2015 to match current market prices per kg of condemned carcass. The purpose was to assist farmers recover quickly after losses from bTB (12).

Regulation of Cattle Movement

On 03 March 2016, as part of the disaster response of the Biosecurity Authority of Fiji post-Cyclone Winston a movement restriction on live animals was implemented to discourage movement of livestock without prior approval from the BAF or the Fiji National Disaster Management Office (18).

Subsequently on 13 January 2017 under section 77 of the Biosecurity Act 2008, the whole of Fiji was declared a biosecurity emergency area for Bovine Tuberculosis (*Mycobacterium bovis*) (19). During November 2017, the BAF and the MOA documented a movement control policy (20) to provide guidance on the implementation of cattle movement control. Movement of all calves and cattle within Fiji is strictly prohibited without prior authorization from BAF. Movement of cattle or calves without authorization is an offense attracting a maximum penalty of FJD 40,000 for individuals and FJD 200,000 for businesses or imprisonment. On 03 February 2018, the declaration was extended for continued implementation for a further 6 months.

National Stakeholder Forum

To clarify the status and extent of bTB infection and the challenges faced by the industry and to promote communication and collaboration in delivery of the BTEC program, a stakeholder forum was held in May 2017 with government MOA, Biosecurity Authority of Fiji (BAF) and Ministry of Health (MOH) representatives, Fiji industry stakeholders and relevant experts from Australia and New Zealand. Presentations highlighted the needs for a clear policy and strategy for bTB eradication and rehabilitation, action to address overlapping and unclear legislative and stakeholder responsibilities (particularly between MOA and BAF), immediate removal of infected cattle from farms, auditing and capacity building programmes, and a data recording system for monitoring, evaluation and learning (4). Stakeholders agreed that the BTEC Program requires further investment from the government to set up a stronger team structure with necessary equipment for disease surveillance and personnel with appropriate legal powers to effectively undertake its field operations. A draft BTEC strategy was developed during the forum and endorsed by stakeholders, and members for the BTEC planning committee designated to finalize the strategy document.

Documentation of Fiji Brucellosis and Tuberculosis Eradication Strategy

The Ministry of Agriculture further refined and finalized this strategy in early 2018. Input to this process included review and recommendations on meat hygiene, bTB control strategies and diagnostic test selection by a technical team under the Government of Chile funded project "Strengthening the institutions responsible for the inspection and certification

TABLE 7 | Factors contributing to this situation and recommendations to strengthen the BTEC program in Fiji.

Factor	Related to	Main recommendations
Insufficient consistency in the number and location of farms tested between years	Changes between years in government budget for the BTEC program eg reduction in 2006–2007 following political crisis in 2005/2006. Changes between years in budget allocation for bTB in the BTEC program eg reduction in 2009–2010 due to response to brucellosis detection after 13-years absence of detections (7). Insufficient number of BTEC field staff to conduct SID testing. No interrogation of BTEC records to inform plans for on-farm testing.	Ensure a consistent, adequate annual budget allocation for the BTEC program and the bTB component of it. Ensure adequate number of BTEC field staff. Implement a planning process for the BTEC program based on regular interrogation of bTB records with veterinary oversight. Establish a national database for data storage, manipulation and reporting.
Standard operating procedure for reading of SID test	Negative designation for any reaction at injection site <4 mm across all farms irrespective of status (unknown, infected, clear) will have led to a false negative result for some infected animals, such as cattle with chronic infection subsequently identified with tubercule lesions at abattoir carcass inspection and have impeded clearance of infection from infected farms.	Review of the SOP for reading of SID test particularly for known infected farms.
Inconsistent application of SOP for SID testing	Inadequate training and supervision of BTEC field staff.	Provide adequate training for BTEC field staff. Ensure adequate veterinarians in the BTEC program to supervise field staff.
Inconsistent application of SOP for test and cull and quarantine on infected farms	Inadequate training and supervision of BTEC field staff.	Provide adequate training for BTEC field staff. Ensure adequate veterinarians in the BTEC program to supervise field staff.
Unregulated cattle movements	Inadequate specification and implementation of cattle movement regulations.	Review of regulations on cattle movement administered by Biosecurity Authority of Fiji. Improve implementation of regulations by Biosecurity Authority of Fiji and consider involvement of harmonization with Ministry of Agriculture in implementation.
Stray cattle	Presence of stray cattle (untethered owned and unowned cattle grazing freely on public land and intruding on private land) acting to maintain infection in known infected areas.	Review of regulations on stray cattle administered by Biosecurity Authority of Fiji. Improve implementation of regulations by Biosecurity Authority of Fiji.

of agricultural products, and the coordination of the national system of food safety in Fiji" (21).

The goal of the Fiji BTEC Strategy is total eradication of bovine tuberculosis and brucellosis by 2037. The documented strategy lays out the direction for future implementation of the Fiji BTEC Program to attain the long term goal of official recognition by OIE of Fiji as free from both bovine brucellosis and bovine tuberculosis and maintaining this disease-free status (4). The strategy document includes specification on testing policy and strategy, zoning, reactor disposal and compensation, governance and operational management including staffing. It states a new role, full-time project manager, recognizing its importance for effective implementation of the BTEC program and an appointment effective June 2018 is being supported by the Fiji Dairy Industry Development Initiative [funded by New Zealand Ministry of Foreign Affairs and Trade (MFAT)].

Further Initiatives

Along with improvements to field testing and cattle movement control, opportunities for simultaneously improving the laboratory diagnostic capacity for bTB early detection and confirmatory diagnosis have been sought. The BTEC veterinarians and managers have established laboratory network links with Australia, India, New Zealand and Thailand to support diagnostic capacity building in Fiji. Under discussion with the World Organization for Animal Health (OIE) is funding for a Laboratory Twinning Program between FVPL and Animal and Plant Quarantine (QIA) Korea for proficiency testing and laboratory management training.

Concerned about the potential contribution of zoonotic TB to the human TB burden in Fiji, the MOA in collaboration with the Fiji Ministry of Health and Medical Services and the University of Sydney funded by the Marie Bashir Institute will undertake geospatial analysis of human tuberculosis cases and bTB-infected cattle farms, pilot TB surveillance of households in identified high risk areas for bTB exposure, and send samples from human extra-pulmonary cases and cattle cases for species determination. This investigation arises from concern about levels of extra-pulmonary tuberculosis (EPTB). During 2016 among the 312 notified human TB cases, 29% were classified as extra-pulmonary tuberculosis, nearly double the 15% of extra-pulmonary tuberculosis cases among the global total of human TB notifications in 2016 (3, 22). The contribution of bTB to these EPTB cases in Fiji is unknown because the routine diagnostics used do not distinguish pathogen species. There is suspicion of involvement due to the practice

of raw milk consumption in some households that own cattle.

DISCUSSION

When the preliminary analysis of 2011–2013 bTB records indicated wide spread endemic infection, the Fijian government acted swiftly in September 2014 to revise SOP for SID testing. This was followed up by actions from 2014 to 2018 that have enhanced identification of infected cattle farms and removal of infected cattle, strengthened implementation of restrictions on cattle movements, and led to the endorsement of a new Fiji Brucellosis and Tuberculosis Eradication Strategy. These are critical steps on the journey to reduce bTB in the national cattle herd, and then subsequently to progress to bTB eradication. This staged process of bTB reduction and containment followed by eradication can be guided by the lessons learnt by other countries on the road to bTB control and eradication, such as Australia, Ireland and New Zealand. The generic components, first of bTB control and containment while ensuring continuity of the industry, and second of bTB eradication and proof of freedom must be contextualized to the bTB situation in Fiji. A policy based on contemporary scientific evidence and international best practice in bTB control needs to be accompanied by specific research in Fiji, given its particular geoclimatic and cultural features. It is crucial for the Fijian government and the dairy and beef industries to be aware that the current policy will need to be modified over time and the commitment to implementation maintained when the BTEC program transitions to the final eradication stage. Industry concern about an increasing proportion of SID test positive cattle with no visible lesions at slaughter is expected with continuation of current SOPs. This provides an example of a situation where technical expertise is required to inform future decisions on test protocol, and where specific research would be beneficial to determine if false positive cases are present and to understand the basis and the extent of these. The international community also needs to consider its role in supporting the Fijian government and industry to attain bTB freedom for the benefit of animal and human health in the Pacific. As Fiji serves as a regional hub, providing live animal stocks and animal products to the neighboring island countries, addressing bTB in Fiji supports the long-term goals of sustainable livelihood and food security in the Pacific island region.

The case study of bTB control in Fiji offers lessons within a Pacific context about the importance of the following technical and social aspects to achieve success in animal disease control.

1. Objective, ongoing assessment of bTB distribution using agreed performance measures (such as bTB farm incidence, reactors per thousand tests, number and proportion of reactors removed) is internationally accepted as essential for critical assessment of progress toward control and eradication (8). This requires a national database for data storage, manipulation and reporting plus data sharing with other national systems for cattle movement and farm registration. It is timely that the NZ MFAT funded project Fiji Dairy Industry Development Initiative has extended its project coverage to include development of web-based database which will link the BTEC geospatial and farm registration information with the agriculture census information of the Economic Planning and Statistics Division of the Ministry of Agriculture. Funding to progress has been approved and the database is now at the planning stage.
2. Robust and accurate diagnostics able to minimize false farm or animal designation given bTB prevalence level at the relevant stage of the control and eradication process must be applied. Selection of the most appropriate diagnostic test/s given the stage of control program and the field conditions for animal testing requires expertise in test protocols. Understanding is needed of the costs of false designation to bTB maintenance and spread (in relation to false negative animals/farms) and to unnecessary loss of productive animals and prohibition to trade (in relation to false positive animals/farms). The Fiji MOA recognizes that early identification of infected farms and infected animals is critical. To date the SID PPD-B in the caudal fold is the single diagnostic applied in the Fiji BTEC program due principally to its low cost and practical suitability to on-farm conditions. While the combination of SID test in the caudal fold (assuming use of potent tuberculin) and carcass inspection at slaughter is reasonable for detection of infected farms, a more sensitive testing regime is needed to support eradication from known infected farms. Thus, the MOA is considering application of other diagnostics, such as the interferon-γ test as a confirmatory test for SID positive animals in known infected farms, and increased use of culture to confirm status of lesions identified at abattoir carcass inspection. A cost-benefit analysis on the use of single intradermal comparative tuberculin test (SICTT) and interferon-γ [with sensitivity when applied in parallel approaching 93% (23)] in place of SID for cattle on known infected farms to aid control and eradication whilst maintaining a milking herd to permit business continuity is recommended.
3. Quality control (QC), the managerial process to compare actual and desired performance of a service or product, will act to ensure an animal disease control program is meeting its objective at the best possible return for the funds invested (24, 25). When disease detection is based on diagnostic procedures with aspects that have subjective interpretation, such as the SID PPD-B and post-mortem inspection (26), quality control will contribute to improve accuracy and consistency in detection. The Irish bTB eradication program with QC applied inputs (personnel, training, SOP, equipment, tuberculin, reagents, computerized recording system), performance (post-mortem surveillance, field surveillance) and outputs (test results, program delivery), provides a model for consideration. For example, the National Handbook of the Irish program that states the national policy and SOP for veterinary management of herds under restriction due to bTB is revised every 3 years to ensure continued improvement and refinement of program activities (27). Given the reliance on SID in the caudal fold and carcass inspection at slaughter for infected farm detection in Fiji, QC

should particularly focus on checking tuberculin potency and standardized training and competency testing of government meat inspectors.

4. Farmer cooperation with control and surveillance activities is vital for the success of animal disease programs. Active participation requires farmer knowledge of bTB risk and impact on cattle production and health, and farmer confidence that BTEC requirements are feasible and effective. Strengthening incentives, such as compensation for culling of positive animals will encourage more farmer cooperation. Effective communication about bTB via farmer targeted and general community campaigns is required to generate farmer action and community support. Clear messaging is proving challenging for bTB due to confusion about tuberculin skin test performance, the involvement of wildlife reservoirs in some countries, and local cultures and beliefs, particularly in countries where despite sustained control programs bTB remains endemic, such as Spain and the United Kingdom. Recent qualitative research involving farmers and veterinarians in Spain articulated the link between farmer non-participation in on-farm testing and distrust of official veterinary services and with farmer perception of little benefit to be gained from bTB freedom (28).

CONCLUSION

The Government of Fiji has demonstrated sustained commitment to reduce bTB in the cattle population. The determination to succeed in a resource limited setting with challenging field conditions is to be commended. The history of bTB control elsewhere shows that the use of tuberculin tests (SID PPD-B and/or SICTT) needs to be relevant to the context (29) and the purpose of their application communicated clearly to avoid confusion and farmer disengagement (28). Guidance from the international animal health community is essential to inform refinement to the Fiji BTEC Strategy on the journey to a bTB-free Fiji.

AUTHOR CONTRIBUTIONS

EB collated and analyzed the data for the retrospective study. RP assisted EB with collation and entry of the bTB data 1999–2014. LB, RP, and TT assisted with interpretation of the findings of the retrospective study. All authors contributed to the content and the preparation of this manuscript.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the Fiji Ministry of Agriculture for granting permission to conduct the retrospective study of the 1999–2014 Fiji BTEC data and the BTEC personnel that performed the data transcription. The first author completed the retrospective study in partial fulfillment for the requirements of the Master of Veterinary Public Health Management degree at The University of Sydney.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer MG declared a shared affiliation, with no collaboration, with one of the authors, EB, to the handling editor at time of review.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 26 March 2019
doi: 10.3389/fvets.2019.00081

Negotiated Management Strategies for Bovine Tuberculosis: Enhancing Risk Mitigation in Michigan and the UK

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Bovine tuberculosis (bTB) is an epidemiologically, politically, and socially complex disease. Across multiple international contexts, policy makers have struggled to balance the competing demands of wildlife and agricultural interests in their efforts to create workable and effective disease management strategies. This paper draws comparative lessons between the cases of Michigan in the USA and the UK to exemplify some of the challenges of developing an effective strategy for the long-term control of endemic disease, particularly reflecting on efforts to "responsibilise" cattle producers and engage them in proactive activities to mitigate transmission risks on their own farms. Using qualitative data derived from 22 stakeholder interviews, it is argued that the management of bTB in Michigan has important lessons for the UK on the role of human dimensions in influencing the direction of disease control. The management of endemic bTB relies on the actions of individuals to minimise risk and, in contrast to the predominantly voluntary approach pursued in the UK, Michigan has shifted the emphasis towards obtaining producer support for wildlife risk mitigation and biosecurity via a mix of regulatory, fiscal, and social interventions. Whilst the scale of the bTB challenge differs between these two contexts, analysis of the different ideological bases for selecting management approaches offers interesting insights on the role of negotiated outcomes in attempts to adaptively manage a disease that is characterised by complexity and uncertainty.

