EPIDEMIOLOGY AND CONTROL OF NOTIFIABLE ANIMAL DISEASES

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EPIDEMIOLOGY AND CONTROL OF NOTIFIABLE ANIMAL DISEASES

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Cattle extensive farm.
Image: VISAVET Health Surveillance Centre.

Surveillance, early detection, control and eradication of notifiable animal diseases is of critical importance for countries in order to maintain or improve their animal health status. This requires the collaboration of all stakeholders involved including animal health authorities, livestock industry and veterinary research institutions among others. Prevention, control and eradication programs must take into account the characteristics of the host (including potential reservoirs), the pathogen (transmissibility, virulence...) and the environment (temperature, animal density...) but also the socio-economic context in which they have to be implemented (highly influenced by funding availability), while at the same time guaranteeing compliance with international trade regulations. This has led to the adoption of a wide range of approaches to address the risk posed by specific pathogens in different countries, and at the same time similar strategies have yielded very different results in different regions. This Research Topic includes a variety of manuscripts focusing on different aspects of surveillance, control and eradication of diseases of critical importance for livestock, including cattle, swine and wildlife, in an attempt to provide an overview of the current situation in different countries.

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EDITORIAL
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Editorial: Epidemiology and Control of Notifiable Animal Diseases

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Editorial on the Research Topic

Epidemiology and Control of Notifiable Animal Diseases

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INTRODUCTION

There are a number of criteria by which an animal disease is classified as notifiable; the most important are typically related with its potential to spread internationally, as well as its impact on the health of domestic livestock, wildlife and, not the least, on human health (1). Because of the above, surveillance, early detection, control, and eradication of these diseases is of critical importance for countries in order to maintain or improve their animal health status. This requires the collaboration of all stakeholders involved (e.g., animal health authorities, livestock industry, and veterinary research institutions). The ability to prevent or respond adequately to the novel introduction of a notifiable disease into a herd, region or country, to control its spread and eventually accomplish its eradication requires the availability of adequate diagnostic tests for a preferably early detection of infected animals, an adequate knowledge on its epidemiology including the potential routes of transmission within or between herds and, ideally, the existence of vaccines to avoid disease dissemination. Altogether this can help to ensure an optimal allocation of the existing resources to minimize the likelihood of a disease outbreak, as well as its potential spread and negative impact. This research topic includes a variety of articles focusing on different aspects of surveillance, control, and eradication of diseases of critical importance for livestock, including cattle, swine, and wildlife, in an attempt to provide an overview of the current situation in different countries of the world.

DISEASE DETECTION

One of the requirements for a disease to become notifiable is the existence of a reliable means of detection and diagnosis that allow a precise case definition (1), something often challenging in the case of livestock diseases. A typical example of such a challenge is bovine tuberculosis (bTB): despite major efforts invested in its control and eradication, the disease is still present in many countries worldwide, due in part to the limitations of the current diagnostic tests and their application. Three articles of this research topic either review or evaluate the performance of the two tests most commonly used for its diagnosis, the tuberculin skin test and the *in vitro* interferon-gamma release assay (IGRA) (Good et al.; Keck et al.; de la Cruz et al.). Good et al. provide an overview of the past and present use of the tuberculin skin test for bTB detection since its first applications by the end of the nineteenth century and describe the current challenges for the development of improved alternative tests. The other two articles provide evidences of the usefulness of the most widely used tool for bTB diagnosis other than the tuberculin test, the IGRA as an ancillary test for maximizing diagnostic sensitivity. Keck et al. demonstrate how the introduction of the IGRA has helped to decrease bTB prevalence in a specific cattle subpopulation in which the tuberculin test is difficult to perform, bullfighting cattle, in France. Finally, de la Cruz et al. compared two different commercial IGRA kits that have been used in Spain for bTB detection, finding large differences in terms of their sensitivity, and suggesting that their usefulness could be optimized by adjusting the cut-off values as recommended by the manufacturer.

RISK ASSESSMENT

Introduction of a non-endemic disease into a region can have catastrophic consequences. These consequences can be particularly dramatic when/if the disease has a high impact on animal health, the need for its surveillance detection complicates the trade of animals and animal products, wildlife species can subsequently become reservoirs of the disease, and tools like vaccination to reduce the susceptible population are not available. Brown and Bevins coauthor two studies that provide a detailed review of the epidemiology and risk of introduction and establishment into the United States of America of two of the most important swine diseases that are currently expanding their geographical range: classical swine fever (Brown and Bevins) and African swine fever (Brown and Bevins).

DISEASE MODELING

Increased knowledge of the epidemiology of a well-established disease can also be of great use, since it will assist in the improvement of current control programmes. This knowledge often depends on the population under study, and therefore different factors may drive disease distribution in different settings. The use of explanatory, predictive and spread models can help to identify those factors and assess their impact. Clegg et al. studied the factors associated with larger bTB outbreaks in cattle farms in Ireland in an attempt to identify parameters that would allow the implementation of better targeted policy measures. Haredasht et al. used slaughter surveillance data to characterize the temporal trends in cattle condemnation in the US in general and California in particular in order to develop tools for detection of changes in carcass condemnation rates that would help to design prevention and mitigation strategies to minimize its impact. Mourant et al. developed a compartmental disease spread model to evaluate the potential impact of mitigation strategies such as vaccination, and validated it using historical data on rinderpest outbreaks as an example.

EXPERIMENTAL STUDIES

In certain cases, however, data derived from simulation models or observational studies may not be sufficient to draw conclusions on the efficacy of preventive and control measures, and additional experimental data are required. Again bTB is a perfect example of such a situation due to its complex epidemiology and chronic nature, which makes predicting and interpreting field data particularly challenging. Gormley and Corner review how the careful evaluation of data from naturally infected badgers can serve as the basis to design experimental infection studies shown to be essential for the evaluation of diagnostic assays and to measure vaccine efficacy in this species. Finally, Serrano et al. hypothesized about the potential beneficial effect of an inactivated paratuberculosis vaccine on bTB progression in calves using data generated through an experimental *M. bovis* challenge.

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

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REVIEW
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The History of *In Vivo* Tuberculin Testing in Bovines: Tuberculosis, a "One Health" Issue

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Tuberculosis (TB) is more than 3 million years old thriving in multiple species. Ancestral *Mycobacterium tuberculosis* gave rise to multiple strains including *Mycobacterium bovis* now distributed worldwide with zoonotic transmission happening in both directions between animals and humans. *M. bovis* in milk caused problems with a significant number of deaths in children under 5 years of age due largely to extrapulmonary TB. This risk was effectively mitigated with widespread milk pasteurization during the twentieth century, and fewer young children were lost to TB. Koch developed tuberculin in 1890 and recognizing the possibility of using tuberculin to detect infected animals the first tests were quickly developed. Bovine TB (bTB) control/eradication programmes followed in the late nineteenth century/early twentieth century. Many scientists collaborated and contributed to the development of tuberculin tests, to refining and optimizing the production and standardization of tuberculin and to determining test sensitivity and specificity using various methodologies and injection sites. The WHO, OIE, and EU have set legal standards for tuberculin production, potency assay performance, and intradermal tests for bovines. Now, those using tuberculin tests for bTB control/eradication programmes rarely, see TB as a disease. Notwithstanding the launch of the first-ever roadmap to combat zoonotic TB, many wonder if bTB is actually a problem? Is there a better way of dealing with bTB? Might alternative skin test sites make the test "better" and easier to perform? Are all tuberculins used for testing equally good? Why have alternative "better" tests not been developed? This review was prompted by these types of questions. This article attempts to succinctly summarize the data in the literature from the late nineteenth century to date to show why TB, and zoonotic TB specifically, was and still is important as a "One Health" concern, and that the necessity to reduce the burden of zoonotic TB, to save lives and secure livelihoods is far too important to await the possible future development of novel diagnostic assays for livestock before renewing efforts to eliminate it. Consequently, it is highly probable that the tuberculin skin test will remain the screening test of choice for farmed livestock for the considerable future.

Keywords: tuberculosis origins, tuberculosis in a social context, zoonotic tuberculosis, tuberculin test in cattle, One Health and tuberculosis, tuberculosis, bovine TB

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ORIGINS OF TUBERCULOSIS (TB)

Tuberculosis is an ancient disease, found in relics from ancient Egypt, India, and China. Gutierrez and colleagues thought it likely that an early ancestor of *Mycobacterium tuberculosis* was present some 3 million years ago in East Africa, and they propose that it may have infected the great apes and ancestral man at that time. TB is largely preventable, treatable, and even curable since the 1950s yet, thousands of years after it ravaged ancient cultures, three people die of TB every minute in the twenty-first century (1.5 million in 2014), and TB continues to thrive despite the fact that the mycobacteria that cause it can only multiply and propagate inside a host (1–4).

Genetic analyses of TB mycobacteria and molecular clock evolutionary analysis dates the ancestral TB strain to approximately 40,000 years ago, which coincides with the period when anatomically modern humans were traveling outwards from Africa to settle in Europe and Asia. This ancient strain split into two major lineages between 10,000 and 20,000 years later and spread worldwide with the expansion of human populations (1, 4, 5). In ancient Greece, Hippocrates saw the disease in his patients, evidence of TB was found in scarred skeletons at various places around Europe and Asia and, during the 1990s, *M. tuberculosis* DNA was extracted from lesions on 1,400-year-old human bones found in Europe and Borneo (5). Soon after that, TB was identified in tissues from a Peruvian 1,000-year-old mummy, thus it became apparent that the disease had arrived in the Americas long before the European colonists (5, 6). The strain found in the Peruvian mummy, differed from the most prevalent strains in modern South America, being most closely related to a strain found in seals, leading scientists to theorize that seals initially picked up *M. tuberculosis* when breeding on African beaches before transporting and transmitting it from Africa to South America (7). Genetic studies of modern animal and human TB strains from around the world suggest that some sequences were shed, approximately 6,000 years ago, from a progenitor strain of one lineage of the human-adapted *M. tuberculosis*, that ultimately gave rise to what we now term the *Mycobacterium tuberculosis* complex (MTBC), and from which *Mycobacterium bovis* evolved. This, in evolutionary terms, may be associated with early farming and animal domestication between 10,000 and 15,000 years ago (5, 8). However, there are also suggestions that ancient cattle TB, with a Holarctic pattern, had reached North America some 20,000 years ago, before bovine domestication, indicating that we may not understand the entire TB spread scenario (9). Rothschilde et al. reported finding lesions resembling TB in the bones of a Pleistocene long-horned bison (*Bison cf. antiquus*) radiocarbon dated as being "17,870 ± 230 years old". Spoligotyping of these lesions revealed pattern plots that contained MTBC segments that cannot be assigned to any individual modern species of the MTBC based on existing spoligotyping data, but which are closest to the *M. tuberculosis* group and not associated with modern *M. bovis* or *Mycobacterium microti* (9).

TB IN A SOCIAL CONTEXT

Outbreaks of TB in humans peaked with a prevalence as high as 900 deaths per 100,000 of population between the eighteenth and nineteenth centuries as primarily farming, rural societies in Europe and America became industrial and urban when, during the industrial revolution, field workers moved to the cities in search of work. This rise in TB deaths reflected the impact of poverty, malnourishment, primitive sanitation, poorly ventilated housing, and overcrowding (10, 11).

Driving forces, like those at work in humans, influenced the prevalence of TB in cattle in the eighteenth and nineteenth centuries. In tandem with the migrating rural human population, in an era without refrigeration, milking cows also moved to cities. There they were kept in close confinement with poor ventilation which led to increased TB prevalence in cows and consequently *M. bovis* in milk. TB prevalence in cattle was further aggravated by the subsequent progressive amplification of cattle production (10). The zoonotic risks of *M. bovis* in milk from an infected bovine population were already known in 1895 when it was reported that milk from animals with TB contained tubercle bacilli visible microscopically even though the cows were lacking detectable lesions of the udder and that such milk could transmit disease orally to guinea pigs, rabbits, pigs, and calves (12). In New York, milk pasteurization began in 1912 and, in the decade following when 50% of milk was consumed pasteurized, the non-pulmonary TB death rates declined by 50%. Furthermore, whereas previously 50% of tuberculous neck glands had been confirmed as *M. bovis*, once pasteurization became the norm only 6 of 50 such glands confirmed as *M. bovis*, 5 of which were found in people who drank raw milk (13). During the late nineteenth and early twentieth centuries, it became increasingly clear that TB could be transmitted through food, particularly milk. By 1914, TB specialists agreed that most human cases of TB in the stomach, neck glands, and throat had been transmitted by the consumption of infected milk (14). *M. bovis* of bovine origin was estimated to be responsible for approximately 15,000 deaths in the US in 1917; three times as many as die from all food-borne illnesses today (15).

It is estimated that in Great Britain (GB), 6% of human deaths due to TB were due to *M. bovis* before the introduction of any effective bovine TB (bTB) control programmes (16). It is now difficult to credit that by championing a campaign for clean and honest milk without adulteration, e.g., added untreated water, or germs of infectious diseases, and the adoption the American style system of milk certification for cleanliness (introduced in the District of Columbia in 1904), Wilfred Buckley became the "*bête noire*" and the enemy of farmers and the milk trade (17). Nevertheless, despite human health implications and attempts by three Royal Commissions, between 1890 and 1911, to define the disease as a public health and food safety problem, it appears to have been the economic consequences of bTB that led to attempts to eradicate the disease in British cattle (18). Discussions on the grading of milk and also pasteurization had been ongoing in GB from around 1914. However, opposition to changes that would have reduced the risk of TB to consumers both from ideologues and vested interests, including the farming industry and parts of the milk trade ensured that the political parties in power took no decisive action and compulsory pasteurization was delayed (17) even though mandatory pasteurization of milk had been introduced in New York city as early as 1910 (19). Action to eliminate bTB was regarded as "too big a problem," "damaging to the farming industry," with "uncertain science" but without any concept of the necessity to act with a duty of care to the consumer or the precautionary principle (17). It was generally accepted that 40% of the British milking herd was infected with TB in the 1920/1930s, nevertheless, pasteurization was only gradually introduced by the industry between the two world wars and then only to increase the shelf life of retail milk (17). It seems strange now that at the time there was no appreciation of the need to engender the trust of consumers in the food chain or that this is compatible with the interest of farmers. It was not until 1935 that it became policy to develop "Attested herds" for TB freedom and, in 1947, Francis reported 1% of tuberculous cows as having TB of the udder which represented roughly 0.5% of all cows in GB at the time (8). It was not until the 1950s that compulsory slaughter of TB-infected cattle was enforced in the UK with the nationwide bTB eradication campaign (17).

In 1992, Hardie and Watson (20) noted that milk pasteurization was not widespread in the UK until the 1930s. It reached approximately 50% of the population by 1939 while raw milk was still supplied from non-attested herds in 1960. Hardie and Watson (20) reference W. A. Lethem (1955. Milk-borne tuberculosis, 1921 to 1953. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* 14, 144–145) to say "In 1955, Lethem used deaths from abdominal tuberculosis in children under 5 years as an index for *M. bovis* infection. In this age group, this form of tuberculosis was thought to be almost entirely due to ingestion of milk contaminated with *M. bovis*. The fall from 1,107 deaths in 1921 to 12 deaths in 1953 was much more marked than the equivalent reduction in deaths from other non-pulmonary forms of tuberculosis. This was attributed to the development of 'safe' milk in the intervening years, due to a combination of pasteurization and control of *M. bovis* infection in cattle." They also demonstrate that in an area where bTB is endemic, raw milk may still be a risk by reporting a clinical outbreak of *M. bovis* affecting three school children in 1959 and attributed it to contaminated milk from a herd infected following a tuberculin test (20).

The benefits of pasteurization of milk, when bTB levels are high, to reduce TB infection particularly in young children are well established. In The Netherlands, pasteurization of milk had spread in the 1940s resulting in a reduced mortality rate per 100,000 in children under 4 years from 3.03 in 1934 to 0.76 in 1945 and, likewise, in children between 5 and 14 years from 2.16 in 1934 to 0.92 in 1945 per 100,000 (21). This represented a remarkable decline when you consider that this period coincided with World War II in Europe when the incidence of TB in people increased in Europe, including in The Netherlands, and among USA military personnel, before falling again (10, 11, 22).

Zoonotic *M. bovis* TB is still a problem even in the developed world. For instance, in the USA, human infection with *M. bovis* has been mostly, but not completely, eradicated with milk pasteurization combined with, the culling of herds with skin test-positive animals from about 1917 (15). Human *M. bovis* cases usually account for <1% of all human isolates in the USA, primarily in immigrants, and predominantly located in extrapulmonary sites (cervical and mesenteric nodes, the peritoneum, and the genitourinary tract), although *circa* 50% of adults will only present with pulmonary TB (23). An analysis of TB trends from 1980 to 1991 (23) and from 1994 to 2005 (24) in San Diego County demonstrated that the annual *M. bovis* culture-positive rate as a proportion of all TB cases increased annually, from 3% in the earlier study to 5% in 1994 and to 11% in 2005 ($p < 0.001$). Between 1994 and 2005, 8% (265/3,291) of culture-positive cases were confirmed with *M. bovis* and 92% (3,026/3,291) with *M. tuberculosis* (24). However, the incidence of *M. bovis* was higher in children <15 years (45%) than in adults (6%). The study authors cautioned that this may be an underestimate as culture was only successful in 80% of cases under national and local clinical case definitions with unsuccessful culture overrepresented in those otherwise most likely to have *M. bovis* TB. Usually, only adults died during treatment with mortality in *M. bovis* cases twice as high as in *M. tuberculosis* cases. Persons of Hispanic ethnicity accounted for >96% of the *M. bovis* TB cases, with 60% occurring in those of known Mexican origin. In addition, factors associated with *M. bovis* TB included having extrapulmonary disease with a normal chest radiograph suggesting that the source of infection was most probably oral with consumption of Mexican unpasteurized dairy products identified as the major risk factor. The authors suggested that to ensure elimination of this zoonotic transmission, regulation of unpasteurized dairy product production, and eradication of bTB in dairy cattle was required in the long term (24). Similarly, in Ireland in 2005, a case was detected involving two children infected by a cow with only a high somatic cell count as evidence of the presence of *M. bovis* in her milk (25). In this case, however, all milk supplied from the herd was pasteurized, and thus raw milk consumption was only a zoonotic risk to the farm family. It was the detection, at a routine Single Intradermal Comparative Tuberculin Test (SICTT), of positive calves that had also been fed raw milk on the farm which was the sentinel in this case that prompted an investigation of the TB status of the family members on farm (25). *M. bovis* has also been detected from the milk of dairy goats both in the bulk milk tank and in milk from an individual TB-infected goat (26). The risk of zoonotic TB is equally applicable to raw milk products from any species in a country or region with endemic *M. bovis* and hence regulation of unpasteurized dairy product production, and the elimination of TB in livestock is a long-term requirement to eliminate this source of infection. Indeed, in an environment with a high bTB prevalence, with both *M. bovis* and *M. tuberculosis* being detected in milk constituting a zoonotic risk (27), and in keeping with the goals of the WHO, and as already suggested by Soxhlet in 1886 (28), should all agencies insist on the pasteurization of milk? This would help to reduce infant exposure to pathogenic mycobacteria, would improve human health, would reduce hospital admissions and treatment costs associated with TB, and would reduce avoidable deaths.