Keywords: bovine tuberculosis, risk mitigation, biosecurity, human dimensions, responsibilisation

OPEN ACCESS

Edited by:
Daniel J. O'Brien,
Michigan Department of Natural Resources, United States

Reviewed by:
Shawn J. Riley,
Michigan State University, United States
Steven Leroy Halstead,
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *03 August 2018*
Accepted: *26 February 2019*
Published: *26 March 2019*

Citation:
Little RA (2019) Negotiated Management Strategies for Bovine Tuberculosis: Enhancing Risk Mitigation in Michigan and the UK. Front. Vet. Sci. 6:81. doi: 10.3389/fvets.2019.00081

INTRODUCTION

Bovine tuberculosis (bTB) is principally a disease of cattle, but there are several places worldwide where free-ranging wildlife are reservoirs of infection, namely brushtail possums in New Zealand, European badgers in the United Kingdom, wood bison and elk in Canada, African buffalo in South Africa and white-tailed deer in the United States (1). Where the disease has become established, it can have considerable economic consequences for livestock keepers and poses challenges for national governments and agencies in devising a workable and socially acceptable eradication plan. The ultimate rationale for intervention is based on the potential threat *Mycobacterium bovis* poses to public health (2); however, the proximate driver for expenditure on bTB management is the potential economic effect of trade restrictions on milk and meat products (3, 4) and the wider ecological concerns associated with potential disease spread into new regions and ecosystems.

The case for eradication has been contested based upon cost benefit criteria and the relative importance of the risk posed to human health [see (5–7)], but it remains the declared goal for many international control programmes [see, for example, (4, 8)].

Experiences from around the world exemplify the challenges faced by disease managers in constructing a coherent, cost-effective, and workable strategy for eradication. Multiple ecological and epidemiological challenges remain [see (9, 10) for a review], but socio-economic and political factors also have a key role to play in influencing the outcomes of disease control strategies; including, the cost-effectiveness of the policies, political will to implement management programmes and the social acceptability of individual control measures. The UK is perhaps the foremost example of the difficulties involved in constructing a control regime under conditions of intense socio-political scrutiny. A primary point of contention has been the decision to cull badgers in England, which are considered to have important cultural associations for the general public [see (11, 12)]. Vigorous debate on the role of badger culling in the control of bTB has resulted in policies that have been considered to lack coherence (13) and a situation where the devolved administrations pursue their own control policies, with differing approaches to addressing the disease in their wildlife populations (14, 15)[1]. This has resulted in what Allen et al. [(10), p. 110] considers this to be part of "the current impasse in bTB control" across Britain and Ireland, with multi-factorial problems inhibiting the national eradication programmes.

Socio-economic and political factors have been highlighted as determinants of success in analyses of international control programmes. For example, Professor Ian Boyd, The Chief Scientific Adviser to the UK government's Department for Environment, Food and Rural Affairs (DEFRA) described bovine tuberculosis as a "sociological problem," stressing the importance of human dimensions in influencing disease outcomes. Similar claims have been made in review papers on the complexity of bTB control (16) and in studies of eradication attempts in the US (1), Australia (17), and New Zealand (18, 19). These determinants tend to focus on three separate, but interconnected factors: the effectiveness of political decision-making; social acceptability of the policies; and the attitudes and actions of affected stakeholders.

This paper focuses on the experience of bTB control in the US state of Michigan to provide a comparison for current and future policy developments in the UK. Whilst the scale of the problem in Michigan is different to the UK, there are interesting comparators in terms of socio-economic and political factors influencing the perceived success of efforts to achieve effective disease control. For example, Carstensen et al. (1) reported, "public tolerance" and political will were considered to exert significant influence on the control measures available to disease managers in the US. The authors also cite a series of temporal, social, economic, and logistical factors that shaped public and stakeholder attitudes towards aggressive disease control strategies, the limitations that these factors placed on management options and the subsequent implications for bTB eradication from the wildlife reservoirs in the USA. Carstensen et al. (1) concluded that, in comparison to the response to a notable outbreak of bTB in Minnesota in 2006, which successfully prevented the self-sustaining establishment of the disease in wildlife, Michigan has lacked the leadership to initiate more "aggressive" bTB management strategies in both cattle (via, for example, buy-out options for herds in areas of high bTB risk) and wildlife (through substantial reduction in deer numbers via intensive culling).

Without the will to institute more "aggressive" responses to controlling the disease in cattle and wildlife populations, the management of bTB often requires a negotiated management response, based upon the level of funding available and the buy-in from the thousands of individual disease managers (e.g., farmers, hunters, and the like) tasked with controlling the disease over a sustained period. As Miller (20) notes, management of diseases at the livestock–wildlife interface often require long-term engagement using a combination of altered livestock husbandry practices, active disease suppression in wildlife, and prevention of transmission using mitigation techniques. Considerable attention has been given to the development of interventions designed to mitigate the risk of bTB disease transmission between cattle and wildlife [see (21, 22)]. Generally, the research concludes that risk mitigation interventions such as deer exclusion fences have great potential but the challenge lies in farmers modifying their husbandry practices and behaviours (20) including maintaining the integrity of fences and keeping gates closed (23, 24). Risk mitigation measures that rely on stakeholder adoption of preventative behaviours [see (25)], therefore, pose challenges for risk managers in formulating measures that will incentivize positive responses.

Similar issues can be observed in the UK relating to the adoption of preventative biosecurity measures at the farm level. Whilst biosecurity is cited as a key part of the Defra's 25 year Strategy to Achieve Officially Bovine Tuberculosis Free Status for England (2014), multiple challenges remain regarding farmers' adoption of measures to reduce the risk of bTB transmission between cattle and between cattle and wildlife. Farmers can be reticent to implement measures because of the limited evidence surrounding the efficacy of many of the interventions (9, 26, 27); the perceived impracticality of implementing measures on their own farms (28), particularly relating to badger exclusion and isolation of bought in cattle, and the uncertain benefits that will accrue in reducing their risk of a bTB breakdown as opposed to the costs of modifying feed and water sources, installing fences to reduce contacts with neighboring herds or establishing isolation facilities for newly bought in animals. Whilst farmers acknowledge the theoretical importance of biosecurity as a preventative measure, this does not always result in taking action to reduce risks on farm (29–31). Such reluctance to act may be associated with farmers' often-reported "fatalistic" belief that there is little that they can proactively do to prevent a bTB breakdown or that "luck" rather than their own actions has more

[1]Animal health is a devolved issue in the United Kingdom. England, Wales, Scotland, and Northern Ireland each have the ability to develop and implement their own control policy for bovine tuberculosis, which is currently subject to oversight and audit by the Food and Veterinary Office of the European Commission. It should be noted that Scotland has been Officially Tuberculosis Free (OTF) since September 2009.

of an influence on the likelihood of the disease entering their herds (32–34).

Currently, the majority of biosecurity measures outlined in Defra's 25 year Strategy are voluntary, with some additional requirements for farms within badger culling areas and for "persistent" bTB herds. Improving biosecurity on and off farm is stated as an important management goal within Defra's Strategy. As the literature indicates, risk managers will need to formulate measures to address the apparent disjuncture between the acknowledged importance yet under-implementation of risk mitigation measures on farm. Using Michigan as a case study, the objectives of this study were to investigate management approaches, policies and interventions designed to engage farmers in adopting and sustaining preventative bTB biosecurity measures and qualitatively assess their impact in contributing to disease control.

The paper will outline some of the comparative lessons that can be learned from Michigan in their attempts to enhance the on-farm risk mitigation element of their disease management strategies and the policies considered most effective in encouraging proactive disease management at the farm level.

METHODOLOGY

The research focused on stakeholder perspectives on eradication efforts, assessing the relative merits of different policy interventions aimed at disease management and appraising the key factors affecting efforts to achieve bTB eradication. The research approach was based upon 22 in-depth face to face interviews conducted at the end of 2014. Non-probabilistic, purposive sampling [akin to (25, 35)] was used to select interviewees with individuals identified based upon their roles as "experts" and "key stakeholders" involved in the development or implementation of bTB policies in Michigan. This research was part of a wider study that included a further set of interviews in Minnesota; the results of which was not reported here. Interviewees were stratified into the following three broad categories: agency professionals involved in bTB management in cattle or wildlife (wildlife managers, programme coordinators, field veterinarians, and communications specialists); university academic and extension personnel; and cattle producer and wildlife stakeholders involved in implementing management practices on the ground. Interviews were conducted in the State capital and in the Modified Accredited Zone (MAZ) in the northeastern lower peninsula (NELP) of Michigan, concentrating on the counties of Alcona, Alpena, Montmorency, and Oscoda Counties.

The research was designed to be a qualitative, in-depth assessment of bTB management approaches in Michigan. As indicated by Naylor et al. [(36), p. 286] "interviewing is the method most often adopted to explore potentially sensitive and controversial issues… and are often commended as a research method for their flexibility and ability to explore difficult issues in a comprehensive and sensitive manner." Unlike the standardised and structured approaches of farmer attitude surveys or Q-Methodology [e.g., (35, 37, 38)] the interviews were semi-structured and discussions were based around a set of themes within an interview guide; this approach has been used in equivalent qualitative studies on bTB and biosecurity [see (32)]. The interview guide consisted of questions relating to the participant's role in bTB control; overview of the factors influencing the relative success of bTB control (including identifying effective policies and interventions); identification of key stakeholders and their positive or negative contribution to disease management; modes of risk communication and the challenges and successes encountered in promoting "best practice" in disease mitigation; and lessons learnt from their experience of managing bTB in Michigan[2]. Each interview was tailored to the expertise and knowledge of the interviewee and so the focus of each discussion was context specific. However, all interviewees were asked about and responded to questions on policies and interventions that were considered to be effective in encouraging disease managers (e.g., farmers and hunters) to adopt positive disease management practices. The results of which are reported here.

Interviews were digitally recorded (with the participants' informed consent) and later fully transcribed. The data was manually coded in order to develop an empirically grounded coding framework, guided by the key research questions. This involved an iterative and in-depth process of "careful reading and re-reading of the data" [(39), p. 258], beginning with an informal reading of the materials to identify an initial set of high-level thematic codes. The approach followed the conventions of Seidel and Kelle (40) quoted in Basit [(41), p. 144] who "view the role of coding as noticing relevant phenomena; collecting examples of those phenomena; and analyzing those phenomena in order to find commonalities, differences, patterns and structures." Categories were developed via a process "data distillation" (42) to organise the coded data into meaningful overarching themes. The themes were based upon concepts from existing literature and from words and phrases used by the interviewees e.g., notions of responsibility and responsibilisation; social networks and peer example; drivers and incentives. These themes are represented as organising concepts in the results section.

Following a broad introduction to bTB management approaches in Michigan, an overview will be provided of the Wildlife Risk Mitigation project, which was identified as being a key development in efforts to enhance on-farm biosecurity activities.

RESULTS

Management Approaches for bTB in Michigan

On-farm Wildlife Risk Mitigation (WRM) is part of a wider approach to bTB management in Michigan, including surveillance, and control measures aimed at reducing the disease burden in both cattle and wildlife (white-tailed deer). The focus

[2]For study replication purposes, the interview guide is included as a **Supplementary Data File**. Full details of the sampling, research approach, and anonymized transcripts can be found within the ReShare UK Data Service repository.

of this paper is WRM, however a brief overview of the control programme is described here.

Michigan was declared free of bTB in cattle and bison in 1979. However, in 1975, and again in 1994, bTB was identified in one wild white-tail deer in the NELP of Michigan. Subsequent testing revealed the disease to be endemic in the white-tail deer population within five of the most north easterly counties of the Lower Peninsula. Since 1995 surveillance and testing has been carried out in the affected area via annual surveillance of hunter harvested deer. To date, the disease has been confirmed in nearly 875 of over 254,000 free-ranging deer tested in Michigan, with 77% of bTB-positive deer found in a core area—Deer Management Unit 452—in the NELP of Michigan, where the counties of Alcona, Alpena, Montmorency, and Oscoda meet (**Figure 1**). Reduction in deer density within the affected area is a key part of the policy, with enhanced measures introduced over successive seasons designed to maximise legal opportunities for the public to harvest deer. These strategies include liberalised hunting seasons; issuing landowners Deer Management Assistance permits to supplement hunting licences; providing disease control permits to cattle producers and non-agricultural landowners in high prevalence areas; and, most recently, the introduction of the Hunter Access Program, to match hunters in search of places to hunt with agricultural landowners seeking additional deer harvest on their land. Deer baiting and feeding bans are also in operation in some of the affected areas.

Following the identification of bTB positive deer in the 1990s, the reinstatement of cattle testing in the affected area revealed the first infected cattle herd in June 1998. Michigan subsequently lost its bTB free status in June 2000 and state-wide surveillance testing was instituted from 2000 to 2003. The Upper Peninsula regained bTB Free status in 2005 and 57 counties in the Lower Peninsula regained bTB Free status in 2011. Surveillance testing identified a core disease outbreak area in 11 counties in the northeastern tip of the Lower Peninsula; since October 2014, seven more of those counties have been declared bTB Free for cattle, leaving 4 remaining. Annual testing of all livestock (cattle, goats, bison) and captive cervids remains in place in the 4 counties (classified as the MAZ by the US Department of Agriculture's Animal and Plant Health Inspection Service-Veterinary Services Branch[3]), with risk-based testing applied throughout the remainder of the State. In the MAZ, the traceability and movement of livestock is regulated through movement permits obtained from the field offices of the Michigan Department of Agriculture and Rural Development (MDARD), electronic identification of animals and annual herd inventories to reconcile discrepancies between animals on farm and official records. Other policies governing livestock movements and limiting deer-cattle contacts will be covered more fully in the following section.