Milk is not the only source of zoonotic TB in humans. For example, in Michigan in 2008, two humans were detected with the genotypically consistent strain of *M. bovis* circulating in Michigan's white-tailed deer. This confirmed that recreational exposure to deer is a risk for infection in humans; therefore, hunters, trappers, taxidermists, venison processors, and venison consumers would potentially be at risk (29). Indeed, interspecies transmission of *M. tuberculosis,* human–animal–human, is a public health concern, especially with close human–animal interaction, notably in places such as circuses, exotic animal facilities, and zoos where there may be contact between TB-susceptible animals and humans (30–37). Where no effective eradication programme operates in cattle, the routine isolation of *M. tuberculosis* from multiple cattle raises the possibility of human-to-cattle-to-human transmission and the specter of increased zoonotic risk if *M. tuberculosis* strains adapt for bovines or other animals. Such findings underline the importance of adopting effective TB control and eradication programmes in humans and livestock alike (38–42). Indeed, the threat from zoonotic TB resulted in the adoption of a resolution by the OIE in 1983 which called for the eradication of *M. bovis* for both public health and economic reasons (43).

Tuberculosis in humans, without any animal involvement, still exists in Europe. A 50% rise of TB prevalence in London from 1999 to 2010 led to London being described by The Telegraph as the TB capital of Europe. At the time, the incidence of TB cases being detected in London rivaled or even exceeded that of Rwanda, Eritrea, Iraq, and Guatemala (44). The most at-risk groups were prisoners, drug users, the elderly, the homeless, refugees, migrants, and those marginalized by society. It is argued that the foreign-born component did not all enter GB having been previously infected but rather that they joined at-risk communities including the poorest in terms of housing, nutrition, and economic status. This reflects that, even in the modern era, TB still maintains its relationship with deprivation (44). The advent of HIV infection led to a dramatic resurgence of TB in humans (11). Today, notwithstanding the development of advanced screening, diagnostic, and treatment methods, one-third of the world's population, or over two billion people, are considered to be TB infected (3, 45). In 2014, 1.5 million people including 0.4 million HIV-positive, died of TB; 140,000 of these deaths were in children (3, 45). Worldwide, it was estimated that during 2014 some 9.6 million people would have fallen sick as a new case of TB (including 1 million children) and yet fewer than two-thirds (63%) of that number or only 6 million were reported to the WHO. This means that there is a 37% worldwide shortfall in diagnosis and/or reporting of new cases. Globally, there has been a gradual reduction in the number of incident cases with a TB prevalence rate which is 42% lower in 2015 than in 1990. However, there were marked differences between high and low TB incidence countries with the African Region having 28% of the world's cases in 2014 and the most severe burden relative to population. There were 834 cases in South Africa and 852 in Lesotho per 100,000 population, i.e., an average 281 cases per 100,000 people in the region or more than double the global average of 133 (3, 45).

TB IN CATTLE

Although cattle are usually regarded as the true hosts of *M. bovis*, TB due to other members of the MTBC, mainly *M. bovis* or *Mycobacterium caprae* but more recently also *M. tuberculosis*, has been reported in many other species of domesticated and wild animals and remains a significant zoonosis (30–42). As with humans, TB in animals is contagious and spreads by contact. The usual route of infection is by inhalation, but oral infection also occurs. Disease progression is protracted, taking months or years to kill an infected animal. In the interim, transmission occurs before clinical signs manifest (46, 47). Symptoms, when evident in bovines, include the following: progressive weight loss, loss of appetite, intermittent cough, swollen lymph nodes, weakness, low-grade fluctuating fever, and diarrhea (48). Infection also leads to less obvious effects such as a reduction in milk yield of 10–20%, reduced fertility, lighter (reduced value) carcase with carcase condemnations at slaughter and restrictions on markets (49). In some animals, lymph nodes, such as the retropharyngeal and others, enlarge and may rupture and drain; if superficial lymph nodes are involved then the drainage will be evident. Swollen lymph nodes may also obstruct blood vessels, airways, or the digestive tract. When involving the digestive tract, bloating, periodic diarrhea, and/or constipation may be seen. In the terminal stages, extreme emaciation and acute respiratory distress may occur. TB can be a major cause of economic loss for both individual livestock owners and countries. Observation of symptoms becomes less evident once an eradication programme, including live animal testing and removal of those infected, commences (46).

TUBERCULIN TEST DEVELOPMENT

In 1720, Benjamin Marten proposed that a microscopic living being able to survive in a new body was the cause of TB, this "being" was termed an *animacula* (50). However, there was wide disbelief of this proposal. In 1882, 162 years later, Koch demonstrated that it was true when he isolated the "tubercle bacillus" (10, 11, 15, 51). At the same time Koch declared that the tubercle bacilli and the human and bovine forms of TB were identical. Koch's declaration apparently ignored the 1868 work of Jean Antoine Villemin, a French doctor, who described the greater virulence of bTB in rabbits as compared with human TB (15). In 1890, Koch cultured the bacillus in a 5% glycerol broth, subsequently evaporated over a steam bath to one-tenth of its volume and filtered. The resulting filtrate, Koch's Old Tuberculin (KOT), contained the soluble fraction of the tubercle bacillus in a 50% glycerol solution. Koch, who himself had TB, demonstrated the properties of KOT, developed as a means of treating and preventing TB; and, having injected himself with his tuberculin, observed that he developed "an unusually violent attack of ague and rise of body temperature"; he also observed that subcutaneous injection in many tuberculous patients had elicited systemic reactions including hyperthermia (10). Almost simultaneously, the possibility of using this property of tuberculin to test cattle for TB was quickly recognized by veterinarians in Russia, Denmark, GB, and the USA, and a tool to help eradicate bTB had been found (10, 11, 15). Nonetheless, Koch initially regarded the skin reaction elicited by KOT as "not to be noteworthy and to be insignificant," nor did he recognize its importance as a diagnostic tool (52). For a long period, it was commonly believed that "the therapeutic value of tuberculins is intimately associated with the tuberculin reaction," as "the physiological response of the sensitized animal organism" (52). However, without the demonstration of any measurable therapeutic success it was soon discredited as ineffective (10).

In February 1891, McFadyean had commenced experiments in cattle; using clinical TB cases as subjects he proceeded to inject various quantities of tuberculin into the chest wall having first established the animal's pulse and temperature (53). He proceeded to monitor the condition, pulse and temperature of the animals every 2 h for 36 h in total. He observed a steady ascent in temperature during the first 14 h following injection when the observed temperature peaked and remained high for a further 4 h and that, at 48 h, the temperature had returned to normal. He also recorded an inflammatory swelling at the injection site, increased sensitivity (Se) of enlarged glands and that, during the febrile phase, the pulse remained elevated with irregular heart action but that otherwise animals appeared as if healthy. He further reported a tuberculous cow which had the "most extensive tuberculous lesions" but which had shown no temperature increase despite receiving the same tuberculin doses as the other subject cases. He observed that in almost all cases "a reaction was obtained in the tuberculous animals, while in no case was there any rise of temperature in the control non-tuberculous animals." He also remarks that "the tuberculous animals appear to have been in rather an advanced stage of the disease, but it still remains to be proved whether any discernible reaction will follow the injection of Koch's fluid when the lesions are of small extent" (53).

Bang introduced the tuberculin test, using KOT, as the diagnostic tool of choice in the first official bTB eradication programme in cattle in Denmark during the early 1890s (54). This was the first programme on a national scale to acknowledge the diagnostic potential of the tuberculin for which Koch had previously been trying to demonstrate therapeutic qualities (51). The so called "Bang method" consisted of repeated, six-monthly tests, to identify test-positive animals. This allowed the separation of test-positive cows from test-negative cows and the culling of all open TB cases and cattle with "TB of the udder." Thus, limiting transmission of infection *via* milk. It was quite an achievement in the early 1900s that he was able to certify the first farms to be test negative for several years and as suitable to sell "superior milk" or milk for infants. Allowing animals to be kept while "profitable" made participation affordable and was an essential factor in gaining support among farmers and dairies (54). Following reports [Ref. (55) as an example] on the achievements using this approach, the "Bang method" was internationally accepted as the major diagnostic tool in the control of bTB. Repetitive use of tuberculin tests remains the basis of all bTB control programs to this day. By the end of 1891, cattle testing using KOT was already operating extensively; and Professor Eber, a veterinarian from Berlin, reported a test specificity (Sp) of approximately 87% having collected statistics on tested cattle (15).

Palmer and Waters provide a concise and interesting review of the origins of the bTB eradication programme in the USA and the many factors of the pathogenesis and epidemiology of bTB, known or at least hypothesized (*surprisingly accurately*) as early as 1899 (15). First tests on cattle involved injecting "tuberculin" subcutaneously in the right scapular region and necessitated the veterinarian making several preinjection temperature measurements followed by regular measurements for 24 h post-injection to monitor any increase in body temperature. However, the variability in temperature change for the subcutaneous test foreshadowed difficulties associated with this application. Between 1892 and 1915, test methods began to vary dramatically, culminating in the lament that "the value of the test depended too much on the ability, competency, and experience of the examiner" (15). Despite these limitations, the subcutaneous tuberculin test, used in a test and slaughter program in the District of Columbia, reduced disease prevalence from 18.87% in 1909 to 0.84% in 1918. Unsurprisingly, the whole idea of testing cattle to eradicate bTB was not without its opponents. Some believed it unnecessary to detect bTB in the early stages of disease instead advocating only a physical examination by veterinarians to determine the high shedders for removal (*redolent of the discussions today pertaining to another mycobacterial disease—Johne's disease*). Others, given the chronic nature of the disease, considered the US approach of test and cull too harsh, or argued that disease control should be managed by herd owners and not by government. At the same time, there were numerous rumors and misconceptions circulating about the test. The most passionate objection being that the test was inaccurate, that (*apparently*) healthy cows tested positive and diseased cows tested negative so that the test would annihilate the cattle population and result in deficits in milk and meat (15). Palmer and Waters describe how unscrupulous cattle dealers and others, including some veterinarians, specialized in circumventing or even failing to perform tests so that infected animals could be sold as having been tested (15). Even today, more than a century later, bTB eradication programmes face remarkably similar challenges including rumors as those described by Palmer and Waters (15). These include misconceptions, careless, illegal or fraudulent activities, demands for greater efficiency (less costly, easier to perform), and more effective tests (100% accurate), complaints with respect to test Sp, more so than Se, indemnity, supervision, lack of funds and of personnel.

The early 1900s saw an explosion in attempts to develop an alternative means to test cattle, presumably reflecting the demands of veterinarians and the industry generally, for a less onerous, more accurate test, not just in the USA as reported by Palmer (15) but elsewhere. In 1908, Charles Mantoux, a French physician, found that intradermally injected tuberculin was effective to diagnose TB (56). Also in 1908, Foth reported that 50% of the infected animals which had not responded to the skin test responded to the ophthalmic test (57). In 1909, Joseph gave a full account of the test in the neck and recorded the results in cattle, based upon measurements of the skin fold at the site of the injection (58). Joseph also discussed some other methods such as the cutaneous method developed by von Pirquet, where the antigen is applied very superficially, Wolff-Eisner's conjunctival method, Escherich's test, and that of Roemer and Joseph where the tuberculin was injected subcutaneously, and the intradermal methods proposed both by Mendel and also Moussu and Mantoux in 1908 (58). With many of the earlier methods, Joseph comments that efficacy depends on the degree of absorption and that, with a highly variable amount of antigen in the tuberculin, interpretation was unreliable. Joseph also noted that the intensity of the reaction is not directly correlated with the amount or severity of lesions and noted that recent infections appear to be associated with particularly strong reactions. He proposed that intradermal injections be made in the lateral neck region and an interpretation for the test. In addition, Joseph elaborated on the advantages of the intradermal test over the subcutaneous test being: no need to determine body temperature, a broader time window for measuring the reaction, reaction lasts longer and is more reliable, no milk drop associated with positive reactions, less antigen required and therefore cheaper (58). In the same year (1909), Römer also published two papers in support of the intradermal test setting out positive, inconclusive and negative criteria for test interpretation (59, 60). Christiansen, in 1910, also commented on the use of the caudal fold by Mantoux, Moussu, and another French author Vallée, but observed that the use of the side of the neck was more convenient and went on to describe the physical performance of the test, the nature and type of response in infected animals using the intradermal test on the neck (61). In 1910, Christiansen and Stub reported on investigations into the use of the ophthalmic tuberculin test, involving 852 animals, being apparently highly specific and easier to perform and to repeat more frequently than the usual test. Although it was not clear what they regarded as the usual test they did state that the usual test was more sensitive (62). They reported that the ophthalmic reactions were observable weakly at 6–12 h and stronger from 12 to 24 h post inoculation. However, with their tuberculin, only 50% of lesioned animals responded to the test leading them to comment on the variation in tuberculin efficacy (Se and Sp) depending on the strain and tuberculin preparation techniques used (62). Following on from the production of KOT several other tuberculins were produced; the *Bacillus* emulsion, the broth filtrate, and the tuberculin residue, all standardized to a definite amount (in milligrams) of solids per volume (52). In 1914, Haring and Bell lamented with respect to the subcutaneous test in California, where it was still being used, that "it cannot be applied satisfactorily to young calves or to wild range cattle, while during the hot season in some of the interior valleys the test has been unsatisfactory even when applied to docile dairy cows" (63). The demise of the subcutaneous test and the adoption of the intradermal test, using the caudal fold method as the screening test for cattle in the USA was imminent. The caudal fold test was increasingly used from 1917 and was adopted as the official means to test cattle in 1921 (15). Further details of these tests and other material on the early use of tuberculin in diagnosis and treatment of TB, from the late 1800s to *circa* 1913, may be found among the forgotten books (64).

In his 1934 President's address to the Royal Society of Medicine, Buxton commented on the various substances referred to as "tuberculin" eliciting either a systemic or local reaction depending on how they were administered (65). He went on to discuss the intradermal tuberculin reaction saying that "the intradermal reaction may be obtained on any part of the surface of the body, but certain regions are obviously to be preferred." Buxton noted that Moussu and Mantoux had suggested the subcaudal fold in cattle which is still used extensively, especially in the USA while others (Ligniéres, Roemer, Joseph, Bang, Jensen, and Christiansen) preferred the side of the neck. Buxton elaborated on the highly specific nature of the tuberculin reaction, that it "is capable of indicating the presence of tuberculous infection in little short of 100% of infected individuals" while at the same time recognizing that not every case of TB in either man or animals would give a positive reaction and that occasionally reactions may be "observed in apparently healthy persons and cattle." He cautioned also that subcutaneous injection of tuberculin may cause desensitization to testing in some animals; that the comparatively low order of the skin response to tuberculin in cattle necessitates the use of a highly potent tuberculin; that there may be a temporary decrease in the reaction in the immediate vicinity of an intradermal injection extending to about a 2″ inch radius of the original injection—an obvious disadvantage in using the caudal fold owing to the restricted area suitable for injection; and that the occurrence of non-specific response can be overcome by the use of a synthetic culture medium and precipitation in the production of tuberculin. At that time, Buxton was already talking about the inheritance of a predisposition to infection as well as that relationship to the post-infection development of disease (65).

In 1942, following on from the work reported in 1939 by Buxton and Glover (66) who attributed a precision of 87–97% to the tuberculin test and recommended the use of synthetic medium tuberculin (*also better purified using precipitation methods*), the instructions to perform the SICTT in GB were that the tuberculin was to be "injected in the middle third of one side of the neck on a line parallel to the spine of the scapula, avian tuberculin is injected about 4″ below the crest of the neck and mammalian about 5″ below the avian" (67). It appears that the British had repeated, for the SICTT, much of the work done by Christiansen and Stub pre-1910, for the SIT and, in 1947, Francis confirmed that the test interpretation for the SICTT and optimal time of reading was "based on a very large number of trials followed by postmortem examination" (8). An allowance of ±4 h for reading on either side of the 72 h is included in the EU Trade Directive (68) but not in OIE (69).

In 1950, Larsen et al. (70) attribute the first use of the neck as a site for intradermal injection to Joseph in 1909 and to Christiansen and Stub in 1910 who, according to Buxton and Glover (66), determined the side of the neck as optimal for the intradermal injection because this site provided the most consistent discrimination for presence or absence of TB infection. Furthermore, Larsen et al. (70) when setting out to assess if an area of skin could be found that would be more sensitive to tuberculin than the caudal fold noted that already, in 1909, Foth (57) had pointed out the deficiencies in the use of the caudal fold. They assessed site Se to johnin (a diagnostic agent for *Mycobacterium avium* subsp. *paratuberculosis* infection, analogous to tuberculin) and tuberculin in steers sensitized to either to paratuberculosis or to TB delineating five regions on each side of each animal with eight injection sites in each region except the caudal fold which had only one site (70). In total, 66 injections were made in each animal (**Figure 1**), and the results were analyzed. The largest reactions occurred in the neck region and showed that the skin on the neck is the most sensitive while the caudal fold was the least sensitive (70).

In 1951, Baisden et al. (71), although they acknowledged that Johnson (72), Wadley (73), and Larsen et al. (70) had individually already determined that different skin areas of cattle differ in their Se to the intradermal injection of tuberculin with the neck being the most sensitive site, reassessed the Se of the neck, the back, the upper and lower sides of cattle to intradermal injection of tuberculin. However, as well as including animals sensitized to *M. bovis* and *M. paratuberculosis*, they added animals sensitized to *M. tuberculosis*, *M. avium*, and *Mycobacterium phlei* to the study

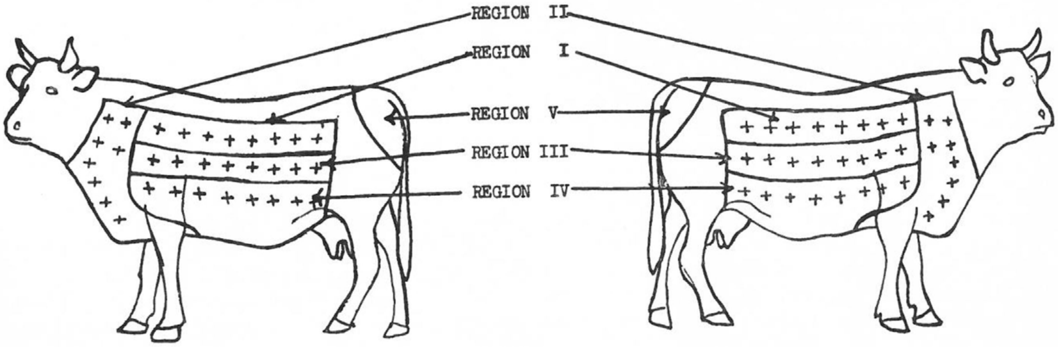

FIGURE 1 | Location of skin regions on right and left sides of animals. Injection sites in each region = +. [Reprinted with permission of the AVMA (Am J Vet Res (1950) 11:301 – Larsen et al. (70))].