FIGURE 1 | Deer Management Unit (DMU) 452, in northeastern Lower Peninsula of Michigan, USA.

Wildlife Risk Mitigation

WRM is now a key element of the bTB management strategy, particularly concentrating on the commercial farms in and around the MAZ in the NELP of Michigan, identified as at risk for bTB transmission from wildlife. The policy began as a series of small scale activities at Michigan State University (MSU) which, from 2008, formalised into a voluntary initiative developed by MDARD, MSU Extension, United States Department of Agriculture (Veterinary Services, Wildlife Services), and the Natural Resources Conservation Service (NRCS), with some input from industry. The objective of the programme was to assist producers in identifying high risk areas and practices on their holding and develop plans to reduce the risk of cattle-wildlife interactions. The approach was designed to form part of the "safety nets" (44) put in place to control the disease, complementing the surveillance testing and movement restrictions in helping to prevent opportunities for infection; the ultimate aim being to draw down the disease incidence in cattle.

The programme required changes to be made to management practices and farm infrastructure in the endemic area. It relied upon the development of a series of interventions to assist and influence the implementation of risk reduction measures on farm, including the introduction of hoop barns and deer-proof

[3]The prevalence of infection a State or zone are classified in five categories: (1) Accredited-free state or zone; (2) Modified Accredited Advanced state or zone; (3) Modified Accredited state or zone; (4) Accreditation Preparatory state or zone; and (5) Non-Accredited state or zone [see (43) for an explanation of the United States bTB accreditation categories].

fencing to protect stored feeds and actions related to cattle accessing feed and water sources. The changes required at farm-level meant that the concept of WRM was controversial from the outset. According to a Michigan policy lead, *"this was probably the most controversial thing that happened in the course of the bTB programme; more so even than testing…there was a tremendous amount of angst and anger about this wildlife risk project."* Producer concerns focused on the practicalities of excluding deer from their property; the cost of implementing the measures and a perceived inadequacy on the part of Department of Natural Resources (DNR) to deal effectively with the disease in wildlife (e.g., through the reduction of deer numbers). Due to the contentious nature of the proposal, the policy making process involved a series of meetings to develop proposals and standards which were an acceptable compromise between what was desired by policy makers and risk managers and what was considered achievable in practice by the agricultural industry. The process was described in the following terms by an individual involved in the development of the scheme:

> *"it's that idea of, okay, if you can't build 20 foot or 12 foot high barbed wire fences all the way round…where are the opportunities to reduce risk most cost effectively? So we got the best available science from [Michigan's bTB Programme] and we started sharing it with our stakeholders, the producers and let them decide."*

The process of negotiation, over a series of three meetings, focused on achieving a balance between an epidemiological ideal and an implementable policy. The process was facilitated by MSU staff as intermediaries and the University published the document.

Implementation

The implementation of the scheme was described by its instigators in terms of a phased approach, based upon the principles of adaptive management [see (45–47)]: phase one was aimed at individuals identified as "early adopters" who were engaged with a prototype version of the WRM intervention; the second phase was an expansion of the programme, designed to appeal to "capable learners"; and the third was regulatory enforcements to draw in those who were "resistant to change." It was also phased in regionally; MDARD concentrated on the outlying areas first, where there was an opportunity to elevate the accreditation status more swiftly (for example, in Michigan's Northwest Region where bTB was not endemic in wild deer) and moved on to the more challenging and higher risk area of the MAZ over time. This incremental approach evolved into an increasingly statutory regime and relied on a number of key push and pull factors designed to maximise participation in the scheme. A combination of one-to-one assistance, co-funding of risk mitigation measures (such as deer fencing) and restrictions placed on market access have been employed to both encourage and enable producer engagement in the scheme, but also to make it challenging for them to stay outside of the system.

The WRM project is designed around a five-step process which aims to bring livestock producers and technical experts together to create a tailored on-farm plan to reduce the risk of infection between cattle and wildlife. Producers are offered an educational meeting before completing an on-farm risk assessment. The risk assessment is conducted between government agency staff and is designed to be both educational (recognising potentially risky areas, and practices on farm) and regulatory, with the implementation of certain mitigation actions being classified as compulsory. Once the WRM Action Plan has been agreed, the producer then indicates a timescale within which they propose to complete the actions. Depending on the risks identified on farm, these actions may include interventions to limit potential infection transfer at sites where cattle are fed (governing where, how often and how much cattle are fed), water sources for cattle and where cattle feed is stored. Each of these sites have been identified as a risk for disease transmission (48, 49) and so require changes to management practices, including fencing off feed and water sources to prevent deer access. Once the plan has been implemented, the work is subject to an annual verification process to check that the interventions and actions are still in place.

As part of the development of the plan, a cost-sharing scheme was introduced to assist cattle producers in implementing the actions. During 2008–2013, over $3.6 m was expended on WRM measures. Government, state and federal funds accounted for $2,637,000 of this figure and a further $1,002,000 was contributed by cattle producers. In the early phases of the scheme, 50% of the cost-share funding came from the state and the USDA, and in the later phases, the bTB programme utilised the USDA, NRCS's Environmental Quality Incentives Programme. The benefit of the latter approach being that mutual aims could be achieved from a single funding allocation and that the conservation office, which already had close historical links to the farming community, could take over the responsibilities for the continuation and annual verification of the scheme.

Drivers and Incentives

The development of the risk assessment and verification process was originally badged as a voluntary approach. However, (dis)incentives were introduced to influence the level of uptake amongst producers. One interviewee described it as, "*incentives on the cattle side were, first of all, it was disincentives, you couldn't move [cattle] if you didn't do it.*" Additional testing and restrictions on market access were the primary levers to encourage uptake of the WRM. The policy stipulated that a pre-movement test be carried out on cattle from non-WRM farms, with a further post-movement test 60 to 120 days after purchase being required of the purchaser at their own expense. The rationale for the approach was described by an individual involved in developing the policy as follows:

> *"So the state used to pay for all that [testing] and in these counties we've said okay, you know, you have an hour, you could get a biosecurity plan and you don't have to do this test, but, you know, if you don't want to do that that's fine, you can do this additional test, but you get to pay for it now and then the guy who buys your cows, unless he gets them slaughtered, has to also do a test at his expense. Well that means that the cattle are discounted, because when people go, oh, I got to do a test, well that's going to cost me something, so*

I'm not going to pay quite so much for these cattle and so that has driven some people and we were trying to use market forces to, you know, move people towards doing the right thing."

Through restricting market access and attaching a financial disincentive to the cattle from non-WRM farms, the aim was to shift producers' assessment of the costs and benefits in favour of enrolling in the WRM scheme. The (dis)incentives were strengthened in January 2015, when regulations were introduced stating that all farms in the bTB core area must be wildlife risk mitigated; otherwise, these producers could only send their animals to slaughter.

Social Networks and Peer Example

In addition to perceived economic (dis)benefits, risk managers involved in developing, and refining Michigan's eradication programme employed a series of techniques to influence the social context into which their strategies were being placed. The approach included the use of existing social networks within the locality to promote sign up to the scheme and peer example coupled with "teachable moments" to encourage producer-advocates of the scheme to explain the benefits, particularly following cases of bTB outbreaks where WRM may have been assistive in preventing disease transmission. The rationale being, as summarised by an extension agent, *"peer example, call it, rather than peer pressure, can be very effective."* The use of social networks was seen as a way of dealing with the negative view towards government officials and enlisting more trusted intermediaries to deliver the message on the benefits of the scheme. This approach is exemplified in the following quotes:

"I think other people have said okay yeah if I'm hearing this from my neighbour and my friend I'm not hearing it from the state veterinarian or, you know, some USDA regulator, but I'm hearing it from, you know, my friends and they tend to take it a little bit more seriously, especially if you're seeing that person every day or at church or in a grocery store or at the bar or whatever, so that makes it a little bit more real".

"So one of the things we did, we had I don't know about maybe 45, 46 of these that were still hanging out here in the farms in here that had not done a biosecurity plan and so back in April I made phone calls to people that work on these farms and just trying to ascertain who is the person that might most effectively communicate things in a positive way, where we would get them actually to do something and so actually some of our guys, you know, are relatives to these people or they've cultivated, you know, decent relationships."

The role of these gate keepers within the producer community was important to facilitate wider implementation, using existing social networks to connect government authorities with producers at the farm level. There were also particular individuals that were highly functional in terms of engaging producers and hunters in disease management efforts, be they as an identifiable, visible, and approachable lead of the bTB programme or as key personnel within the areas most at risk from a bTB outbreak. In the words of one policy maker, "*[t]he policies were supporting the risk mitigation, the policies were making sure you had some local expertise, it wasn't just coming out of Lansing to talk to people."* The division of "distant" government officials in the State Capital of Lansing and the affected communities in the NELP was addressed through convening local meetings, placing the onus on appointing personnel from within the local area and working through MSU extension, which has long-established links with cattle producers via existing research programmes and community outreach.

Sustaining Disease Management Practices

During the development phases, it was recognised that the installation of measures such as deer fencing was only the first part of a successful WRM plan. The second part was the maintenance and continued use of measures by cattle producers, such as keeping gates to feed sources closed. The challenge of sustaining disease management practices at the farm level was described in the following terms:

"How do we get producers to do that, how do we support it, you know, how do we maintain it, because, you know, you can pour a lot of money into fencing and, you know, other mitigation, but if you do it for 1 year and then you say it's too much trouble, you know, to keep the fences maintained and stuff like it doesn't really matter then, so it's not only doing the mitigation, but then maintaining it over time."

To address this challenge, conditions were attached to the grants allocated for co-funding of WRM measures. Producers were required to sign a contract outlining their obligations (e.g., closing gates) and if they were found to be in contravention of those conditions, then the state would be entitled to reclaim the cost-share money and the farm's WRM verification would be withdrawn, with consequent implications for trade and enhanced testing.

Promoting Action and Assessing Impact

WRM began as a controversial policy aimed at enhanced risk mitigation at the farm level. As already noted, the development was controversial because of the implications that the new measures and requirements had for farm management decisions and infrastructure. During interviews, stakeholders reflected on the difficulties involved in introducing and implementing the scheme, but also recognised the perceived benefits that WRM provided in terms of enhanced disease management through reducing risk at the livestock-wildlife interface and the transfer of responsibilities for disease management to producers on their own properties. The following section provides an overview of stakeholder perspectives on the perceived utility and impact of the WRM scheme.

Responsibility

A clear reason for the development of the WRM scheme was to re-centre the responsibility for keeping bTB out of herds back into the hands of the cattle producers. Whilst WRM has been a predominantly government-led scheme (with input from producers and producer organisations), the aim has been to highlight what producers can do on their own holdings to mitigate risk and then, via co-funding and advisory visits, enable

them to implement exclusion measures such as barns and deer fences. This represented a step change in the policy. In the words of a field veterinarian:

> *"I mean before [WRM] it was just test, test, test, test, test, test, find it, where do we find it? And it wasn't until the wildlife risk programme started that we started having something to say hey, let's do something to help prevent it".*

The emphasis on engaging producers in proactive action was driven by a number of considerations: first, the need for producers in the NELP to act in the interest of the rest of the cattle industry in the state of Michigan (to retain interstate market access); and second, the realisation that deer would remain only a partially controllable element of disease transmission due to a perceived—on the part of the cattle industry—lack of social and political will to reduce deer densities. Producers were, therefore, encouraged to look at what they could do on their own holdings to institute some control over the opportunities for transmission within the farm boundaries.

Whilst the aim was to transfer responsibility for mitigating risk to individual producers, the initiative remained government-led. Through the implementation of market-driven interventions, co-funding opportunities and increasingly statutory measures, the onus for compliance came from a regulatory source. Thus, replacing the previous approach of leaving it to individual farmers to assess and institute risk management on farm and relying on peer pressure amongst producers to encourage uptake. When asked about the role of peer pressure, a cattle producer commented:

> *"It's not so much peer pressure as it is pressure on the government or those above to make the policies that'll force them into it, yeah, that's more the pressure than me going over. I don't want to go over to my neighbour and tell him you have to do this, you know, I can go over there and nicely tell him why he should do it, but for me to go tell him he has to do it I don't want to do that, I don't want to put myself in that spot either."*

Engendering greater responsibility for assessing what was possible on individual holdings and underlining producers' ability to exert some control over their own situations was an important driver. This was, however, coupled with a more top-down approach of imposing market and regulatory conditions to promote and embed management changes across areas most at risk from a bTB breakdown.

Assessing the Impacts

WRM was designed as a management strategy to reduce rather than eliminate risk on farm, placing the emphasis on taking greater control over limiting opportunities for deer-cattle interactions and working with producers to focus on the elements within their control to promote effective management of deer-cattle interactions. In terms of benefits, interviewees cited a greater awareness amongst producers of the risks posed to their own farms and enhanced actions around careful storage of cattle feed, with wider general improvements to biosecurity. Whilst being unable to provide evidence for or quantify the benefits of WRM, an assumption was shared amongst interviewees that decreasing the risk of contacts would decrease the number of cases. This opinion is exemplified in the following quotes—the first from a member of the USDA's epidemiological research team and the second from a cattle producer in the high risk area of the MAZ:

> *"Well if the producers are compliant with their plan it has I believe reduced the wildlife livestock interface quite a bit and it's also made people I think more aware of how the disease transmission could occur and what they need to do to decrease the amount of contact that the cattle have with deer."*
>
> *"Well the risk mitigation I believe has worked. It's not foolproof, but it has helped. If nothing else has brought it to the people's attention that these are the focus areas that they should focus on, you know, keeping the feed away and that type of thing. It's brought some attention at least that way and I think some people are becoming more receptive to "agriculture's going to have to take some role in this." I mean when this first started Ag kind of stepped back and said this is their [the DNR's] problem; let them deal with it and it'll work out when they work out their problem. Well obviously, we're not going to reach that point, so we have to step up to the plate and do our part too. Now we have different opinions on what our part is, you know, every person has a different opinion what they're willing to do and capable of doing."*

Both of these quotes raise the issue of producers' implementation of the stipulated measures, and is indicative of a wider theme of discussion on compliance with the control regime. Producers and those involved in the preparation and verification of individual farm plans, stated that WRM tended to be based upon a negotiation between the ideals envisaged by state agencies and the practicalities of what was considered achievable at the farm level. This process was described by producers as a form of "trading" back and forth to find a plan that was acceptable to both parties. Finding this middle ground for WRM was considered to be more constructive than imposing a set of measures that were deemed unattainable by the producer and which may prompt non-compliance. As one producer commented,

> *I'm sure [MDARD] would like us to tighten up a lot of our standards… but then nobody's going to follow through with it.… our standard might not be exactly as high as we want it to be, but if it'll address 50% of the risk and they'll do it 100% of the time; that's better than addressing 90% of the risk and doing it none of the time.*

The same producer stated that, if measures were too onerous, there would be a temptation to make sure that the farm seemed compliant for the winter inspection, but that the effort would not be sustained throughout the remainder of the year.