"because these organisms had been considered in connection with no-visible lesion cases." The findings were that the neck was significantly more sensitive than any other site for all except the *M. paratuberculosis* sensitized group where site was not of significance. They also commented that the caudal fold was "not so likely to elicit reactions from animals of low Se" (71).

In 1959, Richie (67) also described several alternative methods of tuberculin testing, all now discarded from general use in cattle, employed in efforts to find a more efficient, effective, and less time-consuming test than the subcutaneous test which depended on multiple temperature records over time. He described a short thermal test where temperature checks were reduced, the von Pirquet test (tuberculin applied to scarified skin), ophthalmic and palpebral tests (relying on intolerance to light), increased tearing, possibly marked congestion, discharge running down the face in the tested eye, i.e., severe conjunctivitis (*still used in primates*), the Stormont test, and the vulval test. He detailed the double intradermal test (*a second dose of tuberculin injected into the swelling produced by the first usually after a period of 48 h*), also mentioned by Buxton and Glover (66) and Francis (8) as a modification of the 1910 test of Christiansen and Stub, but according to Francis commented that "it has never been satisfactorily demonstrated that it is in any way superior," to the single test and probably tended "to reduce the Sp of the reaction." It was not adopted anywhere as an official test until GB did so after a Medical Research Council report in 1925. The use of the word "single" in the title of the SIT and SICTT was to distinguish these tests from the earlier double intradermal test (67).

The neck was also reconfirmed as the most sensitive site by Paterson in 1959 (74) who also detailed higher Se at anterior (toward the head) sites of the neck and that Se falls off in posterior sites (nearer the shoulder) and in those adjacent to the nuchal crest; thus, he determined the middle third of the neck as the optimal injection site. This in turn was stressed by Ritchie in the same year (67), and it is why the OIE requires that the test for potency assay of tuberculin in cattle rotates through each of the 16 sites "applying eight intradermal injections per animal in both sides of the neck and employing a balanced complete Latin square design" so as to remove any site influence from the calculations as required (69, 75). In 1960, Larsen et al. reported that as the number of simultaneous injections increased, the average size of the reaction that each would elicit decreased (76). This is the reason why the selection of TB-infected animals, for use in potency assay trial performance, is confined to those that show larger reactions. It was only in 1979, on the accession of GB, Ireland, and Denmark, to the EEC that the SICTT was accepted in addition to the Single Intradermal Tuberculin test (SIT) for the Trade Directive (68).

Good et al. (77), when comparing PPDs from different manufacturers, injected control and trial avian/bovine PPDs into the same animal—the control at the anterior border of the middle one-third of the neck and the trial tuberculin at the posterior border and, by using the same tuberculin at both sites in one group of animals, demonstrated that the anterior site readings were greater than at the posterior sites and thus confirmed the earlier work of Paterson (74). Good et al. (77) also showed that it is particularly important for consistent SICTT interpretation that both injections are in the same plane parallel to spine of scapula thus confirming Ritchie's assertion that the injections needed to be made on a line roughly parallel to the line of the shoulder (67, 77). More recently, Casal et al. looked at the effect, in cattle from officially TB free and TB-infected herds, of bovine PPD inoculation site on the skin-fold thickness increase. In TB-infected herds, there was a higher probability of positive results and larger reactions when the injections were performed nearer the head in the neck anterior area—again confirming the site effect (78). Alternative sites/methods are used for tuberculin testing in other species, e.g., pigs: the base of the ear; fowl: in the wattle; dogs and cats: monitoring body temp. However, there is very little data available on standardization of the test methodologies or interpretation criteria for species other than cattle and little or no work done on evaluation of Se, Sp, positive predictive value, safety record, or authorization criteria for use under medicines legislation and thus the reliability of tuberculin skin testing in species other than cattle is debatable.

The OIE (69) now lays down standards, applicable to cattle, for:

(1) The Delayed hypersensitivity test (i.e., the tuberculin test or the SIT) as the standard method for detection of bTB which "involves measuring skin thickness, injecting bovine

tuberculin intradermally into the measured area and measuring any subsequent swelling at the site of injection 72 h later."

(2) Performance of the SICTT where "bovine and avian tuberculin is used mainly to differentiate between animals infected with *M. bovis* and those sensitized to tuberculin due to exposure to other mycobacteria or related genera" (e.g., as used in Ireland and the UK) including providing in detail for

(3) "Test procedure—(i) A correct injection technique is important. (ii) The injection sites must be clipped and cleaned. (iii) A fold of skin within each clipped area is measured with calipers and the site marked before injection. (iv) A short needle, bevel edge outwards and graduated syringe charged with tuberculin attached, is inserted obliquely into the deeper layers of the skin. The dose of tuberculin is then injected." (v) "A correct injection is confirmed by palpating a small pea-like swelling at each site of injection. (vi) The distance between the two injections should be approximately 12–15 cm. In young animals in which there is no room to separate the sites sufficiently on one side of the neck, one injection must be made on each side of the neck at identical sites in the center of the middle third of the neck. (vii) The skin-fold thickness of each injection site is remeasured 72 h after injection. (viii) The same person should measure the skin before the injection and when the test is read."

(4) Tuberculin potency "The recommended dose of bovine PPD in cattle is at least 2,000 international units (IU) and in the comparative tuberculin test, the doses should be no lower than 2,000 IU each"; "In cattle with diminished allergic Se, a higher dose of bovine tuberculin is needed, and in national eradication campaigns, doses of up to 5,000 IU are recommended" and

(5) Potency assay including the necessity to assay in the target species cattle.

In performing a tuberculin test, it is important that the tuberculin used is fit for purpose (46). This was previously recognized in 1908 when it was lamented that "some of the tuberculin on the market is impotent and worthless" and Buxton also commented on tuberculin quality in 1934 (65, 79). Alas such substandard tuberculin, even unauthorized, under any recognized medicines legislation, can still be found on the market and being used to "certify" freedom from disease (80–82). Good et al. (83) compared "the impact of different potencies of a single bovine PPD tuberculin on the field performance of the" SICTT and SIT and found "a significant difference in the number of reactors detected using the high and low potency tuberculins." In addition, "the low potency tuberculin in the SICTT failed to detect 20% of 35 animals with visible lesions," and "11% of animals with visible lesions did not show a positive bovine response (>4 mm) and would have been negative to the SIT" with these latter animals eligible for certification as TB free for export under EU rules (68, 83). In this study, "the potency estimates from the guinea pig bioassay were imprecise" with only "limited agreement between the guinea pig and cattle bioassays" performed in naturally infected cattle (83).

Test Se and Sp should most properly only be assessed under the conditions and in the species in which the test is performed, and caution must be exercised in extrapolating Se and Sp from one environment and/or the tuberculin from one manufacturer and/or one potency, and/or one type of tuberculin test to another (46). However, the focus on "visible" and "non-visible" lesions, and on SIT/SICTT positive animals which are not confirmed as TB infected by a laboratory has resulted in doubts being cast on Sp, positive predictive value and reliability of all tuberculin tests and frequently attempts to "confirm" tuberculin test-positive animals before their removal, using tests of far lower Se. This has, in many places, had an enormous negative impact on TB control programs. In many TB-infected areas, the focus has shifted to maximizing test Sp, even in the face of active infection in a herd, to minimize the number of "non-visible" lesion animals being identified for removal instead of seeking maximum Se to prevent transmission, to rid a herd of TB as rapidly as possible, and to prevent future breakdowns. Goodchild et al. (84) provided some useful data on the Se and Sp of the SICTT in the GB bTB eradication programme namely that in GB, Sp at animal level is 99.983 (standard interpretation) and 99.871 (ultra-severe interpretation), meaning that one false positive can be expected at standard interpretation for every 4,760–7,690 uninfected animals tested; that 91.1–93.7% of reactors are actually infected with TB (i.e., test-positive predictive value); that visible lesion or culture positive results in only 30–40% of reactors "profoundly underestimates the proportion of reactors that is truly infected"; that a small majority (>50%) of NVL/culture negative Rs are infected and that at herd level the Sp is 99.2–99.5% (average test size) and 96.5% for larger (250 animals) herd tests (84). O'Reilly had already assessed the SICTT Se under Irish conditions as 91 and 98% Se (standard and severe interpretation, respectively) (85). Costello et al. obtained similar results, on repeating the study, 90.9% Se (89.6 and 91.2—standard and severe interpretation, respectively) (86). Both these studies slaughtered and examined all animals (221 and 353, respectively) involved (85, 86). Experiments to establish test Se and Sp for a particular environment are acknowledged as expensive and labor intensive and that few involve slaughter of all, including the non-reacting, cattle noting that recently infected animals are unlikely to have visible lesions or to confirm on culture (87). In Ireland, field experience evidences that only a fraction of 1% of the positive reactors to the SICTT on a national basis are false positive (88), the SICTT Sp has been calculated as 99.8–99.9% and demonstrated mathematically in an accepted non-disease-free population, as at least 99.95% (85, 89).

Research continues into the development of new, more accurate, more sensitive tests, less reliant on the measurement of the immune response *in vitro* (bovine interferon-γ release assay or antibody ELISA) or *in vivo* (skin tests) which are less subject to the vagaries of individual operative performance and subjective interpretation (90). The strategic application of the IFN-γ assay (IGRA), which in common with the tuberculin skin test reads the cell-mediated response to antigens and as such is an early-stage means to detect infection, has been found to be a useful adjunct to the tuberculin test (91–97). Thus, in more recent years, the assay has been used in European countries, in accordance with legislation, and also elsewhere to facilitate the early removal of infected animals that are otherwise negative to the tuberculin skin test in problem herds and speed up the clearance of bTB in outbreaks (68). However, the Sp of this assay has, to date, precluded its use as a screening test and research continues into the use of specific antigens to address Sp issues but, to some extent at least, this is likely to be at the expense of test Se (94, 97). While *in vitro* tests, tuberculin based or otherwise, are suitable for domestic species the logistical difficulties with their application, will vary from region to region and between different breeds and farming systems, for example, the availability of suitable laboratories with associated costs may be an issue, and the length of time taken from blood sampling to submission of samples can be critical for IFN-γ assay with laboratories likely to be some distance from infected herds. Therefore, for the considerable future, it is highly probable that the tuberculin skin test will remain the screening test of choice for farmed livestock.

Tuberculin skin tests are rarely, however, suitable for use in wild species due to the necessity to have access to the animal to read the test some days post tuberculin injection. Thus, test methods such as *in vitro* immunological tests need to be developed for use in wild or feral maintenance species as they have for bovines (87, 98). Scientific advances allow new matrices, such as high-throughput sequencing of the peripheral blood mononuclear cells to be evaluated, also the diagnostic potential of immunostaining with anti-MPT64 in various tissue specimens for *M. tuberculosis* infection (99). It remains to be seen if such tests will be added to the armory of tests in the battle against TB in any or all species in which it occurs. There are several reviews of all currently available tests, including the use of ELISA tests to detect a humoral response, therefore a later stage detection test than the IFN-γ, and the effect of a prior SIT or SICTT on the ELISA or IFN-γ response (100–107).

IMPEDIMENTS TO THE ERADICATION OF TB IN BOVINES

While bTB has been successfully eradicated in many countries, others, despite making major efforts, have been less successful (108, 109). Palmer (110) has described TB as a reemerging disease at the interface of domestic animals and wildlife, he cautioned that it will not be possible to eradicate *M. bovis*, or presumably *M. caprae*, from livestock until transmission between wildlife and domestic animals is halted, and he advises that to achieve this will require a "collaborative effort between stakeholders" (110). In 1958, Francis (111) speaking of the difficulties in final eradication of TB, also recommended that TB had to be dealt with in all species to achieve complete success. Therefore, the need to tackle TB transmission between wildlife and domestic animals is not a new concept or suggestion. It is widely accepted that for effective control of TB the disease must be addressed in all species, in which infection establishes and becomes self-sustaining (infected maintenance species) and that tackling the disease in one species alone in an ecosystem with multiple infected maintenance species will not promote a successful outcome (110). Consequently, other species sharing the environment with cattle must be risk assessed to identify potential maintenance hosts, and, where other species will constitute an impediment to final eradication of bTB, appropriate control strategies should be developed and/or adapted (112–115). The experiences in other countries with similar problems should be taken into consideration (113, 116). TB, caused by several members of the MTBC, has been reported in wildlife in several countries in Europe which are laboring to eradicate bTB (109, 117, 118). It is becoming increasingly obvious that endemic TB in wildlife populations is posing a significant constraint to final eradication of disease in cattle. Further, *M. caprae* is now recognized as not being restricted to Spanish goats, as strains of this organism have been isolated from cattle, wild boar, pigs, and humans. Its occurrence has also been reported in France, Austria, Germany and elsewhere (119).

Unsurprisingly, TB has not spread uniformly into wildlife, and it has become more of a problem in those countries and regions where bTB eradication commenced relatively late, where farming involved pasturing of cattle, where they shared the environment with susceptible wildlife, where wildlife density and behavior patterns (not necessarily the same even for the same species in all ecosystems) brought them into contact with infected cattle, and in particular where cattle were not housed. Feed supplied to cattle would have also been available to local wildlife, and this would have put wildlife and cattle into closer contact than they might otherwise have been the case (120). In other ecosystems, drought encourages the congregation of cattle and wildlife species at water holes and/or where pasture/feed is still available. Tuberculous carcases and/or entrails would, even today, be a potential source of infection for wildlife. This is particularly a problem for carnivorous and scavenging species such as lions and lynx in Africa and Spain respectively (121, 122). Thus, TB also has significant conservation implications for some species, e.g., the Iberian Lynx (*Lynx pardinus*) in Spain (123), other species in conservation areas in South Africa (121) and for lechwe antelope in the Kafue basin Zambia (124).

As disease prevalence in cattle decreases, eradication efforts are sometimes impeded by transmission of *M. bovis* and/or *M. caprae* from wildlife to cattle. In epidemiological terms, disease can persist in some wildlife species, creating disease reservoirs, if the basic reproduction rate of the disease and critical host-community size thresholds are achieved. bTB eradication efforts require elimination of *M. bovis* transmission between wildlife reservoirs and cattle where present (125). Some wildlife species, principally the badger in the UK and Ireland, the Australian possum (*Trichosurus vulpecula*) in New Zealand (but not in Australia), and water buffalo (*Bubalus bubalis*) in Australia previously, have been recognized as significant reservoirs of *M. bovis* with endemic self-maintaining infection in these species constituting a major obstacle to disease control programmes (114, 126, 127). In Australia, elimination of wild water buffalo, not a native species, and feral cattle from areas where infection was endemic was a major component of the eradication campaign and Australia is now bTB free (114, 116, 126–128). New Zealand has employed similarly strict population control measures against infected possum populations, resulting in considerable progress (117, 126, 128, 129). Michigan State, USA, had been TB-accredited-free state from 1979, with no tuberculous cattle detected for 5 years, when a hunter found a TB-infected deer in 1994. The local deer population was endemically infected with *M. bovis* and spill back was also detected in local cattle farms (130).

Consequently, on-farm risk mitigation measures against the TB transmission from deer to cattle have been recommended (131). Portugal has reported wild boar (*Sus scrofa*) and deer (mainly red deer—*Cervus elaphus*), both key game wild ungulate species, as being infected with *M. bovis* or *M. caprae* in the important higher density hunting regions where TB prevalence in cattle is also highest (118). Observations in Spain showed that strains of MTBC that originated in bovines and caprines also circulate in the sympatric wildlife populations and that in addition 6 out of 11 spoligotypes resembled types described in human TB cases. The isolation of MTBC strains (belonging either to *M. bovis* or to *M. caprae*), in fenced estates, from cervids and wild boars that have not had contact with domestic livestock for at least two decades, strongly suggest that these mycobacteria are able to survive independently in these populations. Therefore, where they are TB infected, wildlife, including cervids and wild boar, need to be considered in the epidemiology and control of TB (117).

Making use of molecular detection technologies, Santos et al. demonstrated widespread MTBC contamination in environmental samples from the Iberian Peninsula. This supports the occurrence of indirect transmission as a contributor mechanism to maintaining TB in a multi-host–pathogen system (132). MTBC DNA positive samples were proportionately higher in the bTB-infected area than in presumed negative area (0.32 and 0.18, respectively) (132). In 2010, the first detection of *M. bovis* in a feral wild boar was reported in the UK in an area where the same spoligotype had previously been isolated from fallow deer, fox, wood mouse, and polecat (133). Studies under natural weather conditions in Michigan, where *M. bovis* TB had been detected in free-ranging white-tailed deer demonstrated that *M. bovis* bacteria survive sufficiently long to pose an exposure risk for cattle and/or wildlife. This strengthens evidence suggesting that biosecurity on cattle farms and efforts to eliminate supplemental feeding of white-tailed deer will decrease the risk of TB transmission among and between these populations (134). In 2016, French researchers reported finding environmental samples positive for the presence of MTBC and *M. bovis* strains in the environment of farms affected by bTB in a restricted area within the Côte d'Or region where shared genotypes of *M. bovis* circulate in a multi-host system including badgers, wild boar, and deer (135). The persistence of detection over an 8-month period, despite absence of the supposed source of infection, suggested that the DNA detected could belong to viable cells. The detection of MTBC positive signals in 10% of water samples from naturally occurring water springs and accompanying flowing water in pastures where both cattle and wildlife had access is supportive of the role of water in the dissemination of MTBC in the environment and in animal contamination perhaps even by the formation and inhalation of bioaerosols. The average prevalence of detection in badger sett soil and badger latrines was 7.3 and 7%, respectively. These were the highest prevalences detected among 356 environmental samples assessed (135). Similar work in the UK had earlier assessed that correlations between badger social group TB prevalence as determined by the qPCR assay of fecal samples from badger latrines and individual or combined diagnostic test results from trapped badgers suggested that spring was the optimum latrine sampling period, with autumn an acceptable confirmational back up with 100 and 80%, Se respectively (136). Researchers at Warwick University performed parallel qPCR of feces and culture on samples taken from badgers in areas with high bTB prevalence levels in the Republic of Ireland, which indicates that fecal shedding is a good proxy for respiratory shedding (137). In addition, the endangered Kafue lechwe antelopes (*Kobus leche Kafuensis*), in the Kafue basin in Zambia where cattle and antelope graze together during drier months, have been described as feral reservoirs of bTB (124). Wildlife management aimed at reducing the density of susceptible animals within an infected area may contribute to the control of infectious diseases in animals and, if zoonotic, their spillover to humans (125). The problems encountered in tackling disease in the various species involved in disease maintenance and interspecies transmission will be particular to the ecosystem in which they reside, and it is likely that each ecosystem will present its own challenges and indeed socioeconomic influences (46). Wildlife vaccination is an option that is being explored and the UK and Ireland are cooperating in the development of a badger vaccination strategy (138–140) in preference to continued culling, with a view to decreasing TB incidence in badgers to reduce transmission to cattle. In Spain work is ongoing into the development for a vaccine in wild boar (141). If a vaccine can be developed for badgers or wild boar then, in time, it may also be modified for use in other wild species (120).