In addition to reporting that the prevailing opinion had become one of grudging acceptance within the industry, the interviewed producers also raised concerns about what they considered to be the negative consequences of WRM. Issues cited included the reduced carrying capacity of farms (due to restrictions on grazing and availability of land for harvesting winter forage in areas considered attractive to and frequented by wild deer) and the negative implications for smaller producers who were less able to absorb the costs of complying with the

new management regime. Whilst lower stocking densities and removing smaller producers less able to comply with WRM regulations may have positive benefits for the programme as a whole, the social implications of "it hurts some people" was raised as an issue.

A final point of note was the importance of risk perception in sustaining the momentum of the programme. The perception being that, as the sense of risk associated with tackling bTB decreases, the levels of complacency in sustaining disease management efforts increases. The risk of complacency was considered a high priority when developing a control strategy for a disease where endemic infection in the wildlife population persists. Progress towards eradication ultimately depends on a long-term commitment from multiple stakeholders (including producers, hunters, state agencies, and the federal government) to implement mitigation measures, provide adequate economic and political support for sustained management interventions and sustain the policy direction towards a goal that may take decades to achieve.

DISCUSSION

This paper has highlighted that bTB is an epidemiologically, socially and politically complex disease, creating multiple challenges for disease managers in constructing a coherent, cost-effective and workable strategy for eradication. This complexity is particularly pertinent in countries where the disease has become endemic in cattle and wildlife populations, demanding a long-term, multifactorial approach that is dependent upon a comprehensive set of control measures, sustained political will, adequate funding, stakeholder involvement and acceptance of interventions. Michigan and the UK have been highlighted as examples of how this complexity has played out in practice and underlines the case that the development of bTB management strategies need to be viewed as a social as well as scientific undertaking. This argument is in line with the analysis of Gormley and Corner (50) who point to the key role of stakeholders in bTB eradication programmes around the world and underlines calls for interdisciplinary research [e.g., (51–53)] and the development of viable management solutions based upon socio-technical approaches and interventions.

Enhancing Engagement

Human dimensions have been recognised as a key factor influencing the relative success of management approaches (17, 19, 54) with research efforts focusing on the role of public acceptability of wildlife control measures, the attitudes and actions of stakeholders (38, 55, 56) and the adoption of preventative biosecurity measures at the individual farm level. A central research theme, particularly in the UK, has focused on the adoption of biosecurity interventions and efforts to enhance opportunities to limit disease transmission between cattle and between cattle and wildlife at the farm level. Research has highlighted key reasons for the under-implementation of measures, including fatalism, uncertainty and scepticism on the practicality and efficacy of biosecurity interventions and, consequently, an unclear cost-benefit analysis of spend vs. gain. Critically, in an endemic disease situation, progress towards eradication will depend upon sustaining risk mitigation efforts over long periods, depending on the cooperation and buy in of producers and key stakeholders. The research reported here sought to provide an analysis of how risk mitigation became embedded within the state of Michigan's eradication programme and uses stakeholder narratives to identify key components that were considered effective in generating change.

The literature review identified a specific challenge for risk managers: formulating measures that incentivise positive and proactive risk management actions from stakeholders (25). The findings presented here identified Michigan's WRM programme as a step change in the state's approach to disease control. Interviewees identified the programme as a means to transfer some of the responsibility to producers to take a more proactive approach towards risk mitigation, first relying on voluntary uptake and then moving to more statutory measures. Social as well as technical processes were developed to address some of the barriers to change identified in the social scientific literature. For example, WRM was used as a tool to shift the uncertain cost-benefit of instituting biosecurity measures through introducing market and regulatory (dis)incentives; "trusted intermediaries" were identified to communicate with producers, recognising the lack of trust and confidence in government agencies to eradicate the disease (57–60) and finally, questions of practicality and efficacy were addressed by working with individual producers to highlight opportunities for change, facilitating their implementation via co-funding and enforcing change where necessary. WRM is essentially a government-led programme with regulatory backing, but the creation of individual farm plans is based upon a negotiation, balancing the epidemiological ideals of risk mitigation with the willingness and ability of producers to institute what are considered to be practical and acceptable interventions on their holdings. Interviewees could not provide evidence of the effectiveness of WRM, but considered it to be successful in changing the management approach towards more actively involving producers in the control strategy for mitigating their own risks.

When drawing comparisons between Michigan and countries with areas affected by endemic bTB such as the UK, there are limitations that should be recognised when offering any "lessons learnt." First, this is a relatively small qualitative study which was designed to be illustrative rather than representative of stakeholder views. Second, the scale of Michigan's bTB problem is very different to that of the UK, with only 5–6 cases per year in the cattle herd and a prevalence of around 2% in the deer population (47). For example, in 2016, 4 beef herds, 1 feedlot, and 1 dairy herd within the MAZ were found to be bTB positive, which was considered a "spike" in incidence of infected herds (54). By comparison, in the same year, there were 3,753 new bTB incidents in England alone (61). Third, as with any international comparison, there is a difference in the political context for decision-making; particularly relevant in this case is the need for the state of Michigan to conform to Federal requirements established by the USDA, which govern the acceptable level of bTB prevalence and is the ultimate arbiter for restricting or enabling interstate trade of cattle. The different pressures

applied and the balance established between maintaining a viable cattle industry and eradicating bTB are important contextual factors in guiding the policies pursued in charting a course towards eradication.

Whilst recognising these caveats of generalisability, scale and differing political contexts, the Michigan experience does offer an interesting case study in negotiating the challenges of shifting the focus beyond testing and surveillance towards obtaining producer engagement in WRM and farm biosecurity. Defra's Strategy to Achieve Officially TB Free Status for England similarly recognises the need to engage farmers in reducing their risk through careful cattle purchasing and limiting opportunities for transmission between cattle and between cattle and wildlife. However, the Strategy largely remains split between the application of statutory control measures—including continuous surveillance of cattle herds, removal of bTB test reactors and other cattle suspected of being infected with bTB and movement restrictions for bTB breakdown herds—and a predominantly non-statutory (voluntary) approach towards biosecurity implementation. In recognition of the persistent challenges surrounding biosecurity implementation [see (31)], there are ongoing discussions to identify mechanisms to encourage herd owners to take additional steps to improve their purchasing and biosecurity practices, including linking compensation to membership of herd health schemes such as the Cattle Health Certification Standards (CHeCS) scheme (62) and investigating means to give "earned recognition" to farmers for verifiable good biosecurity practices [see (63–65) for context]. This represents a movement towards rethinking the governance of biosecurity, but remains dependent upon the voluntary enrolment of farmers which, to date, has resulted in limited sign-up to the Bovine TB Herd Accreditation element of the CHeCS cattle health scheme. Clearly, as was the case in Michigan prior to the introduction of WRM, the challenge of achieving sustained farmer engagement remains unresolved and potentially requires a rethink of the socio-technical mechanisms by which this could be achieved.

Responsibilisation

Developing a greater sense of responsibility for biosecurity management is an important theme in both the Michigan case study and in policy narratives in the UK. As reported in the work of multiple social scientists, the "responsibilisation" of a wide range of actors beyond government is a process closely linked to the increasing neoliberalisation of animal health management, shifting the onus on to industry and farmers to manage their own risks through enhanced "biosecure citizenship" (66–69). This reflects wider trends in international policy development towards "empowering" citizens to take greater control of their own individual and community well-being in, for example, making themselves less vulnerable to crime through changing their actions and routines to minimise their potential exposure to risk, or making proactive changes to diet and exercise to mitigate future health risks (70, 71).

Whilst the principle of enhanced responsibility is a common theme between the Michigan and UK policy landscapes, the mechanisms to achieve change are different. As Enticott et al. (27) report, the UK model of promoting biosecurity has developed within a political context based upon an ideological reluctance to regulate and has increasingly relied upon theories of behaviour change designed to "nudge" farmers towards taking action via the use of social norms and provision of information to guide choices [see also (72, 73)]. Examples include the introduction of ibTB—a publically available web-based interactive map showing the locations of bTB breakdowns and breakdowns resolved in the last 5 years, in England [see (74)]—and the promotion of the principles of risk-based trading to encourage farmers to make "informed" cattle purchasing decisions and reduce the risk of introducing disease via trade (75–77). This strategy is essentially voluntary, based upon improved communications to heighten awareness towards mitigating risks and operates as a "population strategy" [see (27)] using universal biosecurity principles to convey what should be "best practice" rather than considering applications that are more specific to individual farm contexts. Conversely, Michigan has moved towards a mix of regulatory, fiscal and social interventions that attempt to fit the ideals of standardised biosecurity protocols to specific farm contexts on a one-to-one basis (54).

The neoliberal logic of devolving biosecurity governance to industry and individual farmers has been questioned in the social scientific literature, citing farm-level and institutional factors as reasons why enhanced participation is unlikely to occur [see (78)]. For example, the approach assumes that farmers are willing to take on the additional responsibility and associated actions and that they have the knowledge and resources to implement the changes on their own holdings (ibid). Research suggests that this is not the case, as stated concerns for better biosecurity are not being translated into practice [e.g., (28, 31, 35)]. The reasons cited in Higgins et al. (78) include: farmers considering their biosecurity to already be of a satisfactory standard; concerns over the evidence base underpinning biosecurity interventions and the perceived controllability of the disease [see also (79)]; the applicability of universal biosecurity recommendations to individual farms; and the opinion that biosecurity is essentially a "government issue" with suggested biosecurity actions representing an external solution to an externally imposed problem. Taking each of these issues into account, and adding the unclear cost-benefit of biosecurity applications for bTB, there is a clear lack of incentives for taking voluntary action, often leading to uneven application of measures; the result of which is currently an unknown in terms of its effect on the UK bTB disease control regime.

Incentivising and Sustaining Change

The Michigan case study responds to a number of these critiques through creating a clearer rationale for incentivising changes to biosecurity practices. It also answers concerns about the utility of a one-size fits all set of recommendations that runs counter to farmers' view that these measures are impractical to implement and that they do not solve the complexity and uncertainty that are inherently linked to the disease. In a study of the Biosecurity Intensive Treatment Area (ITA), developed by the Welsh Assembly Government in 2006, Enticott et al. (27) highlighted the limitations of universal biosecurity practices

and the difficulties of inspiring behavioural change with broad-scale knowledge. Instead, the authors advocated for an approach that matches solutions to individual farms via a more discursive process between farmers and advisors. Much like the conclusions reached in the case study presented here, Enticott et al. [(27), p. 334] state that "whilst some biosecurity interventions may make veterinary sense, without the support of the farmer and the wider social environment there is little point suggesting them for they will be rejected."

Incorporating processes of discussion, negotiation and accommodation to individual farm contexts may introduce concerns about diluting potential management outcomes. However, as Enticott (26) and Higgins et al. (69) suggest, finding a balance between standardisation and negotiation may provide options for progressive and responsive solutions that incorporates the challenging component of social complexity into management responses. As multiple authors and policy makers have stated, people and their actions are critically important factors in influencing the trajectory of bTB control and progress towards eradication. Using existing social scientific evidence on the institutional and farm-level factors that both promote and undermine efforts to enhance biosecurity responses should be the first step in devising, implementing, and evaluating different approaches towards embedding interventions that are capable of creating and sustaining proactive management options for bTB.

CONCLUSION

The aim of this paper was to draw comparative lessons between the cases of Michigan and the UK to exemplify some of the challenges of developing an effective strategy for the long-term control of endemic disease, particularly reflecting on efforts to "responsibilise" cattle producers and engage them in proactive activities to mitigate transmission risks on their own farms. The study was designed to respond to prominent themes in the social scientific literature that identified a range of socio-political and economic factors inhibiting the implementation of risk mitigation measures on farm; an issue that is particularly critical in areas with endemic bTB. The results indicate that in contrast to the predominantly voluntary approach pursued in the UK, Michigan has shifted the emphasis towards obtaining producer support for wildlife risk mitigation and biosecurity via a mix of regulatory, fiscal, and social interventions. Whilst there is a common goal of transferring responsibility to producers to exert control over their own transmission risks, Michigan's WRM exemplifies a socio-technical approach that goes beyond highlighting what producers can do (through information and communications campaigns) to incentivising and promoting change via market (dis)incentives, co-funding, utilising social networks and tailoring approaches to individual farm contexts.

Neoliberal approaches designed to "responsibilise" cattle producers have been identified as problematic because the approach assumes that farmers are willing to take on the additional responsibility and associated actions and that they have the knowledge and resources to implement the changes on their own holdings. Taking these issues into account, and adding the unclear cost-benefit of biosecurity interventions for bTB, there is arguably a need to create a clearer rationale for incentivising changes to biosecurity practices in the UK. Whilst the scale of the bTB challenge differs between these two contexts, the development of WRM in Michigan offers instructive lessons in creating a clearer rationale for incentivising changes to biosecurity practices and offers interesting insights on the role of negotiated outcomes in attempts to adaptively manage a disease that is characterised by complexity and uncertainty.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the University of Sheffield ethical review panel with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the University of Sheffield ethics committee.

AUTHOR CONTRIBUTIONS

RL collected the data, came up with the concept for the manuscript and drafted the content.