The above examples illustrate that attributing apparent shortcomings in bTB eradication programmes to failure of the tuberculin test, as often happens, in regions or counties which harbor infected maintenance hosts may be misplaced. Rather, the true value of the tuberculin test is as an indicator of the presence of TB in the population under test. Identification of infected wildlife as the source of such infection is not the function of the test and is rather the function of a sound epidemiological investigation into the source of the TB.

ONE HEALTH

In the early years of the twenty-first century, bTB has largely been reduced to a disease of limited economic importance in the developed world, with controls causing more irritation than the disease itself. Poorer countries are facing a multifaceted impact from TB, which is not merely of significant economic impact, but which also potentially affects the health of livestock, humans, and ecosystems simultaneously and which is likely to increase in the presence of debilitating diseases such as HIV/AIDS and other factors which negatively affect human livelihoods (142). The interplay between humans, livestock, wildlife, and ecology in the epidemiology of zoonotic TB makes TB an ideal target for a One Health approach. Such an approach would enable the development of disease control programs involving both animal and human populations, and allow for expanding scientific knowledge, improving medical education and clinical care, and the development of effective disease control programs for both human and animal populations (143). One Health deals with the very essence of TB as a zoonoses—it is surely axiomatic that the transmission of disease shared between human and animal species must be addressed at multiple levels rather than focusing on humans only or specific animal species only or particular mycobacteria that can cause TB and that environmental, ecological, and sociological factors must be considered in the development of effective disease control programmes.

The One Health concept recognizes the important links between human, animal, and environmental health and provides an important strategy in epidemic mitigation and prevention. It was described by the veterinarian Schwabe (1927–2006) in his book "Veterinary medicine and human health" where he proposed a unified human and veterinary approach against zoonotic disease. The concept is, however, not new. Rudolf Virchow (1821–1902), who coined the term "zoonosis," said "between animal and human medicine there are no dividing lines—nor should there be." James Law (1838–1921) professor of Veterinary Medicine in Cornell educated in the Edinburgh University Medical School and Veterinary College as well as veterinary schools in France believed in "one medicine" where physicians and veterinarians should have close relations. Law's work on TB in the USA had a profound effect on both animal and human health. Likewise, in the early 1890s, one of Bernhard Bang's goals listed as part of the "Bang method" of TB control/eradication in bovines was to limit transmission of TB infection *via* milk, and so to specifically sell safer milk for infants (54). BCG vaccine, developed by attenuating *M. bovis*, of cattle origin, used since 1921 to protect humans from TB was developed by the French physician Albert Calmette and veterinarian Jean-Marie Camille Guérin. Their collaboration demonstrated the "one medicine" or "One Health" concept in action even though Guérin's veterinary background and family TB problems is largely ignored (144). Basil Buxton, Veterinarian, addressed the Royal Society of Medicine in 1934 on the role of tuberculin in the control of TB in the section on Comparative Medicine (65). However, while the concept seems to have been embraced by medical and veterinary communities in the nineteenth century, it seems to have fallen into disfavor during the twentieth century when collaborative efforts between the professions diminished (144). Nevertheless, the "one medicine" concept survived and extended to "One Health" when the Washington Post, in 2003, credited William Karesh who was a veterinarian and president of the World Animal Health Organization (OIE) Working Group on Wildlife Diseases, as saying, "Human or livestock or wildlife health cannot be discussed in isolation anymore. There is just One Health and the solutions require everyone working together on all the different levels."

Tuberculosis does not restrict itself to one host population, and all the members of the MTBC can affect multiple hosts and thus can threaten human and animal health through interspecies transmission. It is of importance to both the animal health and human health sectors as it requires global TB control in all host populations (145). Recognizing the value of cross-sectoral coordination in addressing complex health threats, the FAO, OIE, and WHO formed a Tripartite collaboration in 2010 to develop the concept of One Health and its vision of having a collaborative multidisciplinary work on the health of humans, animals and ecosystems reducing the risk of diseases at the interfaces between them. This FAO–OIE–WHO Tripartite is assessed and updated annually. Their shared "One Health" vision is of "a world capable of preventing, detecting, containing, eliminating and responding to animal and public health risks attributable to zoonoses and animal diseases with an impact on food security through multisectoral cooperation and strong partnerships sharing responsibilities and coordinating global activities to address health risks at the animal-human–ecosystems interfaces" (146). The WHO, in 2014 adopted their "End TB" goals of ending the TB epidemic by 2030, achieving a 95% reduction in TB deaths and a 90% reduction in TB cases by 2035, and have determined that a comprehensive approach is needed which includes new and more effective vaccines, as well as improved diagnostics and treatment (147).

In October 2017, the first-ever roadmap to combat zoonotic TB, i.e., TB in animals and its transmission to humans, was launched at the 48th Union World Conference on Lung Health (148). This multidisciplinary roadmap developed by four groups comprising the WHO, the OIE, the FAO, and the International Union Against Tuberculosis and Lung Disease (The Union) represents a milestone in the fight against TB in both people and animals. It builds on the United Nations Sustainable Development Goals to improve health worldwide including a target to end the global TB epidemic by 2030 as defined by the WHO in the End TB Strategy (147) which acknowledges that people at risk of zoonotic TB are a neglected population deserving greater attention. The roadmap uses a "One Health approach" to address the health risks of TB across sectors to reduce the burden of zoonotic TB, to save lives and secure livelihoods. The roadmap sets out 10 priorities to tackle zoonotic TB under 3 main headings (1) to improve the scientific evidence base, (2) to reduce transmission at the animal–human interface, and (3) to strengthen intersectoral and collaborative approaches. In agreement with others (16, 19, 21), the roadmap acknowledges that the human burden of disease cannot be reduced without management of the animal reservoir, but it also stresses that major technical and scientific obstacles, including the development of novel and affordable diagnostic tests, must be overcome and validated as effective under field conditions.

Indeed, the transmission of *M. tuberculosis* from human-to-animal-to-human still occurs, and is an ongoing risk, especially in countries where there is close interaction of humans with animals and, is of particular public health concern, in places such as zoos, circuses, and exotic animal facilities where there may be contact between TB-susceptible animals and humans (30–37). Where there is no effective eradication programme operational in cattle, the routine presence of *M. tuberculosis* in samples from multiple cattle raises the possibility of human-to-cattle-to-human transmission and possible adaptation of strains of *M. tuberculosis* in bovine or other animal tissues underlining the importance of adopting effective TB control and eradication programmes in humans and livestock alike (38–41).

Discussions on "Does risk to humans justify high cost of fighting bTB?" demands that the "Benefits of stemming bTB need to be demonstrated" and claims that bTB "control in cattle is irrelevant as a public health policy" (149–151) serve to demonstrate that the hard-learnt lessons of history have largely been forgotten and that the "One Health" message on the risk posed to human health (144) is not penetrating to all parties. Pasteurization of bovine milk alone will likely not be sufficient to protect public health if multispecies-based TB controls cease and/or if a strain of *M. tuberculosis* adapted by passage through bovines or some other domesticated or wild species develops that is even more virulent for man and/or has also developed antimicrobial resistance. Strategic exchange of data and discussions involving both veterinary and public health authorities would strengthen TB surveillance in both animal and human populations (152).

Wildlife conservation and ecosystem preservation can also benefit from a One Health approach. The Wildlife Conservation Society recognizes the inextricable linkage between conservation, human health, and the health of wild and domestic animals (146). A single pathogen could wipe out the last populations of an endangered species and, in turn, threaten the stability of local human populations. TB is among its "deadly dozen" potentially lethal diseases that could spread. Economic, environmental and ecological conditions can promote contact between wildlife and livestock and in turn increase transmission of TB at the livestock–wildlife interface. Numerical increases or spatial concentrations of the wildlife population can increase the competition between wildlife and livestock for water and food thus potentially promote the spread of TB directly or indirectly due to the ability of mycobacteria to survive outside a host for a period. Studies have demonstrated that animals in wildlife reservoirs are capable of excreting mycobacteria which can serve as a source of infection to other animals (153, 154). The Wildlife Conservation Society's concept "One World, One Health™" program is a holistic initiative that manages human, wildlife and domestic animal health issues according to a fundamental truth—the "One Health" that affects all is the health of the planet's ecosystems and advises that "the monitoring of wildlife health provides us with a sensitive and quantitative means of detecting changes in the environment. Without wildlife, we may not see what is coming until a crisis has occurred. Wildlife monitoring provides a new lens to see what is changing around us to help governments, world health agencies, and regional communities detect threats and mitigate them before they become health crises" (34, 35, 154).

CONCLUSION

In conclusion, as Sternberg-Lewerin (145) succinctly put it, "A One Health approach is clearly warranted for TB. The disease has similarly serious consequences for humans and a broad range of animal species, and it has been strongly advocated as a One Health issue." TB and specifically zoonotic TB was, and still is, important; ending the TB epidemic in humans and the eradication of TB in cattle and other animals are worthwhile goals for human health, zoonosis, animal welfare and socioeconomic reasons and ideally suited for a One Health approach requiring human medical and veterinary interdisciplinary/multidisciplinary collaborative action. Sharing skills and resources, increasing interaction between public health and veterinarians particularly in resource-limited situations, can raise awareness of the "shared risk" of TB between humans and animals and would help to reduce unnecessary duplication of effort (143, 155). To successfully control TB, all causes of TB, all members of the MTBC must be tackled in all species in which TB occurs. To ignore a reservoir affected species and the lessons of history is to court disaster.

The pioneer scientists who revolutionized the diagnosis of this disease over a hundred years ago were remarkable, indeed so remarkable that it remains a challenge for today's scientists to develop a "better test" or a "better" test reagent. The WHO has determined that "major technical and scientific obstacles will need to be overcome, with validation of effectiveness under field conditions. This will require the development of affordable diagnostic tests in parallel to differentiate infected from vaccinated animals." The target set in the roadmap (148) for the availability of new diagnostics assays for livestock is 2025. However, until this is achieved the tuberculin skin test will necessarily remain the most widely used means of determining the TB infection status of live domestic animals. The necessity to reduce the burden of zoonotic TB, to save lives and secure livelihoods is far too important to await the possible development of novel diagnostic assays for livestock before renewing efforts to eliminate infection in livestock. Tuberculin tests are safe to use and the choice of which type of tuberculin test is determined by the ecosystem in which it will be used. The challenge therefore is to ensure that the tuberculin on the market continues to meet the standards required. Tuberculin potency is critical to test performance and the accurate determination of potency is therefore particularly important. The sale and use of substandard potency tuberculins should no longer be permitted. The skin test needs to remains available, with good Se and Sp, to those far from sophisticated laboratories and with few resources so that it may continue to play a role in TB control in livestock alongside pasteurization of milk for human consumption and public health measures to protect human health and livelihoods. In addition, as suggested by the roadmap for zoonotic TB "the role of wildlife reservoirs, and potential approaches for control through targeted vaccination, could also be further investigated to find sustainable solutions for combatting the disease while safeguarding wildlife conservation."

AUTHOR CONTRIBUTIONS

MG and AD conceived the study. MG, DC, and DB carried out the literature search and compiled the data. MG drafted the preliminary manuscript. All the authors participated in reviewing, editing, read and approved the final draft, and collaborated in producing the final version.

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Successful Application of the Gamma-Interferon Assay in a Bovine Tuberculosis Eradication Program: The French Bullfighting Herd Experience

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In the French Camargue region, where bovine tuberculosis had been enzootic for several years in bullfighting cattle herds, the gamma-interferon (IFN) assay was used since 2003 in parallel with the intradermal test in order to increase overall disease detection sensitivity in infected herds. This study presents the results of a field-evaluation of the assay during a 10-year period (2004–2014) of disease control and surveillance program and explores the particular pattern of IFN assay results in bullfight herds in comparison to cattle from other regions of France. The low sensitivity [59.2% (50.6; 67.3)] of IFN assay using the tuberculin stimulation could be related to the poor gamma-IFN production from bullfight cattle blood cells which is significantly lower than in animals of conventional breeds. The characteristics of the assay were progressively adapted to the epidemiological situation and the desired strategic applications. Data analysis with a receiver operating characteristic curve based on a simple S/P value algorithm allowed for the determination of a new cutoff adapted for a global screening, giving a high specificity of 99.9% results and a high accuracy of the assay. Having regularly risen to above 5% since 2005, with a peak around 10% in 2010, the annual incidence dropped to under 1% in 2014. The positive predictive value relative to the bacteriological confirmation evolved during the years, from 33% in 2009 to 12% during the last screening period, a normal trend in a context of decreasing prevalence. The estimated rate of false-positive reactions during screening campaigns was 0.67%, confirming the high specificity of the test, measured in bTB negative herds, in this epidemiological context. The proportion of false-positive reactions decreased with the age and was higher in males than in females. Although these results indicate that the IFN assay is accurate in the field, it also emphasizes great differences between interferon quantities produced by bullfight cattle blood samples compared to those of classical bovine breeds, which underlines the necessity to adapt the algorithms and combinations of the assay according to local epidemiological contexts.

Keywords: bovine tuberculosis, screening, gamma-interferon assay, field performances, strategic use, eradication

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INTRODUCTION

Bovine tuberculosis (bTB) is a zoonotic disease mainly caused by infection with *Mycobacterium bovis*. Although it is still a major public health problem in some countries, it is more regarded nowadays as an economic problem for agriculture in developed regions of the world where schemes for tuberculosis control and eradication have considerably reduced the prevalence of the disease and sometimes to achieve disease-free status (1). However, these efforts must be maintained, since some countries encounter persistence or reemergence of *M. bovis* infection in their cattle populations (2).

The reasons for these failures are complex and partly due to the difficulties of *ante-mortem* diagnosis of tuberculosis in cattle which remains extremely challenging. In most countries, eradication programmes are based on regular testing and removal strategy by compulsory slaughter of reactors, using the intradermal tuberculin tests with tuberculin purified protein derivative (PPD) to detect infected animals (3). Although this tool has historically been useful in reducing the incidence of tuberculosis, it has shown a lack of sensitivity (4, 5). Furthermore, the skin test is sometimes difficult to use in the field, which may entail operator errors and false-negative results. Limitations in the specificity due to non-specific hypersensitivity reactions in cattle infected with non-pathogenic environmental mycobacterial species are also observed (4). As a consequence, alternative tests were developed to enhance the success rate of infection diagnosis. Particularly, the gamma-interferon (IFN) assay was evaluated and adopted as an official test in several countries (6).

In France, the national tuberculosis surveillance and control program in place since the 1950s and using intradermal tuberculin test has contributed to the drastic reduction of bTB in the country which is recognized as officially "bTB free" since 2001. However, in Camargue, south of France, a region of marshlands where cattle breeding consists mostly of bullfight herds, bTB has been enzootic for several years, with an average annual incidence of 5.5% from 1996 to 2005. Control of the disease has been hampered by the lack of sensitivity of skin test in this population, the free range status of the herds and the breeding techniques which favor contact between animals of different herds. Consequently, the very high level of infection was probably underestimated and 80% of bTB cases were discovered at the slaughterhouse (7). As a consequence of this serious situation, local authorities decided to implement a strengthened bTB control program based on several measures including the use of IFN assay. The decision to use this assay was taken to improve early detection of infection at the farm level (6, 8). In the absence of national or international guidelines for gamma-IFN assay use and interpretation, regional laboratories in collaboration with the National Reference Laboratory had to evaluate and regularly adapt the context of use as well as the interpretation scheme in the context of an ISO 17025 quality assurance system and the regulatory framework for validation of reagents employed for screening notifiable diseases. This study presents the results of the IFN assay field-evaluation during a 10-year period (2004–2014) of disease control and surveillance program and explores the particular pattern of this assay in bullfight herds populations compared to other kind of cattle breeds. The surveillance outcome for the Camargue region is also presented and the strategic use of ancillary tests for the control of bTB is discussed.

MATERIALS AND METHODS

Animal Population Characteristics and Ethics Statement

There are around 250 bullfighting cattle herds in the Camargue region (around 30,000 animals of 2 main breeds, "raço di biou" and "brave"). Animals are either dedicated to bullfighting or to local traditional games (in arena or in villages) and are bred in very extensive conditions. As they are mostly used for entertainment or for reproduction, animals are kept for long time periods within the herd and it is quite common to find animals older than 10 years (up to 11% animals in the herd).

This bovine population is very rarely moved out from Camargue region and has almost no contact with other cattle operations. This explains why bTB is confined to this specific population and does not affect other production systems in the region.

The domestic animals used in this study met the definition of "farm animals", which are not currently covered by French regulations (Décret 2013-118 dated the February 1, 2013, from the French Ministry of Agriculture). The owners of the animals were informed of tests performed on their animals, since all samples were collected during compulsory sanitary investigations.

Field Data

The specificity (Sp) of IFN assay was evaluated in a population of 1,008 animals aged more than 2 years, sampled from six herds considered as bTB free according to the following criteria: (i) no cattle with suspect lesions at the slaughterhouse had been confirmed *M. bovis* culture positive within the last 10 years; (ii) no animals had tested positive to single intradermal cervical skin test (SICT) during the last 6 years (annual screening); and (iii) no epidemiological link with infected herds had been established during the last 6 years. Herds were distributed homogeneously in the Camargue area.

The sensitivity (Se) was evaluated with a data set made of 142 infected animals sampled for IFN assay in 18 depopulated herds from 2006 to 2010.

Field performance of the assay and results of quality controls were also evaluated using data from two global screening programs performed in 2009–2011 (14,199 animals) and 2012–2014 (17,534 animals) in all herds (more than 2 years old animals). The association between a false-positive gamma-IFN response and certain individual characteristics (age, gender, breed) was evaluated using data from herds with bTB-free status in the 2012–2014 global screening period from which individual information was available (11,931 out of 15,532 cattle from bTB-free herds).

The optical density (OD) values obtained with the IFN assay in Camargue were compared to those obtained from 44 infected animals among 24 different herds [comparison of bovine purified protein derivative (PPDB) OD values] and 13,474 animals (comparison of mitogen OD values) from "conventional" cattle from Dordogne (South west France) sampled from 2009 to 2011.

For each infected animal, the macroscopic visible lesions (VLs) were characterized and classified according to the following scoring:

- Level 1: lesions confined to one or several lymph nodes of a same anatomic region.
- Level 2: lesions in an organ with or without lesion of one or more lymph nodes of the same anatomic region, or any caseous lesion of at least one lymph node.
- Level 3: lesions on at least two different anatomic regions.

bTB Case Definition

Herds were considered infected when at least one animal presented one or more of the following criteria for being considered infected:

- observation of clinical signs of bovine tuberculosis associated with a SICT positive result,
- isolation of *M. bovis* by culture,
- association of a positive SICT or IFN assay result with histopathology positive bTB lesions,
- association of a positive PCR result with histopathology positive bTB lesions or a SICT or IFN assay positive result,
- observation of bTB VL in an animal from a previously demonstrated infected herd.

Animals Testing

Blood samples were collected in heparinized tubes the same day SICT was performed, transported at ambient temperature (22 ± 5°C, avoiding extreme temperatures) to the laboratories and processed within 8 h postcollection. Stimulation of whole blood was done with PPDB and avian purified protein derivative (PPDA) (Prionics AG, Schlieren, Switzerland). PPDB and PPDA were prediluted with phosphate buffer saline (PBS) to achieve a final assay concentration of 20 µg/ml. Pokeweed mitogen (PWM) at 5 µg/ml and NIL antigen PBS were used, respectively, to control the blood cells ability to produce gamma-IFN and to detect a non-specific gamma-IFN production. Whole blood cultures were performed with 24-well plates from 2006 to 2009 (1.5 ml of heparinized blood with 100 µl of antigen solution) and 96-well plates from 2009 to 2012 (250 µl of blood with 25 µl of antigen solution, 2 stimulation wells per antigen). Blood samples were evenly mixed before aliquoting. Plates were incubated at 37°C in a humidified atmosphere for 16–24 h, then centrifuged at 500 *g* for 10 min at room temperature. After incubation, approximately 100 µl (96-well plates) or 500 µl (24-well plates) of plasma were removed from above the sedimented red cells using a variable-volume pipette. The absence of significant effect of vessel geometry has been demonstrated by Schiller et al. (9). Plasma samples were tested using Bovigam® (Prionics AG, Schlieren, Switzerland) in duplicate wells. The same analytical protocol was used in Camargue and Dordogne regions. Some animals were tested with the SICT using the official bovine PPD (Synbiotics, France).

PCR and mycobacterial culture were performed on tracheobronchial, retropharyngeal, and mediastinal lymph nodes presenting or not VL at the slaughter house. Lymph nodes were analyzed individually. Culture was performed according to the French *ad hoc* guideline, using solid Lowenstein and Coletsos agar after decontamination with 4% H2SO4 solution neutralized by adding a 6% NaOH solution. After mechanical lysis of tissue, DNA was extracted by using the QIAamp DNA mini kit (Qiagen) or by Magvet MV384 (Life Technologies) with a King Fisher KF96 automate, following the manufacturer's instructions. PCR was performed with a commercial kit (LSI VetMAXTM *Mycobacterium tuberculosis* Complex PCR Kit 2 wells) targeting IS*6110*, which is present in all species of the *M. tuberculosis* complex (10): 5 µl of the extracted DNA was mixed with 20 µl of reaction mix and the reaction was carried out at 50°C for 2 min (1 cycle), followed by one cycle of 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Results were interpreted following the manufacturer's recommendations and by comparison with negative and positive controls. Thermolysates of bacterial isolates were confirmed as *M. bovis* by Luminex spoligotyping as described by Zhang et al. (11).

Data Analysis

The performance of the IFN assay was determined for the following positive cutoff recommended for the Bovigam® kit, designated as the historical positive cutoff: (mean OD PPDB − mean OD NIL $\geq$ 0.1) and (mean OD PPDB − mean OD PPDA $\geq$ 0.1). Results were excluded when the OD value was higher than 0.3 for the NIL antigen-free sample (12) or less than 0.5 for the PWM-treated sample (9).

The Se and Sp were evaluated with their 95% exact binomial confidence interval (CI) at the individual level but also at the herd level, using the following formulas:

- Se herd level = $1 - (1 - \text{Se ind})^n$ considering an average number of $n = 3$ infected animal per herd (based on field data).
- Sp herd level = $(\text{Sp ind})^n$ considering an average number of $n = 76$ animals per herd.

Additionally for all samples, the S/P value was calculated as proposed by Faye et al. (13): (mean OD PPDB − mean OD PPDA)/(mean OD PC − mean OD NC) where PC is the mean OD value of the positive control of the ELISA plate and NC is the mean OD value of the negative controls. The dilution of the positive control was adapted for each batch of ELISA kit to ensure a constant OD value of the positive control for all batches.

These data were used to build a receiver operating characteristic (ROC) curve to examine the impact of different cutoff values on the individual sensitivity and specificity relative to bacteriology and to define an optimal diagnostic cutoff value ensuring a high specificity adapted for global screening. ROC curves were performed using roctab command in Stata (nonparametric ROC analysis). Cutoff values evolved over time in order to be optimized and adapted to the epidemiological situation and the bTb control scheme (**Table 1**).

TABLE 1 | Different cutoff values used over time during the eradication program.

Years	Epidemiological context	Objectives	Cutoff	Algorithm applied	Quality controls
2003–2008	High prevalence	Improve farm level detection and detect new tuberculosis cases	“Historical” positive cutoff	(OD PPDB − OD NIL $\geq$ 0.1) And (OD PPDB − OD PPDA $\geq$ 0.1)	OD NIL < 0.3
2009–2010	High prevalence	Maximize the sensitivity of the global population screening	Positive cutoff	(OD PPDB − OD PPDA)/(OD PC − OD NC) $\geq$ 0.04 And (OD PPDB − OD NIL)/(OD PC − OD NC) $\geq$ 0.04	OD NIL < 0.3 OD PWM > 0.4
			Suspect cutoff	(OD PPDB − OD NIL)/(OD PC − OD NC) $\geq$ 0 And 0.02 $\leq$ (OD PPDB − OD PPDA)/(OD PC − OD NC) $\leq$ 0.04 Or (OD PPDB − OD PPDA)/(OD PC − OD NC) $\geq$ 0 And 0.02 $\leq$ (OD PPDB − OD NIL)/(OD PC − OD NC) $\leq$ 0.04	
2012–2014	Decrease of incidence	Improve the specificity of the global population screening	Positive cutoff	(OD PPDB − OD PPDA)/(OD PC − OD NC) $\geq$ 0.04	OD NIL/(OD PC − OD NC) < 0.125 OD PWM/(OD PC − OD NC) > 0.125

The OD values from infected Camargue cattle were compared to those obtained from “conventional” cattle using a two-sample *T*-test with unequal variances (test command in Stata).

Logistic regression models to assess the association between a false-positive gamma-IFN response and certain individual characteristics (age, gender, breed) were built on data from 2012 to 2014 global screening programs (logistic command adjusting for herd clustering effect in Stata). A false-positive gamma-IFN response was assumed on all IFN positive results from bTB-free herds which were not confirmed by bacteriology and/or PCR.

RESULTS

Performance of the Assay

Characteristics of the Assay

Results for the IFN assay field performance evaluation using historical cutoff values are presented in **Table 2**. The low sensitivity at the individual level was compensated at the herd level considering the high bTB within-herd prevalence during the first years of IFN assay use (average number of three infected animals per herd). The very high specificity at individual level was slightly hampered at herd level but remained above 90% despite the high average number of animals in bullfight herds.

TABLE 2 | Results for the IFN assay field performance evaluation using “historical” cutoff.

Test result	Value% (95% CI)
Se—individual level ($n = 142$)	59.2 (50.6; 67.3)
Se—herd level[a]	93.2 (88.0; 95.5)
Sp—individual level ($n = 1{,}008$)	99.9 (99.4–100)
Sp—herd level[b]	92.7 (63.3; 100)

[a] *Assuming three infected animals per herd.*
[b] *Assuming a mean herd size of 76 animals.*

The relation between (OD PPDB − OD PPDA) and the level of observed lesions of infected animals was assessed using analysis of variance. No significant differences were observed (**Table 3**).

TABLE 3 | Mean (OD PPDB − OD PPDA) values according to the level of visible lesion.

Level of visible lesions	*N*	Mean (OD PPDB − OD PPDA)
Level 1	38	0.1499
Level 2	47	0.1743
Level 3	46	0.1333

Comparison of Gamma-IFN Production between Bullfight and Conventional Cattle Breeds

Figure 1 shows that gamma-IFN production in stimulated whole blood from infected bullfighting cattle was much lower than in infected conventional cattle. Indeed, the average OD value for gamma-IFN produced in response to PPDB antigen in the population of Camargue bullfight cattle (Mean OD = 0.489, $n = 142$) was significantly different ($p < 0.0001$) from the average OD value in conventional cattle (Mean OD = 1.943, $n = 44$). The average PPDB OD value for infected bullfight cattle appeared very near the historical cutoff value. This could explain the low individual sensitivity using that cutoff and suggests that using a test with a better detectability could increase the sensitivity without degrading specificity.

In the whole population of bullfight cattle sampled from 2009 to 2011 ($n = 14{,}199$), the mean OD value obtained in wells stimulated with mitogen was 1.123 (1,091–1,155) while results obtained for cattle from the Dordogne region during the same period ($n = 13{,}474$) showed a significantly higher mean OD value of 2.3 (2.287–2.319). These results indicate that the criteria for

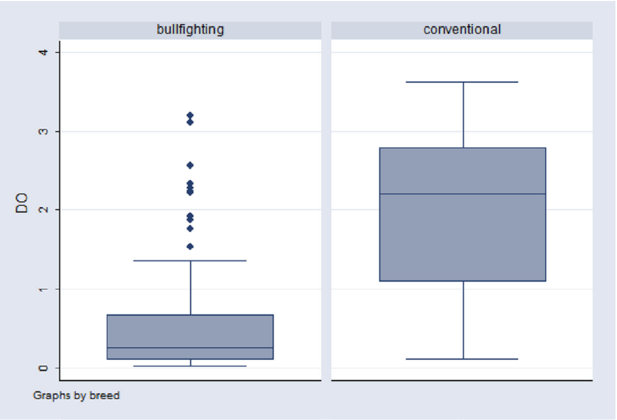

FIGURE 1 | Distribution of optical density (OD) values for PPDB antigen in two infected populations: bullfighting (*N* = 142) and conventional cattle (*N* = 44). The upper lines in the boxes represent the 75th percentile (P75), the middle line represents the median (P50), and the lower line in the box represents the 25th percentile (P25). The ends of the whiskers represent minimum and maximum OD values.

discarding samples with limited gamma-IFN production according to the mitogen's OD value should be determined according to the characteristics of the studied animal population.

Assay Optimization and Definition of New Parameters

Cutoff Determination

Figure 2 presents the ROC curve using the S/P quantitative value, indicating an area under the curve (AUC) close to 0.9 giving indication of a high accuracy of the IFN assay taking into account previously published guidelines (14). The new S/P cutoff, (mean OD PPDB − mean OD PPDA)/(mean OD PC − mean OD NC) = 0.04, presented a high specificity adapted for global screening (**Table 4**). Furthermore this cutoff using a unique value was easier to evaluate than the historical one. As recommended for interlaboratory standardization (15), interpretation of the S/P value enabled to take into account the variability of raw OD values, which are absolute measurements influenced by test parameters and photometric instrumentation.

Quality Control Parameters

The mean OD for NIL antigen was 0.073 (0.071–0.075, n = 14,209) with 97.7% of the values below 0.3 confirming this value as an appropriate cutoff above which the result should be considered uninterpretable. Indeed, we observed that a high OD value obtained in wells stimulated with NIL antigen could entail false-negative results (unpublished results). The criteria previously described by Coad et al. (12) for controlling non-specific reactions was definitively adopted (OD NIL < 0.3 for valid result).

On the other hand, the criteria for controlling the immunocompetence of the blood cells (OD PWM) was adapted to take into account the low reactivity of this local bovine population. Thus, a value of 0.3 was adopted instead of the initial 0.5 cutoff.

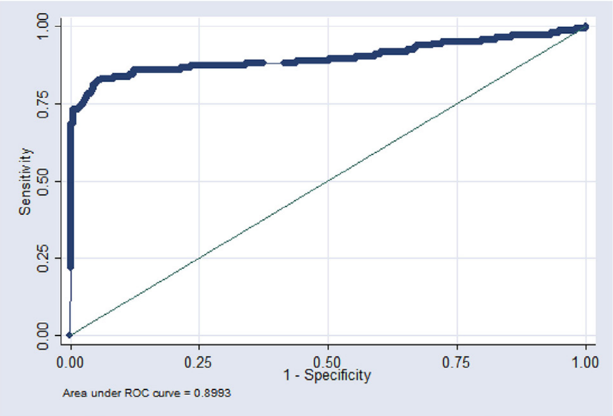

FIGURE 2 | Receiver operating characteristic (ROC) curve established by using the S/P quantitative value.

TABLE 4 | Characteristics of the IFN assay for various cutoff values according to ROC simulation.

	Se (95% CI)	Sp (95% CI)
Cutoff S/P = 0.03	64.1 (55.61–71.96)	99.8 (99.29–99.98)
Cutoff S/P = 0.04	59.15 (50.6–67.32)	99.9 (99.45–100)
Cutoff S/P = 0.05	56.34 (47.77–64.64)	99.9 (99.45–100)

With the introduction of these controls and adaptations, a result was classified as valid if OD NIL < 0.3 and OD PWM > 0.3. From 2012, those cutoff were converted to take into account the control values and samples were excluded when OD NIL/(OD PC − OD NC) > 0.125 or OD PWM/(OD PC − OD NC) < 0.125.

Strategic Use and Field Performances for bTB Control

Context of Use and Evolution of the Incidence Rate

The IFN assay was first used in a limited number of herds and then, between 2006 and 2008, it was applied throughout the region for a screening program in farms considered at risk of being infected (i.e., epidemiologically linked to an outbreak, sanitation program during the previous 5 years, farms in which animals were never or rarely sent to slaughter). In 2008, the use of IFN assay was imposed for premovement controls. From 2009, it was decided to organize a general screening of the Camargue bullfight population. The objective of this first general screening was to maximize the sensitivity to detect as many cases as possible, thus a suspect cutoff was set-up (algorithm shown in **Table 1**). During the second screening program in 2012–2014, this suspect cutoff was eliminated in order to achieve greater specificity. Global screening was systematically organized over two years, starting in September and ending in June. During these periods, half of the farms was screened with IFN assay (cattle over 24 months) each year, while the other half was screened with the skin test (cattle over 12 months). Other actions were also implemented, particularly those concerning the reception conditions of animals in the arenas for reducing risky practices, but also through information and awareness of farmers and veterinarians.

The annual apparent incidence rate increased gradually from 2004 to 2008 (from 3 to 6%) and most of the outbreaks were detected during this period (68 outbreaks detected from 2004 to 2008 vs. 45 in 2009 to 2014). Since the first global screening period (2009–2011), the annual incidence rate shifted from 7% in 2010 to 0.7% in 2014 with only two new outbreaks detected in the field and no detection at the slaughterhouse (**Figure 3**).

Evolution of the Positive Predictive Value

As shown in **Table 5**, the positive predictive value (PPV) of a positive IFN assay relative to the bacteriological confirmation declined significantly between the first (near 20%) and the second screening period (12.5%). This is a normal trend in a context of decreasing prevalence. The number of non-tuberculous mycobacteria cultured from samples of reactor animals increased since 2010, corresponding to this screening pressure and the decrease of PPV (unpublished results). The SICT suspect rate seemed very low which may be linked with the particularly low performances obtained with this test in this specific herd production system.

Evolution of the Specificity Value

The rate of apparent false-positive reactions on bTB-free herds estimated for the 2012–2014 campaign was 0.69% (108/15,532) corresponding to a specificity of 99.31%, thus slightly above the expected rate initially estimated. The available variables that may help to explain the frequency of false-positive reactions were age, gender and breed. In univariable logistic regression analysis, these three variables influenced the occurrence of a positive IFN assay result while in multivariable analysis, only age and gender effects were confirmed (the breed variable effect was coincident with that of sex for "Brave" breed since only cows and bulls were tested for that breed). The proportion of false-positive reactions decreased with the age expressed in years (OR = 0.87, 95% CI: 0.78–0.96, $p = 0.006$) and was higher in bulls (OR = 2.69, 95% CI: 1.52–4.74, $p = 0.001$) than cows.

DISCUSSION

In France, gamma-IFN assay is now regularly used either as a serial test to the skin test where its use is to enhance overall disease detection specificity for screening programs in low prevalence areas (13) or in parallel with the intradermal test in order to increase overall disease detection sensitivity in infected herds (16). This second option was chosen in the Camargue region as the prevalence was high. The IFN assay is now considered to be at least as sensitive as the skin test, but its performance is dependent on a large number of factors, including the antigens used, the treatment of samples, the cutoff values used (17) and

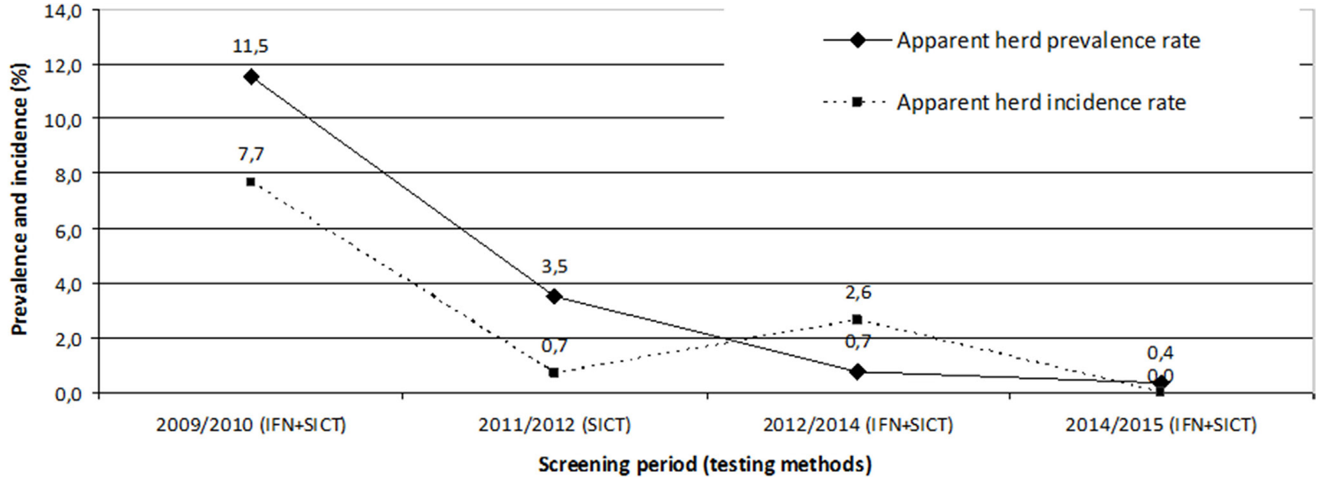

FIGURE 3 | Evolution of the apparent prevalence and incidence rates during the two global screening periods. During these periods, half of the farms was screened with IFN test (cattle over 24 months) each year, while the other half was screened with the skin test (cattle over 12 months), except during the 2011/2012 period when all farms were screened only with the skin test.

TABLE 5 | Results of the two global screening campaigns.

Screening period	Herds tested with SICT	Herds with positive SICT result (%)	Herds tested with IFN assay	Animals sampled[a] for IFN assay (% samples with valid results)	Not valid samples due to NIL criteria	Not valid samples evaluated due to PWM criteria	Herds with positive IFN assay (%)	Herds with positive or doubtful IFN assay (%)	IFN assay positive animals (%)	PPV of IFN assay
2009–2010	232	2 (0.86)	204	14,199 (81.8)	329	2,247	55 (27.0)	102 (50.0)	102 (0.7)	19.6%
2012–2014	274	4 (1.5)	244	17,534 (90.3)	Not available	Not available	58 (23.8)	–	120 (0.7)	12.5%

[a]*Only bovines aged more than 24 months were sampled for IFN testing.*

the interlaboratory reliability (18). The main useful characteristic is that it discloses infection early stages of infection, as early as 14 days post infection (19) but also animals infected with low doses of *M. bovis* (20). One major drawback of the technique could be a lower specificity (21) which can be partially overcome by using recombinant antigens such as ESAT-6 or CFP10 (22) albeit at the cost of reduced sensitivity (23). Since the specificity evaluated with tuberculins was high enough in the Camargue bullfighting cattle population, we decided not to use these antigens in contrast with what is done in other regions in France and elsewhere.