FUNDING

This work was funded by an Economic and Social Research Council Award (ES/K009753/1: Sustainable intensification of UK livestock production: a social scientific approach).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00081/full#supplementary-material

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

HYPOTHESIS AND THEORY
published: 05 June 2018
doi: 10.3389/fvets.2018.00109

Bovine Tuberculosis in Britain and Ireland – A Perfect Storm? the Confluence of Potential Ecological and Epidemiological Impediments to Controlling a Chronic Infectious Disease

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *05 February 2018*
Accepted: *03 May 2018*
Published: *05 June 2018*

Citation:
Allen AR, Skuce RA and Byrne AW (2018) Bovine Tuberculosis in Britain and Ireland – A Perfect Storm? the Confluence of Potential Ecological and Epidemiological Impediments to Controlling a Chronic Infectious Disease. Front. Vet. Sci. 5:109. doi: 10.3389/fvets.2018.00109

Successful eradication schemes for bovine tuberculosis (bTB) have been implemented in a number of European and other countries over the last 50 years. However, the islands of Britain and Ireland remain a significant aberration to this trend, with the recent exception of Scotland. Why have eradication schemes failed within these countries, while apparently similar programs have been successful elsewhere? While significant socio-economic and political factors have been discussed elsewhere as key determinants of disease eradication, here we review some of the potential ecological and epidemiological constraints that are present in these islands relative to other parts of Europe. We argue that the convergence of these potential factors may interact additively to diminish the potential of the present control programs to achieve eradication. Issues identified include heterogeneity of diagnostic testing approaches, the presence of an abundant wildlife reservoir of infection and the challenge of sustainably managing this risk effectively; the nature, size, density and network structure of cattle farming; potential effects of *Mycobacterium bovis* strain heterogeneity on disease transmission dynamics; possible impacts of concurrent endemic infections on the disclosure of truly infected animals; climatological differences and change coupled with environmental contamination. We further argue that control and eradication of this complex disease may benefit from an ecosystem level approach to management. We hope that this perspective can stimulate a new conversation about the many factors potentially impacting bTB eradication schemes in Britain and Ireland and possibly stimulate new research in the areas identified.

Keywords: *Mycobacterium bovis*, Britain and Ireland, eradication, persistence, epidemiology

INTRODUCTION

Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* is a zoonotic disease, primarily affecting livestock, which is of economic importance to the European Union (EU) due to its impact on trade. Indeed, at the inception of the European project, as the European Economic Community (EEC), the first legal initiatives were taken to combat the disease in 1964 with the drafting of council directive 64/432/EEC (1). The latter document foresaw that there was a requirement for animal health legislation

to underpin intra-community trade in livestock and introduced the concept / definition of being "officially tuberculosis free" (OTF), defined as the percentage of herds confirmed as bTB infected not exceeding 0.1% per year for six consecutive years (2). The legislation also defined the goal of the EEC to be disease eradication as opposed to control. Further legislation followed which enshrined the need for member states of the EEC to fund and facilitate test and slaughter schemes for the purposes of bTB eradication (3). In many member states, eradication programmes proceeded effectively, resulting in the granting of OTF status to Denmark in 1980, the Netherlands in 1995, Germany and Luxembourg in 1997, Austria in 1999, France in 2001 and Belgium in 2002 (2). Other states were granted OTF status upon joining the EU – Finland and Sweden 1995 and Czech Republic 2004 (2). In 2009, Poland and Slovenia also attained OTF status, whilst non-member state Norway was recognised as meeting all EU standards around OTF status (4).

Against this backdrop of successful eradication is the contrasting situation observed in the islands at the western fringe of the European continent – Britain and Ireland. Despite dramatic initial success in controlling bTB, England, Wales and Northern Ireland have suffered increasing incidence since the late 1980s. The Republic of Ireland experienced a relatively less dramatic initial reduction in incidence and continues to exhibit an ongoing problem in eradicating bTB, however, recent figures suggest that the situation has stabilised. Scotland is the notable exception, having been granted OTF status in 2009 (4). Data from 2009 indicated that 5–6% of herds from both islands tested positive for the presence of *M. bovis* (4). A contributing factor to the rise in herd incidence in England and Wales can be attributed to the suspension of bTB testing during the 2001 outbreak of foot and mouth disease (5, 6). However it has been recognised that even before the foot and mouth epidemic, bTB herd incidence was on the rise and foot and mouth disease merely exacerbated an already existing problem consistent with an upward trend in incidence since 1986 (6). Indeed, data from the Department of the Environment Food and Rural Affairs (DEFRA) in Britain indicates that '*since 2003 the total number of new bTB breakdowns identified (every quarter) in GB has been doubling at a rate of every 10 years. Prior to the Foot and Mouth Disease epidemic of 2001, the doubling rate was every 5.2 years*' (7). More recently, England, Wales and Northern Ireland have exhibited a rise in herd incidence whilst the Republic of Ireland has experienced a fall (6). Whilst mainland Europe still has a substantial bTB problem in the Iberian peninsula (Spain herd incidence 1.4% in 2009), this pales in comparison to the problems observed in the UK and Republic of Ireland (4).

This begs the question – why, in comparison to continental neighbours, have the territories of the United Kingdom and Republic of Ireland struggled to eradicate bTB? In Europe, diagnostic testing using some variant of the tuberculin skin test methodologies is highly standardised (8–10) following international protocols laid down by EU Council Directive 64/432. Debate around the efficacy of the skin tests are an ongoing matter of concern, with traditional epidemiological approaches estimating wide ranges of sensitivity of 52–100% - median 80% (8). More recently, Bayesian non-gold standard methods applied to data from Ireland have suggested skin test sensitivity is in the region of 50–60% (11), which has been further supported by a Bayesian meta-analysis from studies in the literature from 1934 to 2009 (12). Such relatively poor diagnostic performance may explain a failure to eradicate disease since many truly infected animals will be mis-classified as disease free. However, similar testing schemes using standardised reagents, presumably with similar test performance characteristics, have been used across Western Europe and indeed other parts of the world such as Australasia (13, 14), resulting in disease freedom. Furthermore, the performance improves at the herd-level as a screening test to identify infection, depending on the herd-size (numbers tested) and true within-herd prevalence (15). Also, once infection is identified, supplemental testing with more sensitive tests can be used to clear the within-herd infection (16). One could come to the perhaps overly simplistic conclusion therefore, that even with a poor individual test sensitivity, eventual eradication can be attained. However if this is true, what is confounding progress in Britain and Ireland? Undoubtedly, despite widespread standardisation in the basic diagnostic approach of using injectable tuberculins, there are individual differences in the application of eradication programmes subject to the variations of differing national policies, politics, behaviours and country specific factors (6). Indeed some of these socio-political factors may be some of the most important factors to account for the difficulties encountered in Britain and Ireland (17). Even with such heterogeneity of approach across time and national boundaries, it remains startling that particularly in Britain, which came close to achieving eradication in the 1960 and 1970s (8, 18), bovine TB is resurgent (as discussed above). Therefore, alongside issues of differing diagnostic test application protocols, we propose it is also timely to consider other potential additional factors whose current impact is unknown, but which may be additively preventing progress towards eradication.

Specifically, we hypothesise that there may exist a convergence of detrimental risk factors unique to GB and Ireland that is undermining the bTB eradication effort in these territories. If certain factors do contribute to this hypothesised "perfect storm" underpinning a failure to eradicate bTB, what are they likely to be? Below we discuss some of the likely ecological and epidemiological candidates given current knowledge. These proposed factors are not meant to be a definitive or exhaustive list; indeed we fully recognise that there may be many "unknown unknowns". Rather our intention is to attempt to address the current impasse in bTB control in these islands by adopting a novel perspective which seeks to address the likely multi-factorial problems which afflict our national eradication programmes. We hope that in so doing, we can start a debate on how this *perfect storm* can be investigated and addressed through innovative approaches and methodologies.

THE FACTORS

Heterogeneity in bTB Diagnostic Approaches and Control Programmes

In the late nineteenth century, tuberculins, derived from the culture filtrate of TB causing bacilli (10), were initially produced as potential therapeutic agents by the discoverer of the tubercle bacllius, Robert Koch (10). Their lack of efficacy for this particular task, was superseded by the discovery they could be used in the diagnosis of tuberculosis

(8, 10). In the twentieth century it was discovered that intradermal injection and measurement before and after of skin thickness could be used to detect the delayed type hypersensitivity (DTH) reaction, indicative of bTB infection (8, 10). Since then, "repetitive use of tuberculin tests remains the basis of all bTB control programs to this day" in areas with endemic disease (10), following strict standards laid down by EU council directive 64/432.

Despite such standardisation, there remain differences in application of skin tests and in the programs they support across the EU. We address an overview of these below:

Different Tuberculins and Divergent Potencies

Tuberculin potency for use in all skin tests used in Europe is tightly regulated by EU Council Directive 64/432. It is however recognised that changes in manufacturing and production procedures can result in batch to batch variation in potency (10). Indeed, even with strict regulation, tuberculins of lower potency have been released before (10). Such batch to batch variation and low potency would affect sensitivity of the skin test by reducing the DTH response / skin swelling that underpins diagnosis. Could such heterogeneity underpin the divergent outcomes in Britain and Ireland's TB control programmes compared to mainland Europe? An understanding of the history of tuberculin production may help to partially address this issue. Prior to 1975, the UK produced tuberculin derived from three *M. tuberculosis* strains, which was used in both Britain and Ireland [(8), M. Good personal communication]. Use of this tuberculin, derived from the human pathogen, coincided with the lowest prevalence of bTB in GB suggesting that the testing scheme was very effective. In 1975, both the United Kingdom and Republic of Ireland switched from using an *M. tuberculosis* derived PPD to one derived from the GB AN5 strain of *M. bovis* (8) as this exhibited superior sensitivity and specificity to the *M. tuberculosis* PPD (8, 19, 20). During the period after the 1970s, England and Wales experienced the well documented rise in bTB prevalence despite using this apparently superior Weybridge tuberculin (6). In 1980 the Republic of Ireland switched to using tuberculin produced from the AN5 strain in Lelystad (M. Good personal communication) with the UK following suit in 2008 (21). Downs et al. (21) went on to compare Weybridge and Lelystad tuberculins for bTB breakdown data in GB between 2005 and 2009, finding that the Weybridge formulation exhibited a slightly higher sensitivity and lower specificity. Given this data, in the context of increasing prevalence of bTB in the UK, the Weybridge tuberculin could have resulted in the reduction of false negative animal / herd detection whilst increasing the false positive rate – a combination in conflict with the hypothesis that tuberculin differences underpin the prevalence rise in GB. Whilst it is impossible to know the quality of all Weybridge tuberculin batches produced between 1977 and 2009, it is pertinent to note that batch to batch variation is not just a feature of GB production from the 1970s onwards (21, 22).

Differing Test Formats Across Europe

The most obvious difference is related to the exact test used. In Continental Europe, the single intradermal test (SIT) / cervical intradermal test (CIT) is used, involving the inoculation of *M. bovis* derived purified protein derivative (PPD) to detect skin thickness increases indicative of infection (8, 9). Conversely, in Britain and Ireland, owing to problems with environmental members of the *Mycobacterium avium* complex of bacilli cross reacting with *M. bovis* PPD and reducing specificity (increasing false positives), a comparative test is used (8). The single intradermal comparative cervical test (SICCT) injects both *M. bovis* and *M. avium* derived PPD into separate locations of an animal's neck, and uses the resulting difference in skin thickness between both sites as a diagnostic metric (8). The SIT has been noted to have an increased sensitivity (fewer false negatives) compared to the SICCT (23): SIT sensitivity range 80.2–91.2%; SICCT sensitivity range 52–93.5% (8). It is possible that this difference in sensitivity in the test used in Britain and Ireland may contribute to the comparative difficulty in clearing infection these islands experience, compared to continental neighbours. However, if this were the sole cause of the latter stark difference, it fails to explain why the Republic of Ireland and the United Kingdom exhibit such divergent contemporary and historic herd prevalences (6) despite having used similar tuberculin preparations (see above).

Differing Approaches to bTB Programs Across Europe

In continental European countries, which do not have the same problem as Britain and Ireland, bTB testing programs can have radically different outcomes. For example herd de-population upon the detection of reactor animals was common in France (9) – effectively using skin test as a herd screening test (8). In the continental context however, there is now a move away from whole herd de-population measures because of the costs involved and also animal welfare concerns (9). Economic consequences of whole herd depopulation in Britain and Ireland are of even greater significance owing to the much higher infection prevalence in these regions, and consequently are generally rarely deployed (with notable exceptions: (24, 25). A movement towards more pre-movement testing, to prevent spread of infection to new areas, has been proposed as an alternative to depopulation (9), Pre-movement testing in the Republic of Ireland was a required part of the TB eradication scheme up until 1996 (20). Since then, an evaluation of reviving the practice in 2005 revealed no significant cost benefit, with the suggestion being that very few outbreaks were being caused by onward movement of animals (20). In Britain, pre and post movement testing was introduced in 2006 (APHA, 2017) at direct cost to individual farmers. In Northern Ireland, pre-movement testing has been considered (26), but is currently not an active part of the eradication scheme.

Deployment of gamma interferon, as a higher sensitivity ancillary test, in Britain and Ireland is variable – in Northern Ireland, participation in testing has been voluntary, with no statutory powers in place to remove test positive animals (16). In the Republic of Ireland and in Britain gamma interferon is now conventionally used in problematic herds to increase sensitivity in an attempt to clear infection, but their application and the basis for animal removal has varied widely (6).

Other European countries such as Switzerland also use the practice of taking inconclusive reactors from skin test positive herds (9); however it should be noted, Switzerland who undertook biennial testing (1960–1980), now resort to passive abattoir surveillance

from 1980 onwards (9). Such inconclusive reactor animals have been shown to be at elevated risk of developing infection in the future (27) and could potentially lead to the retention on farm of high risk animals. The economic cost in removing all inconclusive reactor animals is an order of magnitude greater in countries like Britain and Ireland which have a higher disease prevalence.

Compartmentalisation of regions within European countries, based on relative prevalence has been used to contain infection and prioritise resources (2). However, the restrictions used to control spread of infection across these has not been implemented uniformly. Over a number of years, Italy compartmentalized some regions with higher prevalence (28), applying greater movement restrictions between regions on the basis of risk. In Britain, compartmentalization has only been implemented in recent years, and has does not completely prevent movement – relying on pre and post movement tests (29, 30). It is notable, this regionalization approach is not effective in places which have widespread infection without a single locus, such as the island of Ireland (31).

It is conceivable that the program differences discussed above have compounded the ongoing problem Britain and Ireland have with bTB, however it is currently difficult to quantify the magnitude of these impacts.