The sensitivity at the individual level seems very low compared to other studies (4, 5, 13, 24). This could be related to the gamma-IFN production failure by bullfight cattle stimulated blood cells compared to other breeds. Moreover, most of the herds were presumed infected for a long time and might contain some anergic animals, which are difficult to detect with *ante-mortem* tests. However, comparisons between studies must be careful as variations such as different cutoff values, the number of animals, the populations, and sample treatment occur between them. Indeed, to obtain accurate sensitivity estimates for a test, all included animals should be slaughtered and tested for confirming infection, whether or not they react to the evaluated test. This is why we only selected data obtained from completely depopulated herds for this study. Furthermore, some meta-analysis estimated a sensitivity of 67% (5, 25) which is a value situated within the CI determined for the sensitivity of the test in bullfights. Finally, even if the individual sensitivity of IFN assay was not optimal, it especially improved the detection at the herd level, because the within-herd prevalence was high (assuming that an average number of three infected animals were present in bTB infected herd). The individual sensitivity of IFN assay remains also much higher than that of the SICT, which is very low in the population of bullfight cattle herds, evaluated around 10%, most probably because of the difficult logistics of administering this test on wild animals. Actually the sensitivity of detection increased more than 30% by using IFN assay in parallel with the SICT (7). As a result of the low sensitivity of SICT in the Camargue region conditions, we observed very low SICT positive rates during the two screening campaigns. Due to the improvement of the epidemiological situation Camargue, a decrease in the proportion of chronically infected animals can be expected. It is therefore possible that the sensitivity of the tests will be higher in this context. However, this lack of individual sensitivity remains an obstacle for the effectiveness of partial cull. This could be counterbalanced by using the IFN assay in parallel with serology, although the first results obtained in Camargue with this latter test have been rather disappointing (26).

No relationship has been observed between (OD PPDB – OD PPDA) and the level of VLs on key organs (e.g., respiratory) of infected animals and thus of the more or less important excretory status of the animals and their risk of infecting other animals in the herd.

Surprisingly, the specificity of IFN assay in this population is higher in comparison with values obtained in other studies (4). These good results allowed a wide approval of the strategy by farmers at early stages of the use of the IFN assay in the field. This high specificity could partly be due to the low production of gamma-IFN by T-cells of this type of animals, but also to the lower exposure of cattle to non-tuberculous mycobacteria in the quite dry environment at the period when animals are sampled for screening (mostly from September to November). Indeed, the proportion of IFN assay false-positive reactions seems closely related to farming area (16, 27). We also observed a higher frequency of non-specific reactions in young animals, which contrasts with results from Gormley et al. (27). Additionally, in Northern Ireland Lahuerta-Marin et al. (16) found increasing risk of false negatives with age. These results could be explained by the presence of higher proportion of natural killer cells in the peripheral blood from young animals (<18 months), which can be a source of innate gamma-IFN production (28). With regards sex, differences of husbandry conditions between males and females could explain the observed more frequent nonspecific results in males. Indeed males' pastures, which are usually of better quality than those used for females, may be situated in more humid zones where non tuberculous mycobacteria leading to these non-specific reactions are frequent (29). An intriguing question for farmers is the significance of non-VL reactors, which gives the impression that the test has a low specificity and makes its results less credible. This impression was somewhat attenuated by the demonstration of confirmed infected animals (using bacterial culture or PCR) without showing lesions at the slaughterhouse (unpublished data) and results from other studies showing the increased bTB risk when keeping an IFN assay positive animal in a herd from an infected area (6, 8).

The analysis of the ROC curve allowed adapting the characteristics of the assay to field situations and to implement an evolutive control strategy. This work was made easier by simplifying the interpretation criteria, using the S/P ratio which takes into account the interplate absorbance variations and allows a better definition of the required level of detectability for the method. However, the decision area is narrow due to numeric values which are very close to the cut off, for making the difference between infected and non-infected populations. This could be compensated by the use of a method with a higher detectability for a better discrimination of the two populations and maybe a compromise between sensitivity and specificity.

The effect of environmental factors on gamma-IFN production and in particular the breed has already been studied by Schiller et al. (9) who showed that blood sample response to PWM was significantly lower in bullfight herds than classical bovine productions. This could be explained by the influence of stress in this particularly wild breed which is rarely manipulated and selected according to its aggressive behavior for entertainment. This may also explain the lower response to tuberculins more generally during testing (including skin testing) in this population. One of the differences might also be differing levels of T cells between bullfighting and conventional cattle but this has not been shown in this study.

While bovine tuberculosis had been enzootic for several years in bullfighting cattle herds, annual incidence dropped below 1% in 2014 after having regularly risen to above 5% since 2005, with a peak at 10% in 2010. Moreover, all new outbreak detections are now carried by *ante-mortem* testing whereas before the use of the IFN assay 80% of bTB cases were discovered at the slaughterhouse (7). This demonstrates that IFN assay testing on infected herds, in parallel with SICT, is a valuable tool in a bTB eradication program, as already observed by Sinclair et al. (30) and Lahuerta-Marin et al. (16). This was due to the good IFN assay performance in this population but also by facilitating factors in the specific context of the Camargue region: low number of herds, circumscribed geographical area, little exchange with other areas (except few animals from Spain), possibly few environmental non-tuberculous mycobacteria and good communication between stakeholders. Indeed, the acceptability of the measures implemented for animal disease control programs (and particularly for bTB) has an influence on their performance, underlining the importance of a participative approach for their success (31). In contrast, some factors may have limited the speed and effectiveness of sanitation: presence of eventual anergic animals, large size herds, keeping old animals in the herd, poor husbandry practices. The possibility of a wildlife reservoir has also been investigated albeit without demonstrating any case until today, although it appears to be a problem for boars and badgers in other parts of France (32). None of the possible wildlife vector species sampled around cattle herds have been found infected (33), however, the infection pressure was sometimes very high in intensively infected herds as demonstrated by the finding of a case of bTB in a horse sharing pastures in close contact to cattle (34).

Global screening will be continued for 5 more years with two approaches. The first one is a random approach (1/5 of the herds required each year) to demonstrate that the level of prevalence is below 1% (assuming a herd detection sensitivity of 90% and a specificity of 100% by combining the *ante* and *post-mortem* diagnostics). It will be expected that no herd is detected infected among the 200 herds randomly selected every year. The second one, a targeted approach, will increase the chances of detecting the last outbreaks or relapse of infection in herds at risk. The targeted herds will be determined according to the following criteria: former outbreaks (remediated by partial cull), epidemiological link to an outbreak, missing or partial screening, limited abattoir monitoring. Moreover, pre-movement controls will remain mandatory with SICT and IFN assay for all herds.

CONCLUSION

The use of gamma-IFN assay in parallel with the single intradermal cervical skin test in a highly infected geographical zone is a valuable tool for a bTB eradication program provided that a good communication between committed stakeholders exists and that the employed screening tests are adapted to the local epidemiological context. In our particular case, the eradication program was also eased by the high specificity of the test in this particular epidemiological context as in other places one of the major issues with IFN assay is the lower specificity, which can have a significant impact when all animals are tested annually. Tuberculosis is now considered under control in the Camargue and Brave races in the Camargue region, particularly through improved early detection in the field. After 10 years of struggle, stakeholders are mobilized and a surveillance system has been designed for the next five years, the objective being to ensure a level of prevalence below 1% before considering progressively lowering of the screening pressure.

ETHICS STATEMENT

The domestic animals used in this study met the definition of "farm animals," which are not currently covered by French regulations (Décret 2013-118 dated the February 1, 2013, from the French Ministry of Agriculture). The owners of the animals were informed of tests performed on their animals, since all samples were collected during compulsory sanitary investigations.

AUTHOR CONTRIBUTIONS

NK conducted the study, was responsible for laboratory work, analyzed data, and drafted the manuscript. M-LB analyzed data and drafted the manuscript. FS and VV participated to the conception of the surveillance program and assisted with data collection. J-LM assisted with data collection. SD conducted the study, analyzed data, and drafted the manuscript.

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Evaluation of the Performance of the IDvet IFN-Gamma Test for Diagnosis of Bovine Tuberculosis in Spain

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In Spain, the national bovine tuberculosis (bTB) eradication program is based on yearly skin testing of every $\geq$6 weeks old animal using the single or comparative tuberculin test and parallel use of the interferon-gamma (IFN-γ) assay as an ancillary diagnostic test in infected herds. There are several versions of the latter. Recently, a new commercial IDvet IFN-γ assay has been authorized for use in the program, but there is limited scientific evidence about its performance in different epidemiological settings. Therefore, two studies to evaluate the performance of the IDvet assay were conducted. In study 1, a concordance analysis between the new IDvet and the Bovigam IFN-γ assay in use in Spain for over 10 years was conducted. In study 2, results from the IDvet assay when applied in tandem with a single intradermal tuberculin (SIT) test were used to evaluate the concordance between both tests and to estimate their sensitivity (Se) and specificity (Sp) using a Bayesian latent-class model. Field data from cattle herds located in Madrid and Castilla y Leon (Spain) were collected. For study 1, herd selection was based on a high expected prevalence of reactors to the IFN-γ assay, while herds were selected at random to estimate Se and Sp of the new IDvet assay in study 2. Agreement between the results obtained with both kits for IFN-γ assay was poor (Kappa = 0.20), and a receiver operating characteristic (ROC) analysis indicated a low Se of the new IDvet relative to the Bovigam in a heavily bTB infected population. The Bayesian latent-class analysis estimated the Se of the IDvet assay to be 36.7% [95% probability posterior interval (PPI) 14.7–78.8%] with estimated Sp close to 100% when the cut-off recommended by the manufacturer (35) was applied. At the alternative cut-off values of 16 and 4, the estimated Se of the IDvet assay increased to 49.0% (PPI: 24.8–94.1%) and 56.0% (PPI: 30.8–96.3%), respectively, while maintaining a high specificity. The results suggest that the new IDvet assay may have lower sensitivity than the Bovigam for diagnosis of bTB in cattle herds in Spain, and that adjusting its cut-off might be considered.

Keywords: bovine tuberculosis, cattle, diagnosis, interferon-gamma, bayesian modeling

INTRODUCTION

Bovine tuberculosis (bTB), caused by members of the *Mycobacterium tuberculosis* complex (mainly *M. bovis*, and to a lesser extent, *M. caprae*) is an important zoonotic disease with a global distribution that has major implications for both animal and human health (1). Implementation of control and eradication programs has led to a significant decrease of the bTB prevalence and to disease eradication in many industrialized countries (1–3). However, eradication efforts have not been uniformly successful, in part because currently available diagnostic tests cannot correctly determine the *M. bovis* infection status of all tested cattle (4). *M. bovis* infection in cattle is usually chronic, can remain subclinical for a long period, and infected cattle can become infectious long before they exhibit clinical signs of bTB (4). When present, the clinical signs of bTB are not pathognomonic. As a result, control strategies have been based on early detection and removal of infected animals from a herd by applying ante-mortem diagnostic tests and routine post-mortem surveillance in abattoir. Hence, determining the accuracy of diagnostic tests for bTB is of paramount importance (5).

Diagnostic tests used for detection of bTB in cattle are mainly based on detecting the cellular mediated immune (CMI) response, which is triggered in the early stages of infection (6). A popular diagnostic technique for this purpose, the single intradermal tuberculin (SIT) test, is based on the inoculation of the bovine purified protein derivative (PPD) in the skin of the neck or in the caudal fold (7). In certain settings the response to the avian PPD inoculated in the other side of the neck is also measured and compared with the bovine response in what is known as the single intradermal comparative cervical tuberculin (SICCT) test, used as the routine screening test in countries as the UK and Ireland. In the last 25 years, an additional diagnostic tool for measuring the CMI response, the interferon-gamma (IFN-γ) release assay, has been increasingly used (8). This test is based on the detection of IFN-γ produced by lymphocytes present in blood samples stimulated with specific antigens (typically bovine and avian PPD); blood samples are then centrifuged after the stimulation and the resulting plasma is analyzed using a sandwich ELISA (8). As an ancillary test to skin test, the IFN-γ assay has led to increased sensitivity when used in infected herds (9), in part because it can identify animals in an earlier infection stage than the SIT test (10). Therefore, in the European Union its ancillary use is recommended in infected herds located in areas with endemic bTB (annex B of Council Directive 64/432/EEC), such as certain areas of Spain (11).

The first commercially available IFN-γ test (Bovigam, Thermo Fisher Scientific, Waltham, MA, USA) (12) has been used in multiple European countries, including Spain, and its performance has been extensively evaluated under field conditions (4, 9, 13–19). Recently, another version of the IFN-γ assay has become commercially available (ID Screen® Ruminant IFN-γ, IDvet, Grabels, France), but due to its recent development there is limited information available about its performance in different epidemiological settings.

The assessment of test accuracy (i.e., sensitivity, Se, and specificity, Sp) is challenging when infection status cannot be determined because, for instance, a perfect reference test does not exist or is too invasive for widespread use, as is the case for bTB, in which the usual reference test, isolation of the causative agent, is costly, slow and has a very limited sensitivity in a large proportion of the infected animals (4). Latent class analysis is a modern approach to estimating the accuracy of diagnostic tests in the absence of a perfect reference test (20, 21). In Bayesian latent class analysis, parameters (e.g., sensitivities, specificities, and prevalences) can be estimated by combining prior knowledge with information from the current data. Bayesian latent class models have been used to estimate the diagnostic performance of bTB tests (5, 22–27), often revealing important disagreements between the prior knowledge and the study data.

In Spain, a national bTB eradication program is in place since the 80's, and since 2006 contemplated the addition of the IFN-γ test as an ancillary test to increase diagnostic sensitivity according to the European and Spanish regulations. During this period the bTB herd prevalence has decreased from >10 to 2.87% in 2016, but the progress in the last decade has been more limited (11). Here, we conducted a study to evaluate the performance of the new commercial IFN-γ test (called thereafter IDvet test) that has been recently authorized for use as part of the Spanish bTB eradication program. Field data were collected to accomplish two goals, namely to conduct a concordance analysis between the new IDvet test and the Bovigam IFN-γ assay (called thereafter Bovigam test), which has been used in Spain for over 10 years (study 1) and to assess the concordance between the IDvet and SIT tests and estimate their Se and Sp in the absence of a gold standard (study 2).

MATERIALS AND METHODS

Study Population

Study 1. Evaluate the IDvet Test Using the Bovigam Test as a Reference

The new IFN-γ assay (ID Screen® Ruminant IFN-g, IDvet, Grabels, France) and the pre-existing IFN-γ test (Bovigam®, Thermo Fisher Scientific, Waltham, MA, USA) were compared using results from 1,181 cattle from 18 herds located in the regions of Madrid [884 cattle (74.9%) from 11 herds] and Castilla y Leon [297 cattle (25.1%) from 7 herds], in central and west-central Spain, respectively. These herds were selected from among those being tested using the IFN-γ assay during the bTB eradication program in 2015 and 2016 based on a high expected prevalence of reactors to the IFN-γ assay. A detailed explanation on the epidemiological situations in which the test is implemented in Spain is available elsewhere (28). The majority of the sample (84.1% of the animals and 88.9% of the herds) was represented by beef herds (993 animals and 16 herds), followed by bullfighting (103 animals and one herd) and dairy herds (85 animals and one herd).

Study 2. Estimate the Se and Sp of the IDvet Test in the Field

Test results were obtained from 8,426 cattle (78 herds) subjected to the SIT test and IDvet test during 2016. Herds were again located in the Madrid [22 herds, 1,550 animals (18.4%)] and Castilla y Leon [56 herds, 6,876 animals (81.6%)] regions, and they were randomly selected among the bTB confirmed infected herds in the two regions in 2016 in which the IFN-γ assay had been implemented according to the Spanish eradication program (11). Beef was again the predominant production type (19 herds and 1,204 animals in Madrid; 41 herds and 4,382 animals in Castilla y Leon), followed by dairy (one herd-44 animals and 10 herds-1676 animals in Madrid and Castilla y Leon, respectively), bullfighting (two herds-300 animals and two herds-339 animals in Madrid and Castilla y Leon, respectively), and mixed farms (3 herds and 481 animals, all in Castilla y Leon).

Diagnostic Tests

Single Cervical Intradermal Tuberculin Test (SIT)

The SIT test was performed according to European and Spanish regulations (RD2611/1996, transposition of annex A of Council Directive 64/432/EEC) by field practitioners in all >6 week-old animals at the herd by intradermal inoculation of 0.1 ml of the official bovine PPD (CZ Veterinaria, Porriño, Spain) in the anterior neck area (29). After 72 h, animals with a >2 mm increase of the skin fold thickness (or with presence of clinical signs at the inoculation site) were considered reactors (severe interpretation) following the Spanish National Bovine Tuberculosis Eradication Program (11) and culled within 15 days.

IFN-γ Assay

Heparinized blood samples were collected from every animal prior to intradermal injection of the PPDs, and delivered to the laboratory in Madrid or Castilla y Leon within 8 h of collection at room temperature, according to the Spanish National Bovine Tuberculosis Eradication Program (11). Stimulation with bovine and avian PPDs (CZ Veterinaria) at a final concentration of 20 μg/ml, and nil antigen phosphate buffer saline (PBS) was carried out as described elsewhere (8). Plasma samples were harvested after centrifugation and stored at−20°C until testing for detection of the IFN-γ with one or both sandwich ELISA evaluated here.

Bovigam® IFN-γ

The Bovigam test was carried out following procedures described elsewhere (12). An animal was considered positive when the optical density (OD) of the aliquot stimulated with bovine PPD minus the OD of the nil (bovine IFN) was ≥0.05 and greater than the OD of the sample stimulated with avian PPD minus the nil (avian IFN), and negative in any other case (11).

ID Screen® Ruminant IFN- γ, IDvet

. The IDvet test was performed according to the manufacturer instructions (IFNG ver 0617 ES). Briefly, samples were divided into three aliquots and incubated with PBS (blank), bovine (activated sample) or avian (control sample) PPD. When OD values>2.5 were obtained in the blank or both the control and activated sample (suggestive of unspecific reactions) samples were diluted 1:5 in order to bring OD levels into the linear region of OD measurement and reanalyzed as indicated by the manufacturer. Results were then transformed into sample-to-positive ratios (S/P):

$$\frac{S}{P} = \left(\frac{OD\ activated\ sample - OD\ control\ sample}{\begin{array}{c} OD\ mean\ kit\ positive\ control - OD\ meankit \\ negative\ control \end{array}} \right) *100$$

Samples were considered positive when the S/P ratio was ≥35 according to the manufacturer instructions. In addition, alternative cut-off points were evaluated, as described below.