Summary

In summary, these differences in tuberculin potency, application of skin test formats and heterogeneity in downstream choices in program management could have had a divergent effect on the bTB outcomes observed in Britain and Ireland vs Continental Europe. However, it is extremely difficult to untangle their relative importance (as is the case for all of the hypothesised factors), especially against the background of such differing epidemiological (and ecological) contexts. Future research efforts, including "big data", could assess differing interventions in settings with similar prevalence. Such approaches are predicated on better data harmonisation and sharing.

WILDLIFE

Wildlife Hosts Are a Significant Impediment to Eradication

The presence of wildlife hosts of bTB has been found to be a major impediment to eradication in a number of countries worldwide [e.g., Michigan, USA, New Zealand, UK and Ireland; (32)]. Spill-back infection from wildlife to cattle can seed infection into cattle herds (33). In Michigan the wildlife host is the white tailed deer [*Odocoileus virginianus*; (34)], while in New Zealand there is a multi-host problem with the most significant reservoir being the non-native brushtail possum [*Trichosurus vulpecula*; (14)]. In continental Europe, recent research has suggested that wild boar may act as a reservoir of infection, causing increased risk to cattle herds in parts of France and Spain (35–37). Deer may be a widespread, but relatively localised, problem in a number of countries across Europe (38). It has been suggested that European badgers may also play a role in the epidemiology of bTB in cattle in Spain and France (39, 40), however, it is only in Britain and Ireland where there is strong evidence of their impact on the control of bTB in cattle (41–44).

Evidence That Badgers Are Implicated in the Epidemiology of bTB in Cattle - Culling

Badgers are a host species for *M. bovis*, and have been implicated in the epidemiology of cattle bTB in UK and Ireland (41, 42, 44)]. Culling trials have demonstrated significant reduction in risk to cattle herds in areas where badger densities were significantly reduced (41–44). The magnitude of this effect has been shown to be larger in the ROI than in (Randomised badger cull) trials in GB [compare (43, 45) with (41, 42)]. In GB, badger culling was associated with a temporarily increased risk also to herds found at the periphery of cull sites (41). This was hypothesised (the "perturbation effect" hypothesis) to be as a result of increasing frequency-dependent transmission amongst badger populations, causing increased spill back infection to cattle herds (41). This suggests that badgers can play a significant role in spilling back infection to cattle over short duration. However, this peripheral increased risk was transient [<2 years post-cull (46)]; and was not demonstrated during badger cull trials or government-led culling operations in ROI (43, 44, 47, 48). The apparent beneficial effects of proactive culling to farms in cull areas have been maintained for up to 5–11 years after GB cull trials (49–52), and up to 10 years post-cull trial in ROI (53).

Cattle and Badgers Share bTB Pathogen Strains Which Cluster in Time and Space - Strain Typing and Whole Genome Sequencing

Strain typing of *M. bovis* has demonstrated that both badgers and cattle share similar strains with geographic clustering across hosts indicative of interspecific transmission at local scales (48, 54–56). The best evidence for this ongoing transmission has been demonstrated at the genomic level (57, 58). Biek et al. (57) demonstrated that at the farm level, badgers and cattle shared the same or highly similar pathogen sequence type consistent with frequent and recent transmission events – however, the direction of transmission could not be established during that study. It is likely that transmission occurs in both directions (53, 59, 60); however, the force of infection may be greater from badgers-to-cattle than cattle-to-badgers owing to the continual removal of infected cattle through test-and-slaughter (61–63). It is conceivable there may be regional variation in the latter as a result of animal and wildlife densities. Furthermore, culling experiments (see above) have demonstrated that the cycling of infection can be interrupted with beneficial effects for reducing bTB prevalence in the target host population.

Britain and Ireland Have Higher Average Badger Densities Than Elsewhere in Europe

The islands of Britain and Ireland have the highest average recorded density of badgers compared to any other country in Europe (64). Median badger densities across badger study sites suggest a median density of 4.3–5.4 badger km^{-2} for the

British Isles, while studies from across Europe suggest that median badger densities are 0.29–0.55 badger km^{-2} [(64, 65), Byrne, unpublished]. Furthermore, in England and Wales there has been a significant increase in badger social group and population densities in recent decades (66, 67). These figures mask the wide variation in density at lower spatial scales (66, 68–70) – for example, badger densities in Spain can vary from <0.3 to 3.4 badgers km^{-2} across habitat types (71). Similarly, badger densities in Ireland can vary from 0.7 badgers km^{-2} in poor upland habitat (72); in ideal conditions on a wooded island, densities up to 37 badgers km^{-2} have been recorded (64). However, what is important is that badgers benefit from a benign temperate climate in the British Isles (73, 74), and have thrived in areas where woodland and pasture abound (66, 68–70). These habitats can maintain badgers at mean densities of 3–5 badgers km^{-2} in Britain and Ireland (64, 75) and are also the contact point for potential direct and indirect interaction between badgers and grazing cattle (76). While badger density *per se* may not be related directly to risk in a linear fashion, large-scale epidemiological studies in the Republic of Ireland (53), Northern Ireland (77) and in Great Britain (78) have found significant and positive associations between metrics of badger density and increased bTB herd breakdown risk.

There is considerable variation in the societal attitudes to the management of wildlife across Europe [e.g., see (65, 79–84)], and this results in significant variation in the actual management practices implemented across Europe. This may relate to the apparent conflict between conservation, animal welfare, and management goals, as well as cultural differences in the acceptability of pursuits such as hunting. Hunting is more common, and arguably more socially acceptable in many continental European countries [e.g., (82, 83)] than it is in the UK and Ireland. Badger hunting is widespread and relatively intense in a number of countries across continental Europe (65), despite the badger being listed under the Bern convention. In Germany, the annual recorded hunting bag for badgers has been between 50–70,000 per annum, within an increasing trend in the hunt bag in recent years – for example, the bag for 2016 was 71,168, a 11.98% increase from 2015 (85). Similarly, in Finland 8,600–14,000 badgers have been reported in the national hunting records per annum (86, 87) and an increasing trend is reported in Poland where recent game bags are in excess of 4,000 badgers (88). Badger hunting is common and widespread in France, though there are limited available data on the national badger status (89), but hunters have been used recently in bTB outbreaks to sample badgers (39, 90). In Britain and Ireland, the badger has been protected by legislation since the 1970's. It is likely that this protection status has had beneficial effects on population size (68, 70, 91, 92) and may have influenced the considerable variation in the estimated densities of badgers across European countries, and between the British Isles and continental Europe. Furthermore, this broader issue of the "palatability" of wildlife management within society, and the relationship between this effect and the interventions undertaken may have been a significant factor in the bTB epi-system within the UK and Ireland (93).

Badgers Exhibit Significant TB Prevalence

Prevalence of *M. bovis* infection in badger populations may be sought as base-line data, although it is likely to vary by region and over time and is recognised as being difficult to quantify accurately (94–96). Standard pathology investigations have limited sensitivity (42, 97, 98) with the result that prevalence is likely to be underestimated.

Recent investigations indicate that more detailed post-mortem examinations result in the detection of microscopic lesions that would otherwise evade detection by standard procedures (97–99). Badgers killed in road traffic accidents (RTA) have proven to be a useful source of data in attempting to determine badger TB prevalence at a county-wide scale (42, 56, 100). The ISG reported that standard post mortem examination revealed that 15% of GB RTA badgers had TB (42). The ISG cautioned, however, that at a localised level below county size, owing to reduced availability of RTA badgers, this method may not be sufficient for surveillance (42). Similar RTA data collected in NI indicated that ~15% of badgers were infected (101). In GB, the ISG reported that in proactive cull regions, 16.6% of badgers were tuberculous (42) whilst in reactive cull regions this figure was 14.9% (102).

Similarly, studies in the ROI indicated that, by the standard protocol, culled badger TB prevalence was 12.1% (98) and largely in agreement with RTA figures. More thorough post mortem examination of culled badgers led to the detection of an increased number of infected animals. Cranshaw et al. (97) demonstrated that, in GB, proactively culled RBCT badgers had a true prevalence of TB infection of 24.2%. Similarly, in the ROI, more detailed post-mortem examination of culled badgers from across the country revealed a prevalence of 36.3% (98). However, other studies found higher prevalence up to 43%, indicating the variation in estimates depending on sampling and laboratory methods (103). Using cage trapping, anaesthesia and live sampling of badgers Drewe et al. (104) used latent class analysis to estimate the outcome of multiple tests on live badgers (culture, gamma interferon and Stat-Pak ELISA), in the absence of a perfect gold standard diagnosis. Sensitivity of diagnostic testing was estimated at ~93% and badger TB prevalence was estimated subsequently as 20.8% in Woodchester Park, Gloucester (104). Intra-regional, inter-regional and temporal differences in badger TB prevalence are to be expected owing to the potential differences in ecology and population dynamics of both cattle and badgers in different areas as illustrated recently in the ROI (105). Indeed, Byrne et al. (74) demonstrated large spatial variation in badger infection risk based on a sample of over 5,000 badgers across the Republic of Ireland. Using standard PM techniques and bacteriological culture confirmation, there was an order of magnitude difference in the worst infected counties to the lowest prevalence counties. Furthermore, there was a significant decrease in prevalence from 26 to 11% positive, at a period where TB was declining in the local cattle populations.

Regardless of "true" prevalence, these studies indicate that a significant component of the badger population across the UK and Republic of Ireland is infected with *M. bovis*, where bTB is also prevalent in cattle.

CATTLE AND HUSBANDRY

Britain and Ireland Have Some of the Highest National Cattle Densities in Europe

As has been described above for the European badger, the density of host organism available for infection by the TB causing bacilli seems to be of critical importance to ongoing transmission of disease and persistence. By analogy to bTB, Human tuberculosis, caused by *M. bovis*' close relative, *Mycobacterium tuberculosis,* is typically associated with overcrowding in confined spaces (106, 107). It is not surprising therefore that as with badgers, cattle densities will probably have an influence on bTB transmission dynamics. The countries that make up the islands of Britain and Ireland have notably high cattle densities in comparison with other European countries. In 2010, out of 27 countries within the EU, Northern Ireland had the highest mean cattle density of any country at 112 cattle per km^2 (108, 109). The Republic of Ireland was third (84 cattle per km2), with Wales and England ranking 6 and 7th respectively (54 and 40 cattle per km2). Scotland, who are now officially bTB free, are ranked 13th with a mean density of 22 cattle per km2. To illustrate the differences amongst countries, the national herds are large relative to their area in Britain and Ireland, for example, both the Republic of Ireland and Spain have approximately 6 million cattle (109), yet Spain is 7.2 times the size of ROI (504,645 Spain/70,273 ROI km^2). Research suggests that both the size of herds [e.g., (53)] and the intensity of farming (110) can be associated with increased risk of bTB breakdown in endemic countries [for reviews see (54, 111, 112)]. Larger herds may constitute greater risk as it may be more difficult to clear infection, once identified within the herd, due to the poor sensitivity of skin tests (113). The density of cattle within farms can be a proxy measure for the intensity of agricultural production, and has been associated with increased risk of bTB (110). At a macro-scale the risk of bTB increases with increasing intensity (111), primarily due to closer proximity between animals and potential infectious contacts (110, 112). In Britain and Ireland there has been a move towards intensification, with a trend towards larger farm sizes, yet a decline in the absolute number of farms (113, 114). Recent changes in dairy production at the EU level may exacerbate this pattern in the future. However, interestingly, Acevedo et al. (115) did not find a relationship between host density and bTB prevalence when investigating European island as discrete bTB ecosystems.

Britain and Ireland Have Farming Characteristics That May Cause Difficulties in Managing Infectious Disease

Trade is a significant characteristic of cattle farming in the UK and Ireland, with significant patterns of movement that transcend national boundaries (116, 117). Indeed, for example, in 2015 there were 55,285 live animal exports from ROI to Northern Ireland (118). Gilbert et al. (116) showed that, at a GB level, there were significant flows of animals traded over long distances, and also showed that movement metrics were a significant risk factor for bovine TB. Ashe et al. (117) visualised the movement of a cohort of animals from one county in one year in Ireland; a remarkable pattern of movement that encompassed all regions of the island was apparent. However, cattle movement and trade is a scale dependent phenomenon. While long distance movements occur and can potentially link disparate areas epidemiologically, the majority of trade moves are local (119) – the movement kernel is long tailed (120). Recent analysis of trade networks in Northern Ireland has demonstrated that farms are extremely well connected, forming a robust network that is resistant to random and targeted node removal. Essentially, this indicates that the interconnectedness of this herd network makes it difficult to manage spread on an individual basis.

Small movement networks can contribute to the local risk of bTB (121), however they also may explain the strong clustering of pathogen genotype patterns at a local level in Northern Ireland (54). Furthermore, there is a phenomenon of farm fragmentation, whereby herds are made up of a number of spatial fragments. These fragments can have large footprints (122), relative to the home premises, allowing for increased exposure to neighbours or environmental reservoir risk. The long-established practice of seasonal rented grazing, known in Northern Ireland as "conacre," adds to the potential impacts of fragmentation. Furthermore, there are little data available to assess within herd movements of animals – a potential for dispersal of infection both to neighbouring herds, but also spread of infection into the environment, including wildlife hosts. The movement of animals, the spreading of slurry and the sharing of farm equipment could all increase the likelihood of maintenance of TB (92), furthermore the constituent nodes within these networks (e.g., specific farms, marts, auctions) can have disproportionate effects on diseases spread (120, 123). On the other hand, the fact that islands are disconnected to the continent, raises the perspective that this insularity could prove beneficial towards the longer term control of the pathogen (115). Currently, there is a lack of harmonisation of data pertaining to animal movements within Western Europe, to allow direct comparisons between EU member states in terms of network structure and connectivity. Attaining this harmonisation should be a major research goal going forward. Anecdotally, the very dense within and between herd movement networks in Ireland and the UK, are different compared to the rest of Europe. However, without detailed comparative data, this makes direct comparisons challenging.

THE PATHOGEN: *M. BOVIS*

M. Bovis Strain Heterogeneity

The population genetics of the *M. bovis* bacillus in these islands is relevant to investigate the current epidemic and is an ongoing source of interest for many researchers. From a phylo-geographic point of view, such research can inform on the population history and can potentially inform on probable routes of entry into Britain and Ireland in the distant past (124). From a more practical and less academic point of view, phylo-geographic differences in pathogen demographics and evolution may have an outcome that is of importance to disease control and epidemiology. Such region-specific evolution and adaptation can result in differing pathogen phenotypes that result in differing disease outcomes and dynamics as has been well-documented for *M. tuberculosis* infection in humans (125–128). For example, in Vietnamese populations, specific strains of a Beijing lineage of *M. tuberculosis* have been

observed in a number of cases to result in a meningeal form of the disease, rather than the expected pulmonary disease pattern (129). Indeed the Beijing lineage of *M. tuberculosis* is recognised as exhibiting a hypervirulent phenotype distinct from other phylogeographically distinct lineages (130). The latter evidence indicates that certain lineages of a tuberculosis-causing bacillus can vary in their phenotype in epidemiologically meaningful ways. So, the obvious question arises, could something similar happen with bovine tuberculosis caused by *M. bovis?*

Consequently, it is pertinent to attempt to understand the population structure and history of *M. bovis* in Britain and Ireland. The extant *M. bovis* population is almost exclusively dominated by a single clonal complex – the Eu1 clonal complex (124). This is indicative of a genetic bottleneck occurring at some point in the pathogen's history on these islands, an event which led to a contraction in diversity, resulting in greater homogeneity. Such bottlenecks are features of clonal pathogens like *M. bovis* (131). Whether this bottleneck occurred at the time of colonisation or subsequently is unknown. While this Eu1 lineage is present on other parts of Western Europe it is nowhere near as dominant as it is on these islands (124). Eu1 is also found globally among former trading partners and members of the British Empire, suggesting wider dissemination during colonial times (124). It has been shown that this Eu1 lineage spread throughout Britain and Ireland, leading to a homogenised *M bovis* population potentially brought about by the free movement of infected animals between territories (132). More recent cattle movement controls as part of national TB eradication schemes may have subsequently isolated regions one from another and driven more local evolution of specific strain types (132). Previously, Smith et al. (133) had sought evidence to support a hypothesis that a test and slaughter bottleneck of the *M. bovis* population in the 1950 and 1960s may have constituted selection pressure for the evolution and clonal expansion of a "fitter" clone that exhibited some form of advantage with respect to evading the test and slaughter scheme. Quite what the latter advantage could be, if it existed, is still a matter of debate. An ability to evade diagnostic testing is one possibility whilst invasion of / adaptation to a new host / niche such as a wildlife reservoir is another possibility (133).

The host range of the TB- causing bacilli is an intriguing puzzle (134). The human pathogen, *M.* tuberculosis, disseminates in human populations but appears not to be transmissible between non-human animals (134–136). Conversely, *M. bovis* appears to be able to disseminate among many non-human animal species, but humans are generally a dead end host (134–136). However, there is an interesting exception. Recently, Gonzalo Ascenzio *et al.* (137) demonstrated that a subtle mutation in the virulence genes of *M. bovis* can cause it to freely disseminate in humans. This is evidence that a small genetic change in a pathogen can radically expand maintenance host range. Could the Eu1 lineage of *M. bovis* have undergone a similar transition to become a better host generalist? It is noticeable that wherever Eu1 strains are found around the globe, there is a wildlife reservoir problem (124, 134). However, some caution is required here. This may just be an effect of recent demography and trade (124). The Eu1 lineage may just be a "lucky clone", dispersed by chance events. Additionally, the countries which inherited its diaspora are mostly developed world nations likely to have good disease surveillance infrastructure. Therefore, perhaps apparent increased propensity for wildlife adaptation is purely confirmation bias? The fact that many of these countries have had much greater success in bovine TB eradication than Britain and Ireland is also perhaps indicative that there is nothing obviously fitter about the Eu1 clonal complex, and that the wide dissemination of this lineage may purely be a matter of demography and international trade (124). Additionally, other European lineages of *M. bovis*, distinct from Eu1, have been observed to infect cattle and wildlife populations in Spain (138), Portugal (139) and France (140). However, in the absence of empirical comparisons between multiple *M. bovis* lineages, the pathology they induce across multiple hosts and their epidemiological characteristics, it is perhaps premature to rule out the hypothesis of the Eu1 lineage being in some way fitter. This bears further investigation (134). It is not inconceivable that whilst multiple *M. bovis* lineages have similar host ranges, the relative efficacy and virulence within similar hosts may be different owing to genotypic and phenotypic divergence as has been seen with *M. tuberculosis* lineages (127). Similarly, whilst the global diaspora of Eu1 *M. bovis* strains arising from historical trade and colonialism (124) are undoubtedly genetically similar, there remains the potential for region specific evolution since introduction. Different ecological contexts and applications of control schemes could have resulted in phenotypic divergence from a similar ancestral stock of bacilli.

Further work looking for an *M. bovis* strain phenotype in Northern Ireland has yielded limited evidence of an advantageous adaptation with regard to ability to evade detection. Wright et al. (141) demonstrated that field isolates of differing strain type exhibited no significant difference in response to the tuberculin skin test at the animal level. Allen et al. (132) raise the caveat that Wright *et al*'s study was confined solely to Northern Ireland which contains strains from only the Eu1 lineage, and a geographically distinct sub population of Eu1 at that. Given the likely genetic homogeneity, would one reasonably expect to find stark differences in disease outcome / pathogen phenotype in such a setting (132)? Ideally, comparison of the epidemiological characteristics of strains extant in the recent past, predating test and slaughter schemes, within Britain and Ireland would also have been very interesting. However these strains are unavailable as their presence predates molecular characterisation and sample storage. Indeed, our knowledge of the *M. bovis* population in these islands is currently limited to that which is extant, and we have no definitive way of knowing whether Eu1 strains have always predominated or supplanted another lineage(s) of the bacillus. The fact that Eu1 strains were exported during the time of Empire suggests this lineage may have been at the very least, common for a considerable period of time in Britain and Ireland. Therefore any speculation on a fitter phenotype evolving within Britain and Ireland may be moot. Allen et al. (132) suggest that casting the net wider and comparing Eu1 to non Eu1 lineages in Western Europe or further afield may yield more fruit in this endeavour. In line with this hypothesis, it is perhaps telling that differences in disease outcome in *M. tuberculosis* have been observed at the level of major lineages – see previous. It is of note however that in a study, again confined to Northern Ireland and Eu1 only strains, Wright et al. (142) did find evidence for a difference in strain virulence, and Milne et al. (143) have observed that certain strains are associated with chronic, ongoing infections in certain herds over many years.

Given the contrasting views and evidence discussed above, the null hypothesis, in which there is nothing inherently "special" about *M. bovis* strains in Britain and Ireland, remains inherently plausible, but worthy of greater study. Much of the argument that Eu1 is not special seems to hinge on anecdote in the absence of data. Where empirical evidence is available, there are limitations in our ability to infer wider trends from geographically restricted findings as discussed previously. It would therefore in our view, be pertinent to attempt to address issues of pathogen lineage and its potential effects on bovine TB diagnosis and host range with well-designed studies and analyses. Regarding effects on TB diagnosis, we have already suggested above that intra lineage comparison across a wider geographic area is a better way to definitively settle this question. Overall then, it is our opinion that it would be better to empirically affirm or reject these hypotheses around evasion of diagnosis and host adaptation rather than dismiss them on the basis of gut feeling in the absence of hard evidence. If either or both hypotheses proved to be have some grounding in fact, then this could have implications for the application of control schemes in Britain and Ireland.

CO-INFECTION – EFFECTS ON BTB DIAGNOSIS

Co-infection dynamics are increasingly being recognised as a driver of the heterogeneous response of hosts to infection, and for persistence of diseases over time (144, 145). *Mycobacterium bovis* infection may be modulated by the presence of other infections (146–148), especially where severe infections immunocompromise the host. There is some evidence that *Mycobacterium bovis* infection progression can be impacted by viral infections such as Bovine Viral Diarrhoea (BVD), with the immunological response compromising tests used to disclose infected animals (149, 150), but see (151). Similarly, exposure to other infectious *Mycobacterium* species such as *M. avium paratuberculosis* (MAP or Johne's disease) can confound the immunological diagnosis of bTB through cross-reactivity (151–153). Furthermore, environmental mycobacteria can also affect the performance of bTB immunological tests, for example *M. hiberniae* (154, 155). Some of these environmental mycobacteria have been closely associated with bogs and peaty soils and subsoils (156), which is a significant habitat type within the British Isles (157), and could potentially impact on tuberculin skin test performance. Indeed, the potential for cross reaction is one of the reason why in Britain and Ireland the comparative skin test is used (bovine and avian tuberculin), which is not the case in other jurisdictions where such cross-reactions are rare (8).

MAP is now endemic in Ireland and Britain (158, 159), and suffers from similar diagnostic problems to bTB. Recent research from Ireland has highlighted the potential nexus between MAP and bTB (151, 160, 161). MAP exposure can affect the correct diagnosis of bTB, hindering the disclosure of truly-infected animals. However, it should be noted that MAP is now widely distributed in Western Europe, and similar problems have been described there [e.g., Spain; (162)]. Britain and Ireland may be particularly vulnerable to interference owing to the fact both territories use the comparative tuberculin test.

Recently, liver fluke (*Fasciola hepatica*) infection has been associated with a negative impact on the disclosure of bTB using the experimentally-infected cattle model and SCIT testing (146–148, 163). The prevalence of fluke infection in GB has also been associated negatively with the probability of dairy herds breaking down for bTB after whole herd tests. The size effect was large, with an estimated under-ascertainment of 33% (148). Co-infection, therefore, represents a mask potentially hiding the true infection status of both animals and herds, making clearance and ultimately eradication very difficult.

Liver fluke is endemic in Britain and Ireland, with high prevalence of infection (148, 164–166). At farm level, prevalence has been estimated as 86% in Wales, 83% in Ireland and 48% in England (164, 165). At the animal level, >60% animals exhibit some evidence of fluke damage in the livers of slaughtered cattle in Ireland (167). Using surveillance data, Byrne et al. (166) showed that >60% of herds had some infected animals in Northern Ireland, while herd prevalence approached 100% where at least 100 animals were sampled over a three year period. Given the results of Claridge et al. (148), these levels of infection may have a significant impact on the disclosure of bTB-exposed animals using immunological tests like SCITT. However, recent research from Northern Ireland failed to show a large size effect of co-infection on tuberculin reactions from field data (161), but did find associations between fluke co-infection and TB pathology mirroring other studies (146, 161, 168). But how different are the British Isles than other countries in Europe in terms of fluke exposure?

Recent spatial analyses and comparative studies across Europe have suggested that there are significantly lower levels of infection in continental Europe than in the British Isles (169–171), with particularly high levels of infection in cattle throughout the island of Ireland (170).

The distribution and abundance of liver fluke in the environment is strongly affected by climate and habitat types, through exposure and survival of intermediate hosts (164). This is in part due to the wet, temperate climate within the north-western Europe (169, 170). The exposure of livestock in the British Isles is also affected by farming practice (field based grazing), soil type, high soil moisture level and the abundant access to fresh water sources (172, 173).

While recent research has found equivocal evidence for the mechanism (168), there has been no comparative analysis of data derived from low and high fluke prevalence areas (i.e., international comparisons). One suggested hypothesis in Ireland is that such a high proportion of animals are exposed that there is a general depression of tuberculin reaction sizes (161).

CLIMATE AND ENVIRONMENT

Climate Adaptation and Change - Effects on Fluke

Future forecasts of fluke infection risk paint a depressing picture for parts of Europe, with especially significant predicted increases in risk for Britain and Ireland (169, 174). These forecasts have been primarily derived from climate projections, which for the most part are suggesting that Britain and Ireland will become warmer and wetter on average, but also more climatologically variable. Fox

et al. (174) forecast significant increases in fluke in all regions of the United Kingdom, with a projected epidemic for parts of Wales by 2050. Similarly, Caminade et al. (169) also forecast significant future risk for increasing fluke in Ireland and Britain up to the year 2080, but also increasing risk for north-western parts of continental Europe. This increasing parasitic risk, coinciding with the troubling emergence of fluckicide resistance, indicates that screening tests such as the SCITT and surveillance data based on post-mortem pathology for bTB, may become even less robust for disclosure of infected animals.

Environmental Contamination with *M.bovis*

Despite recent advances in epidemiological analyses, molecular typing and whole genome sequencing of *M. bovis* (54, 57, 60), surprisingly little is known about the exact transmission mechanisms that spread infection within and between cattle and wildlife populations. Previously, owing to the work of UK government appointed ISG, who administered the Randomised Badger Culling Trial (RBCT) in Britain, it was assumed that direct contact between animals was required to facilitate disease transmission by aerosolised *M. bovis* bacilli leaving the respiratory tract (42).The cited evidence for the primacy of this suspected transmission route was the preponderance of tuberculous lesions observed in the upper respiratory tracts of both badgers and cattle that underwent post mortem examination (42, 175). However, more recently, studies which made use of proximity logger collars fitted to sympatric (42) cattle and badgers in Northern Ireland and England, failed to detect very close contact between species that would facilitate direct respiratory transmission (176–179). These findings do not preclude the hypothesised existence of super-spreaders in cattle and badgers. The ISG however were sceptical that many such super spreader badgers existed (42). The latter may however be controversial, with some evidence of badger super-shedding in a high density English badger population (96), and indeed the precedent of super shedding in other wildlife species (180). The latter opinion, in concert with the apparent lack of meaningful direct contact between species, has raised the possibility that a contaminated environment may potentially be playing some indirect role in disease transmission between species (42, 177, 179, 181, 182). This hypothesis has been raised before, with studies in the past focusing on the potential role badger urine and faeces may play in contaminating soil, pasture and feed (76). Renal lesions have been observed to be the second most common type of tuberculous pathology in infected badgers in some earlier localised studies (183, 184). More recently, 13–14% of culled badgers exhibited such lesions in GB (185), with a similar 15% lesion presentation rate seen in Northern Ireland (101). Badger urine has been observed on occasion to contain 250,000–300,000 bacilli per millilitre (186, 187). Badger faeces deposited at latrines close to territorial boundaries have also been observed to be potential sources of *M. bovis* in the environment (188). These bacilli are believed to enter the GI tract via ingestion of respiratory mucus (51). In one gram of badgers faeces, 75 colony forming units have been observed (187). It is conceivable that badgers and cattle inspecting urine trails or faecal latrines left for territorial marking (75, 188) could aerosolise bacilli from these sources and seed a respiratory tract infection. Other prominent veterinary pathogens have been observed to be aerosolizable from an environmental source - *Coxiella burnetii*, which causes of Q Fever, has been observed to infect animals and humans exposed to contaminated wool (189) and *Mycobacterium avium paratuberculosis*, the causative agent in Johne's Disease has been observed to be aerosolised in dust particles derived from bovine faecal material in animal housing (190). An intriguing recent study demonstrated that *M. canettii*, a pathogen predicted to be a common ancestor for the *M. tuberculosis* Complex, could produce pulmonary infection, indistinguishable from aerosol-mediated pulmonary infection, in mice fed spiked material (191).