Statistical Analysis

Concordance Analysis

Agreement between the qualitative results obtained from both IFN-γ kits (study 1) and between the IDvet test and the SIT test (study 2) was measured using the kappa statistic. Receiver operating characteristic (ROC) curves were used to evaluate the performance of the IDvet test at different cut-offs in relation to the Bovigam (study 1), with Youden's index used to assess optimal cut-off values. The same tests were performed using only the data from beef cattle. These analyses were carried out using SPSS V. 20 (IBM Inc., Chicago, IL, USA) and the "pROC" (30) and "ROCR" (31) packages from in R 3.4 (32).

Latent Class Analysis

We followed the guidelines for reporting of diagnostic accuracy in studies that use Bayesian latent class models (STARD-BLCM) (21).

A Bayesian latent-class model was used to estimate the Se and Sp of the SIT test and the IDvet test in infected herds (study 2), in the absence of a gold standard (33, 34). Samples were considered to belong to two different populations based on the region of origin (Madrid and Castilla y Leon) of herds and the two tests were assumed to be conditionally dependent (33) since both are based on the detection of the cell-mediated immune response (10).

Prior beta distributions for the Se and Sp of the SIT and IDvet tests were built according to reported/estimated values (5, 7, 35–38) (**Table 1**) based on the most likely value and a low 95% credibility interval using ParameterSolver V3.0 (University of Texas MD Anderson Cancer Center). For the IDvet test, larger prior standard deviations were used to reflect the uncertainty in its Se and Sp due to the lack of prior information for this kit and the findings from study 1 (see Results). Covariances between the SIT test and IDvet test for infected and non-infected subpopulations were specified as previously described (39), and these parameters were modeled with uniform prior distributions related to the Se and Sp of the tests (40) (**Supplementary File 1**).

Based on data collected in 2014-2015 from infected herds in Castilla y Leon and Madrid, the common prior distribution for the bTB prevalence was assigned a mode of 5% and a 95th percentile of 20% [specifically, beta(0.99, 13.4)] for both regions.

A sensitivity analysis to evaluate the impact of the priors on the results of the model was conducted using diffuse uniform (0, 1) distributions alternatively for the Se and Sp of each test. Model estimates (posterior medians and 95% posterior

TABLE 1 | Prior estimates of sensitivity and specificity of the SIT test and the IDvet test.

Diagnostic test	Performance measure	Prior estimates		Reference	
		Mode and 5th percentile	Beta distribution	Authors, year	Reported/estimated values
SIT test	Sensitivity (%)	69 (>40)	alpha: 5.65 beta: 2.71	Alvarez et al. (5)	69.4 (40.1–92.2)
				de la Rua-Domenech et al. (4)	83.9 (63.2–100)
				Monaghan et al. (7)	68–95
				Wood et al. (37)	68.1
				Wood et al. (36)	65.6 (56.6–73.9)
	Specificity (%)	95 (>75)	alpha: 8.65 beta: 0.73	Alvarez et al. (5)	99.4 (98.7–99.9)
				de la Rua-Domenech et al. (4)	96.8 (75.5–99.0)
				Monaghan et al. (7)	96–99
				Wood et al. (37)	96.7
IDvet test	Sensitivity (%)	90 (>50)	alpha: 3.35 beta: 0.62	Alvarez et al. (5)	89.3 (77.5–97.2)
				de la Rua-Domenech et al. (4)	87.6 (73.0–100)
				Gormley et al. (35)	88
				Nunez-Garcia et al. (38)	67 (49–82)
				Wood et al. (37)	81.8
				Wood et al. (36)	80.8 (72.8–87.3)
	Specificity (%)	90 (>80)	alpha: 33.1 beta: 3.97	Alvarez et al. (5)	85.7 (84.4–87.6)
				de la Rua-Domenech et al. (4)	96.6 (85.0–99.6)
				Gormley et al. (35)	95
				Nunez-Garcia et al. (38)	98 (96–99)
				Wood et al. (37)	99.1
				Wood et al. (36)	90

probability intervals, PPI) were compared with those obtained using the informative priors. Models were also run only using the data from beef cattle as an additional sensitivity analysis. In addition, alternative cut-offs for the IDvet test (S/P ratio= 16 and 4) were evaluated.

Three Markov chain Monte Carlo runs were implemented in order to visually assess convergence and mixing of the chains. Convergence was also assessed using the Gelman-Rubin diagnostic (41). Posterior inference was based on 5,000 iterations after discarding the first 2,500 as burn-in. Autocorrelation was eliminated through thinning the chains by collecting one in 10 consecutive samples. All analyses were conducted using OpenBUGS V. 3.2.3 (42) called through R, version 3.4 (32) using the "R2OpenBUGS" package (43). The OpenBUGS code is provided as supplementary material (**Supplementary File 1**).

RESULTS

Concordance Analysis

The number of reactors to each test in both studies are shown in **Table 2**. Agreement between the results obtained with the Bovigam and the IDvet tests (study 1) was poor (Kappa = 0.20), with most of the discordant results being positive in the former and negative in the new test (**Table 2**). The ROC analysis indicated that at the manufacturer recommended cut-off of 35 for the IDvet, Se relative to the Bovigam test was low (Se = 15.1% and Sp = 99.9%). Better agreement was achieved when lower cut-offs (16 and 4) were used (Kappa = 0.52 and 0.71, with Se = 38.8 and 65.5, respectively, and Sp > 98.5%). A high value of 92.5% (95% CI: 89.6–95.3%) was obtained for the AUC (**Figure 1**). The optimal cut-off point according to Youden's index in relation to the Bovigam was 1.3 (yielding Se = 81.3% and Sp = 94.2%). When only beef cattle were considered, very similar Kappa values were obtained regardless of the cut-off used (0.20, 0.52, and 0.70 for cut-offs 35, 16, and 4, respectively), with also a very high AUC (90.9%) and a similar optimal cut-off point (1.7).

Agreement between the IDvet test and the SIT test (study 2) was slightly higher (Kappa = 0.34), although important differences were still observed (**Table 2**). When the results were analyzed separately by production type, the agreement was higher for beef cattle (Kappa = 0.43) and close to zero for dairy and mixed herds (Kappa = 0.08 and −0.01, respectively), whereas it could not be calculated for bullfighting cattle due to the lack of SIT reactors. For cut-off values of 16 and 4, the agreement between tests decreased to Kappa values of 0.27 and 0.14, respectively (**Supplementary Table 1**).

TABLE 2 | Number of reactors to the IDvet test and to the Bovigam test performed on 1,181 cattle (study 1), and number of reactors to the IDvet test and to the SIT test (severe interpretation) performed on 8,426 cattle (study 2) from Madrid and Castilla y Leon (Spain).

				IDvet test (35)		**Total**	
				Negatives	**Positives**		
Study 1		Bovigam test (0.05)	Negatives	1,087	1	1,181	
			Positives	82	11		
Study 2	Madrid	SIT test (severe int)	Negatives	1,509	8	1,548	8,426
			Positives	22	9		
	Castilla y Leon	SIT test (severe int)	Negatives	6,738	76	6,878	
			Positives	35	29		

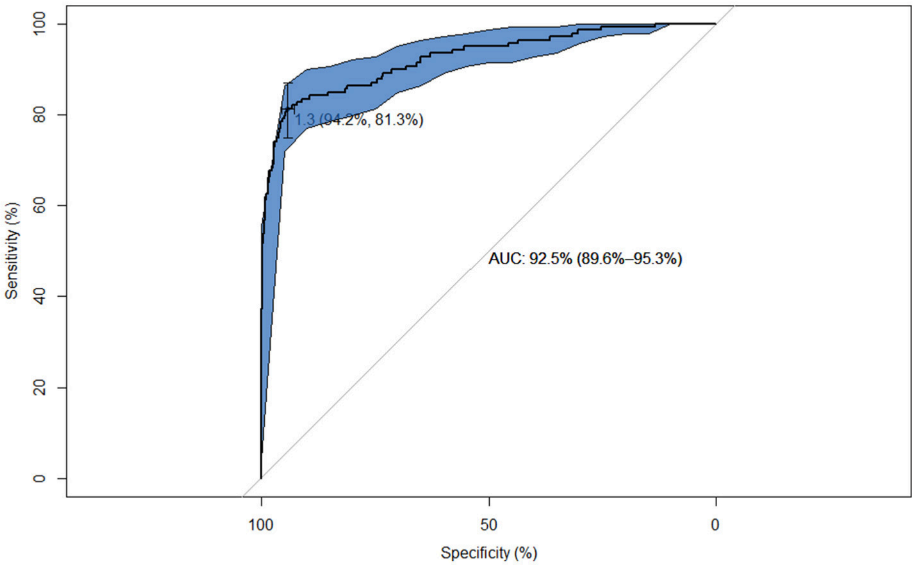

FIGURE 1 | ROC curve of the performance of the IDvet test relative to the Bovigam test on 1,181 samples from animals analyzed with both tests. The cross points the location for the optimal cut-off for maximization of the Youden's index for the IDvet test (1.3) along with the Sp and Se at this cut-off.

Latent Class Analysis

The Bayesian latent class analysis yielded posterior estimates (median and 95% PPI) for the IDvet test of Se = 36.7% (14.7–78.8%), with very high Sp (**Table 3**). The posterior distribution of Se for the IDvet test was shifted below its prior distribution (**Supplementary Figure 1**), whereas the opposite was true for its Sp. In contrast, the posterior estimates of the performance of the SIT test were largely in agreement with the prior information.

Conditional dependence between the SIT test and the IDvet test was estimated to be very low in both the infected (correlation coefficient of positive results: −0.002, 95% PPI −0.13 to 0.09) and non-infected (correlation coefficient of negative results: 0.002, 95% PPI 0.00 to 0.004) populations, suggesting a possible conditional independence between the results of each test. Note that, in general, conditional independence occurs when Sp's are close to 100%.

A sensitivity analysis (**Supplementary Table 2**) resulted in no major changes to the posterior distribution of Se and Sp for both tests (the magnitudes of all percent differences were < 8%), except when a uniform prior was used for the Se of the IDvet test assay where a percent decrease of 41% was observed for the posterior median (36.7 vs. 21.6). Prevalence estimates were similar across analyses that used different priors (e.g., overlapping 95% PPI's), and were 2–3 times higher in the region of Madrid than in Castilla y Leon. When only values from beef herds were used in the analysis, the 95% PPI for the sensitivity of the IDvet test was similar (19–88%) but a higher median value was found (60.9%), while changes in the posterior estimates for the sensitivity of the SIT test and the specificities of both techniques were small (12% of median estimates). Similar estimates were obtained when using a burn-in of 20,000 posterior iterates and 50,000 total iterates.

At cut-off points of 16 and 4 for the interpretation of the IDvet test, the estimated Se of the IFN-γ assay increased (as expected) to 49.0 and 56.0%, respectively, while maintaining a high specificity (**Table 3**). The posterior estimates for the performance of the SIT test and the bTB prevalence were not affected by the cut-off applied in the IDvet test (**Table 3**).

TABLE 3 | Posterior estimates (median and 95% posterior probability interval) for sensitivity, specificity and the mean of the prevalence distribution (%) obtained for the combination of IDvet test and SIT test on 8,426 cattle from Madrid and Castilla y Leon (Spain), for different prior distributions and IDvet alternative cut-off points.

Model	Priors	Diagnostic test	Sensitivity	Specificity	Prevalence	
					Madrid	Castilla y leon
Original	Original priors (Table 1)	SIT test	78.68 (49.28–95.00)	99.53 (98.95–99.98)	1.85 (0.51–3.34)	0.64 (0.04–1.43)
		IDvet 35[a]	36.69 (14.66–78.81)	98.78 (98.41–99.18)		
Alternative cut-off points for IDvet	Original priors (Table 1)	SIT test	76.59 (47.06–94.51)	99.55 (98.95–99.99)	1.90 (0.59–3.50)	0.69 (0.04–1.56)
		IDvet 16[b]	49.03 (24.85–94.13)	97.86 (97.38–98.36)		
	Original priors (Table 1)	SIT test	76.41 (46.11–94.41)	99.61 (98.99–99.99)	1.96 (0.70–3.62)	0.78 (0.06–1.71)
		IDvet 4[c]	55.98 (30.76–96.34)	93.89 (93.25–94.57)		

[a] *Cut-off recommended by the manufacturer.*
[b] *Cut-off for interpretation = 16.*
[c] *Cut-off for interpretation = 4.*

There was no evidence for lack of convergence of the Markov chains used to simulate from the posterior distribution (**Supplementary Figure 2**), as indicated by graphical assessment of the chains and the Gelman-Rubin statistic <1.001 for all parameters.

DISCUSSION

Multiple factors related with the host, the pathogen and the environment may affect the performance of a diagnostic test, and, therefore, extrapolation of results obtained in different epidemiological settings may lead to biased and misleading conclusions. For this reason, in the studies presented here we aimed at estimating, by using a variety of analytical approaches, the performance of the new commercial IDvet test under field conditions in bTB-infected herds of Spain. Even though both Bovigam and IDvet tests share the same target (IFN-γ produced by lymphocytes stimulated with bovine PPD), when the results obtained in both assays were compared (study 1), the agreement was poor (Kappa = 0.20). In addition, the Se of the IDvet test relative to the Bovigam test in a population formed by heavily bTB infected cattle herds was very low (15.1%), although given the limited specificity of the Bovigam (with estimates between 84.4 and 99.6%) (4, 5, 35–38) a proportion of false positive reactions to this test could be expected, and therefore this figure could be an underestimation of its true sensitivity. Nevertheless, the high AUC values obtained when the quantitative readings obtained in the IDvet test were compared with the qualitative response in the Bovigam test (92.5, 90.9% when only beef animals were considered) suggested that there was in fact a close relationship between the response measured in both tests. Moreover, the ROC analysis suggested that decreasing the cut-off in the IDvet test (thus requiring a lower difference between the response recorded in the sample stimulated with bovine PPD compared with the avian PPD to define an animal as a reactor) could lead to a substantial increase in the agreement between both tests (**Figure 1**), despite the different calculations used to define the positive status (see Material and Methods). In addition to the comparison of the responses after the *in-vitro* stimulation with avian and bovine PPD to define a reactor considered in both the Bovigam and IDvet tests, the protocol of the IDvet test includes an extra step (dilution of samples) to avoid false positive reactions due to sensitization with other cross-reacting microorganisms such as *Mycobacterium avium* subsp. *paratuberculosis*, that are known to affect the performance of IFN-γ based assays in domestic ruminants (44). Although this could have theoretically contributed to the observed differences between both tests, less than 1% of the samples included in study 1 had to be diluted because of high readings in the control and activated samples, and therefore this was not a major source of variation in our study.

A limited agreement between the IDvet test and the SIT test (Kappa= 0.34; study 2) was also observed. This is consistent with a field scenario in which animals are subjected to frequent skin testing and SIT-reactors have been already removed when the IFN-γ assay is introduced for the first time in infected herds (7, 10, 12, 45–47). This limited agreement between the SIT test and the IDvet test, coupled with the low posterior estimates for the codependence terms obtained in the latent class analysis (correlation coefficient range: −0.13 to 0.09 and 0.00 to 0.004, for infected and non-infected animals, respectively) are in agreement with estimates obtained for the Bovigam test (5), and reinforce the potential usefulness of the application of IFN-γ based assays in parallel to the SIT test to maximize the diagnostic sensitivity (45, 48, 49). In the case of the IDvet test, however, results from the Bayesian latent class model (study 2) confirmed the apparent lower Se of the IDvet test compared with the Bovigam test observed in study 1, since posterior estimates of the IDvet Se were significantly lower than those recently estimated for the Bovigam test using a similar—though wider—prior (36.7, 95% PPI 14.7–78.8, vs. 89.3, 95% PPI 77.5–97.2 estimated previously for the Bovigam test) (5). The very large uncertainty in the IDvet Se posterior estimates may be also influenced by the small number of IDvet reactors in the sample (**Table 2**). In contrast, the results obtained for the SIT test in this study and the previous one were comparable, with higher median estimates obtained here but very similar PPI (78.7, 95% PPI 49.3–95.0, compared with 69.4, 95% PPI 40.1–92.2) (5). When only data from beef herds were analyzed, a higher posterior median value for Se of the IDvet was obtained (60% compared with 37% when analyzing all animals) although the 95% PPI was similar, and values were nevertheless still lower than previous estimates obtained for the Bovigam test as well as the priors used in the analysis. In fact, the sensitivity analysis revealed a conflict between the prior information used for the Se of the IDvet test in this study and the data, because when a non-informative prior was used the posterior estimates for its Se were even lower compared to the use of the informative prior using all animals (**Supplementary Table 2**) or only beef cattle (data not shown). This confirms the hypothesis that the IDvet test had a significantly lower Se compared to the early test (**Table 3**).

Our results contradict those obtained in experimental studies carried out in France, Belgium and Mexico, in which high values of Se (88.3, 95% CI: 81.1–95.5) and Sp (99.0, 95% CI: 98.4–99.6) were reported (50). Those trials however involved a relatively limited number of animals ($n = 77$) already positive to either PCR, culture or the SIT test, thus representing a potentially biased subpopulation of all infected animals present in an infected herd, what could lead to an overestimation of the sensitivity of the test (51).

Similar to the observation of study 1, results from study 2 suggest that a decrease in the cut-off value in the interpretation of the IDvet test could substantially increase the sensitivity of the test (see **Table 3** and **Figure 1**), in agreement with a previous study (47), while maintaining a high specificity (median posterior values $>93.9\%$). Given that in the EU the Bovigam test is applied to maximize the number of infected animals detected, this may be a reasonable approach when samples from infected herds are analyzed. However, further validation of this hypothesis may be required.

Here, a two-population approach was used because infected herds located in the same region were expected to present similar prevalence levels, as reflected in the official bTB reports for the previous years (52). In fact, the ratio between the estimated bTB prevalence in each region (Madrid/Castilla y Leon; **Table 3**) and the reported in 2016 is similar, 2.9 and 2.6, respectively, even though posterior estimates were below the most likely prior values.

The use of a latent class analysis allowed overcoming the limitations of the gold-standard approach, since all available reference tests for bovine tuberculosis have low sensitivity particularly in early stages of infection, when its detection is most critical (4, 53). However, for the comparison between the performance of the IDvet and Bovigam tests a latent class model was not used because the population had been selected based on an expected high prevalence of infection. Hence, the assessed population was not representative of the field situation, so that it only allowed a comparison of the performance of the tests in that very specific context. Still, results obtained in that potentially biased population suggested that the IDvet test could have a lower Se compared with the Bovigam test. For study 2, animals were selected randomly from infected herds in which the SIT test and the IDvet were being implemented routinely, and hence were considered truly representative of the situation in which the performance of the test was intended to be determined.

In conclusion, our results suggest that the IDvet test may have a lower sensitivity than the Bovigam for diagnosis of bTB in cattle herds in Spain when the cut-off recommended by the manufacturer is applied. Decreasing the cut-off may result in a substantial increase of the sensitivity while maintaining a high specificity, although generalization of that result would require verification under alternative epidemiological settings and conditions.

ETHICS STATEMENT

Animals included in this study were only subjected to the regular tests performed in the framework of the Spanish official program for eradication of bovine tuberculosis, and therefore no experimental research on animals was conducted in this study.