A crucial question for the viability of this hypothesis is how long can *M. bovis* persist in the environment? Previously, bacilli in badger urine were observed to survive on pasture for ~3 days in the summer and ~14 days in the winter (186) potentially due to the differing intensity of solar UV radiation, which can kill the bacilli. A number of studies in different countries indicate that the survival of *M. bovis* in environmental matrices is variable (192). *M. bovis* in faeces or faeces-contaminated soil appears to remain viable for up to ~6 months in some studies (193, 194). More recently, Barbier et al. (195) have undertaken *in vitro* experiments in which differing soil types were seeded with *M. bovis* and incubated at 4 and 22˚C. Their findings indicated that *M. bovis* persisted for longer (up to 150 days) at the cooler temperature, whilst results for differing soil types were inconclusive (195). It may also be worth investigating whether *M. bovis* strain variation may have a role to play in adaptation to environmental persistence. Within the Eu1 major lineage that dominates the UK and Ireland (as discussed above), it has been noted that there is considerable heterogeneity in cell wall content as detected by Fourier Transform Infra-Red Spectroscopy (196). Indeed, the major genetic deletion which is a hallmark of the Eu1 lineage removes a gene responsible for trehalose biosynthesis – an important component of the glycolipid rich hydrophobic cell wall (124). Recently it has been observed that hydrophobic cell wall components, which are a feature of the pathogenic bacilli in the *Mycobacterium tuberculosis* complex, aid aerosolisation and pathogenicity (197). Conversely, environmental mycobacteria appear to have more hydrophilic cell wall components (197). Could the Eu1 lineage, or some of its descendants have evolved a phenotype that retained pathogenicity but permitted environmental persistence? Comparison to other lineages in the type of experiments Barbier et al. (195) have performed may be a useful way of addressing this hypothesis.

Current and Future Climate Effects on *M. Bovis* Persistence in the Environment

Also pertinent to this debate is the climate of the UK and Ireland compared to continental Europe. Britain and Ireland inhabit a zone of the globe whose predominant weather tends to be mild and wet without experiencing extremes in temperature – classified under the Köppen system as a Cfb climate; temperate with no dry season and 10 or more months of the year exhibiting temperatures above 10 ˚C (198). Much of Western Europe, including northern Spain, most of France, Germany, Belgium and Holland are also categorised as belonging to this Köppen climate category (198). However, it is noted that whilst the Köppen system is useful for

broad inferences of year on year regional climate (199), it can miss intra-regional variation, particularly in European locales (198). Britain and Ireland are a case in point. Both territories are islands, surrounded on all sides by the Atlantic Ocean and various seas. Indeed, the North Atlantic Oscillation is the primary driver of the maritime climate niche within the broader Cfb category that Britain and Ireland occupy (200). Whilst there is variation across Britain and Ireland in climate - a general west to east cline in temperature, precipitation and sunlight is observed (200) - the general trend (even for south east Britain, which is most like the continent) is that both islands exhibit milder winters and cooler summers than continental neighbours (201). Indeed, records show that these islands receive less sunshine (202) and more precipitation (203) than other Western European countries. Given what we have discussed above about the factors effecting viability of *M. bovis* in the environment, could these contemporary conditions influence *M. bovis* survival in the environment of these islands, or specific regions of them, compared to continental nations? The obvious counterpoint to this is that Britain in particular came close to eradicating bTB through the 1950 and 1960s (18). So, if a contaminated environment was important for regional persistence, shouldn't that have prevented the scale of decline in prevalence during that period? It depends on the likely scale of importance an environmental reservoir would constitute, and whether that importance has changed with time. Perhaps the changing circumstances in wildlife abundance, farming practices, strain effects etc since the 1970s could have conspired to make a contaminated environment more of a contemporary problem in contrast to the past? Alternatively, if the role of environmental contamination is relatively small regardless of the point in time it occurs in, then it could still be an important factor in regional persistence. For instance, Britain did not completely eradicate bTB after initial success in reducing prevalence throughout the 1950 and 1960 s. Low level infection in cattle remained a problem with eventual recrudescence through the 1970–2000 s leading to the current impasse (18). It is plausible that an environmental reservoir may have played a role, alongside other factors, in preventing that final push to complete eradication.

With changing climate in the future, the UK and Ireland are predicted to see even milder, wetter weather (204, 205), with intra-regional variation in exact outcomes. Predicted general trends are for drier, hotter summers (205, 206), potentially with less cloud cover in southern Britain (205), and milder, wetter winters with increased probability of extreme precipitation events [Sweeney et al, 2001; (205)]. It would be pertinent therefore to begin to address whether contemporary climate effects and predicted future effects are likely to have any impact on the survivability of *M. bovis* in the British and Irish environment. These questions could perhaps be addressed in the future using field data and *in vitro* experimentation. The effect of weather conditions have been correlated with variation in *M. bovis* risk in cattle (116, 207–209), and such weather variation has significant impact on the population dynamics of wild reservoirs also (74, 210) potentially impacting patterns of infection (95), adding to the complexity.

Emerging Environmental Hosts

Alongside general environmental contamination with *M. bovis*, and potentially contributing to it, is the role that soil based organisms may play in dissemination of bacilli and their persistence. Specifically, protozoa have been implicated as potential reservoirs of *M. bovis*. It has in the past been hypothesised that the benign environmental bacteria that went on to become virulent, intracellular pathogens, may have evolved many of their intracellular persistence apparatus within the "nurseries" of environmental protozoa (211–214). Initially, Mardare et al. (215) had suggested that amoeba predation of bacilli was more likely to result in inactivated bacilli and reduced persistence in environmental samples. However, more recently it has been shown that protozoa containing TB causing bacilli, when fed to mice can result in active tuberculous infection (216).

Earthworms have also recently been observed to ingest *M. bovis* from cattle faeces and disseminate bacilli in castings across the wider landscape (217). From regional sampling and regression of soil content data, predicted earthworm abundance and species diversity across Europe have recently been determined (218). These data demonstrate that earthworm abundance is greatest in Denmark, Holland, Britain and Ireland compared to other Western European countries and that Ireland and Britain display one of the highest diversities of species across the continent (218). Earthworms have also been noted as a major component of the diet of badgers, particularly in Britain (219) and to a lesser extent in Ireland (220). Greater investigation of the potential role these ecosystem engineer species play in the epidemiology of bTB may shed light on environmental persistence and transmission dynamics.

What About Scotland? Is It the Exception That Proves the Rule?

Scotland poses an apparent challenge to the paradigm we have attempted to develop within this manuscript as an Officially TB Free (OTF) territory within Britain – it is part of the British isles, badgers reside there (there are also other potential wildlife hosts, with relatively large deer populations) especially in the lowlands, there is a significant cattle industry, and similar tests and testing regimes have been employed as in the rest of the British Isles. However, the relative magnitude of these characteristics is worth dwelling upon.

Badger density is significantly lower than in the rest of the British Isles (66, 67, 69, 70, 221). Badger abundance can be estimated using the density of main setts, representing the number of social groups within an area, allowing for reasonable estimation of abundance at large national scales (222). Comparing the mean social group density across countries of the British Isles, Scotland has the lowest mean density of 0.11 social groups km^{-2} (to the nearest thousand, 9,000 social groups (221), whereas in England and Wales the average value is 0.49 km^{-2} [72,000 social groups (66)], the Republic of Ireland has 0.25 km^{-2} [(19,000 social groups (69)]; and Northern Ireland, with the highest estimated density, of 0.58 km^{-2} [8,000 social groups (70)]. While there is debate as to the linearity of the relationship between social group density and abundance [(223); but see (222)] the magnitude of the difference would suggest a significant difference in average badger population

density between the countries (69). This is most likely related to the low proportion of the most suitable badger habitat in Scotland (much of Scotland is exposed, and/or upland).

Similarly, Scotland's cattle density is significantly lower than other countries within the British Isles [see above (6)]. However, the cattle industry is concentrated in the lowlands, meaning that similar intensity of farming may be occurring at local scales, with large herd sizes being reported (6). Scotland has a lower proportion of dairy farms, relative to the rest of the UK and ROI, and dairy herds can represents an increased risk relative to other farming types [(6, 224); but see (225)].

Furthermore, there are significant trade links between Scotland and the rest of the UK and ROI, including from relatively high TB risk areas (225, 226). This trade represents a risk for Scottish farmers who trade in from high incidence areas (225, 226), but the relative risk has been diminished significantly through the introduction of pre- and post-movement testing of traded animals (226). These restrictions have reduced the amount of international trade and also reduced the risk to infection (re)introduction (226).

In terms of liver fluke, recent spatial models would suggest that much of Scotland (especially the highlands) is high risk and future climate changes may exacerbate this problem (174). The confounding effects of co-infection in low prevalence situations seem not to mitigate against the maintenance of disease freedom.

Scotland is on a quadrennial testing regime, with herds tested at least once every four years (225), therefore, the testing regime is at a lower frequency than much of the rest of the UK and Ireland.

An argument could be made that Scotland reduced historic bTB levels to a low enough level to allow cattle measures alone to achieve freedom. Perhaps then, the historical reduced wildlife and cattle densities were then sufficient to act as a bulwark against recrudescence and the establishment of endemic disease? In fact, GB was very close to eradication in the 1980s before bTB re-emerged significantly in the 30 years since (18). During this period high bTB levels were largely confined to the south and south east of Britain, from where the epidemic has slowly expanded (227). In the intervening period badger population density has increased significantly (66–68), the intensity of cattle farming may also have increased, coinciding with policy changes, and there may be potential interactive effects of concurrent infections, all of which would have been exacerbated by the 2001 foot and mouth crisis (6).

CONCLUSIONS

In this article we have attempted to propose potential reasons why the British and Irish experience in eradicating bovine tuberculosis has been so fraught compared to that of other jurisdictions in recent times. Our suggestions have arisen from a broad comparative approach which contrasts landscape, ecological, animal husbandry and molecular epidemiological characteristics within Britain and Ireland to those primarily observed on the wider European Continent. We note, with caution, that correlation is not causation. However for all proposed factors, we have endeavoured to present a coherent narrative, supported by published evidence, which links each to pertinent aspects of bTB epidemiology. We do not propose that these potential factors are exhaustive, merely that they may be worthy of further investigation, and individually or collectively may constitute novel hypotheses that go some way to explaining the comparative lack of progress in bTB eradication in these islands.

Our hypothesis is that owing to their history, ecology and geography, Britain and Ireland may occupy a "goldilocks zone" for bovine TB. Factors highlighted in this review include the presence of a sufficient wildlife reservoir, a potentially amenable environment for *M. bovis* maintenance, a number of endemic infections that could impact on the diagnosis and transmission of bTB, an evolutionary lineage of the pathogen unique to Western Europe and a large, highly connected, dense network of farms where the movement of infected animals could be facilitated, partially due to the limitations of the statutory test at the individual level.

As regards further investigation, we propose a wider scale comparison of all listed factors across Britain and Ireland, and their association with risk of bTB persistence and other pertinent epidemiological outcomes, contrasted to territories / regions with lower bTB prevalence. The latter may help to ascertain if any of the factors have a significant impact on bTB eradication efforts and also to quantify their relative importance. The latter type of investigation could be achieved in two ways:

1. Aggregating all retrospective information for the listed factors across multiple patches of interest across Britain and Ireland into a single data resource that could contrast intra-regional differences and find potential associations and effects – in effect a meta-analysis.
2. Prospectively, across Britain, Ireland and Western European countries, identify regions with varying burdens of disease and actively measure / catalogue the stated factors for statistical analyses.

A caveat is that both strategies would bring their own inherent problems. Both would require harmonisation of retrospectively and prospectively collected data, to control for differences in bTB eradication scheme administration and data collection methods.

However, efforts to survey broader vistas of the bTB landscape may make these efforts more worthwhile, identifying novel mechanisms amenable to control. There may have been a tendency to restrict one's horizons when investigating bTB persistence in Britain and Ireland – a parochial approach, that whilst understandable with a complex disease affecting many herds and animals on a national scale – may miss some important epidemiological drivers. Owing to potential intra-national homogeneity in the characteristics of risk factors, their relative importance at a wider scale could be masked – for example: since the lineage of *M. bovis* found in Britain and Ireland lacks diversity, intra-national comparison of potential effects of strain variation would be difficult to detect, since everything seems so genetically similar.

A potential criticism of our focus on some of these factors, is that even if they did have a significant effect on bTB epidemiology, that effect may be very small and therefore, any intervention would potentially not be practical or cost efficient. However, in the absence of firm evidence either way, this criticism could appear to be somewhat pessimistic. The reproductive index (R0) for bovine TB between cattle in Britain has been estimated to be low – 1.1 (228). Between badgers, R0 has also been observed to be low – ranging

from 1.03 to 1.19 (223). Between species R0 has recently been estimated to be in the region of 0.05 (60). These results suggest that not much effort may be needed to tip the R0 (of the two-host system) below one and drive the epidemic to extinction. It may well be that targeted intervention on multiple factors of small effect, when combined with the larger effects of the nationally managed eradication schemes, could help achieve this goal. In effect, we are suggesting that addressing some of the potential factors identified here, may result in an aggregation of marginal gains that takes the standard eradication scheme protocol as its base line, and applies an ecosystem management approach to drive down remaining infection.

AUTHOR CONTRIBUTIONS

AA and AB came up with the concept for the manuscript and drafted it. RS made additions to the text, edited existing text and gave advice.

FUNDING

Authors' work is funded by the Department of Agriculture, Environment and Rural Affairs, Northern Ireland (DAERA-NI)

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