AUTHOR CONTRIBUTIONS

MdC, AB, PP, and JA conceived and performed the statistical analyses. MdC, and JA drafted the manuscript. JN, EP, AG, JS, RD, and OM participated in the generation, collection and curation of the data, and collaborated in interpretation of the results. AP, LdJ, and JA designed the study and coordinated the work. All authors revised critically the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2018.00229/full#supplementary-material

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A Review of African Swine Fever and the Potential for Introduction into the United States and the Possibility of Subsequent Establishment in Feral Swine and Native Ticks

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African swine fever (ASF) is caused by African swine fever virus (ASFV), which can cause substantial morbidity and mortality events in swine. The virus can be transmitted *via* direct and indirect contacts with infected swine, their products, or competent vector species, especially *Ornithodoros* ticks. Africa and much of Eastern Europe are endemic for ASF; a viral introduction to countries that are currently ASF free could have severe economic consequences due to the loss of production from infected animals and the trade restrictions that would likely be imposed as a result of an outbreak. We identified vulnerabilities that could lead to ASFV introduction or persistence in the United States or other ASF-free regions. Both legal and illegal movements of live animals, as well as the importation of animal products, byproducts, and animal feed, pose a risk of virus introduction. Each route is described, and current regulations designed to prevent ASFV and other pathogens from entering the United States are outlined. Furthermore, existing ASFV research gaps are highlighted. Laboratory experiments to evaluate multiple species of *Ornithodoros* ticks that have yet to be characterized would be useful to understand vector competence, host preferences, and distribution of competent soft tick vectors in relation to high pig production areas as well as regions with high feral swine (wild boar or similar) densities. Knowledge relative to antigenic viral proteins that contribute to host response and determination of immune mechanisms that lead to protection are foundational in the quest for a vaccine. Finally, sampling of illegally imported and confiscated wild suid products for ASFV could shed light on the types of products being imported and provide a more informed perspective relative to the risk of ASFV importation.

Keywords: African swine fever, viral introduction, emergency preparedness, surveillance, domestic pigs, feral swine

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KEY POINTS

- African swine fever (ASF) is caused by African swine fever virus (ASFV), which is the only known arthropod-borne DNA virus.
- Currently, ASF is not present in the United States, but it is a high consequence, foreign, notifiable swine disease, and the economic consequences associated with an introduction could be catastrophic.

- The virus is endemic in many parts of the world, including most of sub-Saharan Africa, the island of Sardinia, and parts of the Caucasus region and Eastern Europe.
- The routes of concern for the introduction of ASF into the United States are the legal or illegal importation of live animals (or their products) or a bioterrorism event.
- Introduction or spillover events from domestic swine into feral swine populations would substantially complicate the eradication process as would infection in native *Ornithodoros* tick species.
- Currently, there is no ASF vaccine approved for use.
- Future research should involve (1) laboratory feeding experiments to evaluate multiple species of North American *Ornithodoros* ticks that have yet to be characterized, (2) expanded analyses to explore the distribution and host preferences of competent soft tick vectors in relation to high pig production regions as well as high densities of feral swine, (3) characterization of antigenic viral proteins that contribute to a host immune response and determination of immune mechanisms that lead to protection, (4) expanding classical swine fever slaughter surveillance and random blood collections to include screening for ASFV, in the event of an increased risk of viral introduction, and (5) sampling of wild suid products that were illegally imported and confiscated for the detection of ASFV.

INTRODUCTION

African swine fever (ASF), first described in Africa in the 1920s, is caused by African swine fever virus (ASFV). Infection results in high morbidity and mortality in swine and has drastic implications for global domestic swine production (1). This disease is reportable to the World Organisation for Animal Health (OIE), and viral infection in swine can have severe economic consequences associated with production losses, trade limitations, and eradication programs (2). Currently, the United States is ASFV free. This article outlines what is known about ASFV and aims to describe existing gaps in knowledge. Finally, a summary of US vulnerabilities for viral introduction and persistence is provided. Countries with endemic ASF in domestic swine likely have a different set of challenges compared to the United States and other ASF-free regions and may benefit from the development of disease control methods that are commonly used, such as enforceable quarantine zones, diagnostic assays, and culling of infected animals. However, eradicating a disease that is established in a wild population, such as wild boar, is highly complex and depends on a deep understanding of the disease ecology within a specific epidemiological context.

VIRUS DESCRIPTION

African swine fever virus is a large, enveloped virus in the Asfarviridae family that causes hemorrhagic diseases in domestic pigs and several species of wild swine (3). The virus is a genetically complex double-stranded DNA virus that contains a series of genes used for virulence, immune evasion, and cell process modulation (4). Twenty-three genotypes have been described based on the partial sequences of the p72 gene (5, 6). All 23 genotypes are present in Africa, whereas only genotypes I and II have been found outside of that continent. The virus primarily infects cells of the mononuclear phagocytic system (monocytes and macrophages) and replicates in the cytoplasm. The endoplasmic reticulum is believed to play an important role in viral assembly and ASFV envelopment (3, 4, 7).

TRANSMISSION AND CLINICAL DISEASE

African swine fever virus can be transmitted *via* direct contact with infected animals, either domestic swine or wild boar, indirect contact *via* contaminated fomites or uncooked meat from infected animals, or through arthropod vectors, particularly soft tick species in the genus *Ornithodoros* (1, 8). The virus is highly stable in proteinaceous environments and quite resistant to high temperatures, requiring 60°C for 20 min for inactivation. Domestic pig-to-pig transmission is thought to occur primarily through infection of the upper respiratory tract as domestic pigs have been shown to shed infectious virus from all secretions and excretions, with particularly high concentrations in the oronasal fluid. ASFV is very persistent in blood and tissues after death; thus, an opportune vehicle to transmit infection is feeding uncooked swill. Environmental contamination following necropsies, pig fights that result in bloodshed, or bloody diarrhea following infection may also serve as a route for new infections. Airborne transmission has been demonstrated in a laboratory setting where animals were densely housed (9).

Ornithodoros ticks have also been found to serve as biological vectors for ASFV, with documented transstadial, transovarial, and sexual transmission (10). In some regions of Africa, ASFV cycles between juvenile common warthogs and *Ornithodoros porcinus porcinus* ticks, which inhabit their burrows. In Europe, *Ornithodoros erraticus* have been found to vector ASFV and were involved in the disease epidemiology on the Iberian Peninsula between the 1960s and 1990s; however, *O. erraticus* are not involved in the current ASF scenario in Eastern Europe and Sardinia. Biting flies, particularly *Stomoxys spp*, have been found to be capable of mechanical transmission for ASFV (11).

Domestic swine, Eurasian wild boar, warthogs, bushpigs, and giant forest hogs are all susceptible to infection with ASFV; however, warthogs and bushpigs generally develop asymptomatic infections and serve as a viral reservoir, in what is often referred to as the sylvatic cycle (12). Peccaries are thought to be resistant to infection. Neonatal warthogs develop a sufficient viremia to infect new ticks but do not develop clinical disease, and adult warthogs are impervious to the pathogenic effects of the virus although the virus can be often extracted from their lymph nodes (13). ASFV has a predilection for lymph nodes near the head, and warthogs remain infected for life (14). Neither horizontal nor vertical transmission has been documented in warthogs, with soft ticks serving as the sole route of transmission between infected and susceptible warthogs (1, 15, 16). Sexual transmission is not indicated in warthogs; however, the virus is found in genital secretions and so it remains a possibility (1). To date, there has been no conclusive data suggesting a long-term carrier state; however, a survey conducted in central Kenya found ASFV [detected *via* polymerase chain reaction (PCR)] in asymptomatic domestic swine and warthogs (17).

Experimental infection of bushpigs demonstrated the absence of clinical disease despite a robust viremia lasting 35–91 days following infection with ASFV, which was sufficient to infect *O. porcinus porcinus* ticks that fed on the bushpigs during their viremic period (18). Infected ticks were able to transmit ASFV to naive domestic pigs. Certain strains of ASFV in experimentally inoculated bushpigs were capable of transmission *via* direct contact with domestic swine, whereas other strains were not. Infected domestic swine were not able to transmit the infection to in-contact bushpigs, suggesting that they are not as readily infected *via* direct contact compared to domestic swine.

Clinical disease can manifest in multiple ways ranging from death with no signs (peracute, mortality ~100%) to an asymptomatic infection; however, most isolates of ASFV cause acute hemorrhagic fever in domestic pigs and result in mortality nearing 100% (1, 19). All age groups of pigs have been found to be equally susceptible to ASFV infection, as opposed to classical swine fever virus (CSFV) where young pigs are much more susceptible (20). Acute infections are caused by highly virulent strains and are typically characterized by a high fever, anorexia, lethargy, weakness, recumbancy, diarrhea and/or constipation, abdominal pain, hemorrhagic signs, respiratory distress, nasal and conjunctival discharge, and abortions in pregnant females. Death often occurs within 7–10 days after the onset of clinical signs. Depending on the virulence of the ASFV strain, acute infections are often the predominant form at the beginning of an outbreak in disease-free regions; however, once established, the disease often progresses to subacute clinical forms that can be sustained over time (20). It is important to note that this pattern has been previously observed although it is not the established truth. Moderately virulent strains result in subacute infection (often with high mortality in young animals and much lower mortality in older animals) where the clinical signs often include abortion, fever, and transient hemorrhaging with death or recovery occurring within 3–4 weeks. Chronic infections (mortality is very low) are characterized by intermittent or low fever, appetite loss, and depression and, in some instances, result in a fatal infection. Animals that remain persistently infected for months, such as survivors or subclinically or chronically infected pigs, may play a role in disease persistence in endemic regions. Also, it has been speculated that they may contribute to sporadic outbreaks and introductions to ASFV-free zones (20).

Domestic pigs are most infectious during the incubation period and may shed virus for >48 hours prior to the presentation of clinical disease (1). Recovered pigs may shed infectious virus for 1 month after the disappearance of clinical signs. Pig populations that have developed a degree of resistance to the virus are better able to maintain and circulate ASFV as the disease is not self-limiting (21, 22).

GLOBAL DISTRIBUTION AND EPIDEMIOLOGY

African swine fever was restricted to the African continent from its first description in the 1920s until 1957 when an outbreak was reported in Portugal (23). This outbreak was effectively controlled and eradicated until a second recurrence in 1960, which resulted in ASF being endemic in the Iberian Peninsula (Portugal and Spain) until 1995. During the 1970s and 1980s, ASF emerged in several parts of the world, including other European countries (the Netherlands, Italy, France, and Belgium) and the Americas (Cuba, the Dominican Republic, Haiti, and Brazil) (16). This global spread is thought to be due largely to feeding domestic animals contaminated pork products that entered each region *via* international air and seaports. After establishment in domestic swine herds, infected pigs and pork products became the primary source of infection.

On the basis of the ability of ASFV to be transmitted *via* direct and indirect contacts and through an arthropod vector, Sánchez-Vizcaíno et al. (23) outline five epidemiological scenarios and examples of regions where each type of situation occurred, depending on the existence of wild reservoirs and competent tick vectors. The first scenario involves the original natural cycle and describes transmission in Eastern and Southern Africa in which a sylvatic cycle occurs between wild suids, especially warthogs, and *O. porcinus porcinus* ticks. Spillover into domestic swine is typically associated with infected tick bites or ingestion of contaminated warthog meat. A second scenario describes transmission occurring primarily through direct contact between infected and susceptible domestic pigs and indirect contact between susceptible pigs and contaminated pork products. Ticks are not involved. This describes ASF dynamics in many West African countries. Third, as was observed on the Iberian Peninsula, both wild boar and domestic pigs were infected, and transmission primarily occurred *via* direct contact between infected and susceptible animals and *via* the consumption of infected meat. *O. erraticus* contributed to transmission in outdoor production systems; however, this tick species is only capable of transstadial transmission but not transovarial, and therefore, their vector competency is lower than *O. porcinus porcinus*. Between 1968 and 1980 in Central and South America, a fourth scenario was observed in which the disease only affected domestic pigs and neither wild suids nor ticks were involved. This scenario is much easier to eradicate compared to all others. The fifth scenario occurred in Russia and the trans-Caucasian countries where both wild boar and domestic pigs were involved in transmission but ticks were not found to be involved. Most outbreaks were found in domestic pigs and were linked to movements of affected animals and their products. Understanding the epidemiology of disease, specific to the region of interest, is crucial as the development of emergency control and eradication plans are dependent upon disease transmission patterns and risk factors.

IMMUNE RESPONSE TO ASFV

Infection with ASFV is characterized by severe immunosuppression and apoptosis, primarily replicating in monocytes and macrophages, and is believed to enter cells *via* receptor-mediated endocytosis (24, 25). Activated macrophages release IL-1, IL-6, and TNFα, which all contribute to acute-phase reactions, inflammation, activation of endothelial cells, and apoptosis (26). Similar cell tropism and organ distribution have been observed across all strains of ASFV; however, more severe tissue destruction is associated with strains of increasing virulence. Neutralizing antibodies and $CD8^+$ T cells and natural killer cells are believed to play an important role in the host immune response against ASFV. *In vitro* experiments suggest that some cellular mechanisms are regulated by ASFV *via* the encoding of specific regulatory genes and by interaction with viral and cellular proteins; however, most cellular functions altered after infection remain unknown (25). Proteomic evaluation demonstrated that ASFV shuts down the majority of protein synthesis, affecting approximately 65% of cellular proteins. Specific cellular proteins were found to be overexpressed after ASFV infection, and most were involved in redox homeostasis, programmed cell death, and coagulation.

The role of neutralizing antibodies has been evaluated, and results are variable. Passive transfer experiments performed in domestic swine by Onisk et al. (27) found that 85% of pigs that received the anti-ASFV IgG survived challenge compared to 0% of unimmunized controls. Treated animals underwent transient fever but otherwise appeared clinically normal. Viremia in pigs that received the antibody transfer was found to be delayed and reduced.

Viral neutralizing epitopes were identified on three viral capsid proteins—p30, p54, and p72—and domestic swine were immunized using a baculovirus expressing each of these proteins prior to challenge with a homologous virus (28). Immunized animals were found to have a 2-day delay in the onset of clinical disease and a reduced viremia, but there was no effect on disease development, progression, or outcome. The authors concluded that neutralizing antibodies to these ASFV proteins are insufficient for antibody-mediated protection.

The findings by Onisk et al. (27) and Neilan et al. (28) appear to be in stark contrast to one another, and differences are believed to be due in part to variations in virus strains (and subsequently, virulence) and challenge doses. The relative role of neutralizing antibodies may be dependent on the virulence of the ASFV isolate used, with neutralizing antibodies providing a more protective response against less virulent strains. However, large differences in study design between the two experiments make comparison very difficult as Onisk and colleagues used passive transfer, which is a mixture of numerous antibodies compared to Neilan et al. (28) who immunized swine with specific epitopes. Much further characterization of the role of antibodies is required.

Interestingly, in northern Mozambique, a region endemic for ASF, a population of domestic pigs were found to have high levels of circulating antibodies to ASFV (29). A group of pigs from this population were collected and their offspring were evaluated through experimental ASFV challenge for the heritability of this resistance to ASF. The offspring were acutely susceptible to challenge with a virulent strain of ASFV, suggesting that the ASFV resistance in the parental population was not heritable. The authors hypothesize that this observed resistance is resultant from (1) prior exposure to a less virulent but antigenically similar field virus prior to exposure to a virulent strain, (2) maternal antibody resistance, (3) exposure to small quantities of infectivity that may result in a sublethal infection that confers immunity to a subsequent challenge (29).

VECTOR BIOLOGY

As stated previously, several soft tick species have been implicated in ASFV transmission in endemic and outbreak regions. It is important to note that the taxonomy of the *O. porcinus porcinus* ticks has changed over time on the basis of both morphological and biological characteristics. Prior to 1979, *O. porcinus porcinus* ticks were often referred to as *Ornithodoros moubata porcinus* or simply *Ornithodoros moubata*. The *O. moubata* complex was then split into four distinct species, including *Ornithodoros porcinus*, which was further divided into *O. porcinus porcinus* and *Ornithodoros porcinus domesticus* (30). However, in much of the current literature *O. moubata* and *O. porcinus porcinus* are used interchangeably.

Plowright et al. (31) demonstrated that *O. porcinus porcinus* could be infected with multiple strains of ASFV and develop a persistent infection although the minimum infective dose varied between strains. Furthermore, experimental challenges confirmed that infected ticks could readily transmit ASFV to domestic pigs. Later studies determined that *O. porcinus porcinus* could transmit the infection transovarially; however, there was tremendous variability between egg batches from different ticks and between successive egg batches from the same tick (32). Interestingly, it was found that the prevalence of infected eggs increases after each successive infected blood meal. *O. porcinus porcinus* ticks have been found to maintain high ASFV titers over time, and no cytopathological lesions have been observed in these ticks, suggesting that *O. porcinus porcinus* ticks and ASFV are co-adapted and likely represent a co-evolved system (33). ASFV follows a common virus–tick pathway upon ingestion of an infective blood meal, viral replication in the midgut, escape into the hemocoel, and infection of the coxal and salivary glands (34).

While *O. porcinus porcinus* ticks are involved in the sylvatic cycle of ASFV with warthogs, other *Ornithodoros* species are capable of transmitting infection. *O. erraticus*, found in the Mediterranean and Middle East, was implicated in ASFV transmission, and longitudinal monitoring found higher titers over time, which is suggestive of viral replication (35).

Several *Ornithodoros* species are indigenous to North and Central America, as well as the Caribbean. Experimental infections in *Ornithodoros coriaceus, Ornithodoros parkeri,* and *Ornithodoros turicata* (Americas) and *Ornithodoros puertoricensis* (Caribbean) have been performed with multiple ASFV isolates (33). *O. coriaceus* ticks were infected with five different isolates of ASFV, and viral persistence was found to range between 77 and 463 days, with transmission to domestic swine demonstrated at 502 days postinfection with the DR II strain. *O. parkeri* were challenged with one strain of ASFV and found to be infected for 46 days postinfection, whereas *O. turicata* were found to be infected for 23 days postinfection. *O. puertoricensis* ticks were infected with a single isolate of ASFV and demonstrated transmission to domestic pigs at 239 days postinfection.

In addition, transovarial and transstadial transmission were demonstrated; however, transmission rates decreased with each molt. Importantly, despite the presence of *O. puertoricensis* in Haiti and the Dominican Republic, it did not appear to complicate ASFV eradication in 1978, likely due to the lack of contact between infected pigs and ticks (36). For a comprehensive overview of vector competency with different isolates of ASFV, please see the study by Kleiboeker and Scoles (33).

ASF AND EUROPEAN SPREAD

African swine fever is endemic in much of Africa but was first introduced outside of the African continent into Portugal in 1957 and again in 1960 (37). The most likely route of introduction was *via* ASFV-contaminated swill as this is a very effective means of spreading the virus over long distances. The disease was first discovered in swill-fed swine near the Lisbon airport, which furthers the hypothesis that the virus was introduced *via* this route. ASFV then spread to Spain and remained endemic on the Iberian Peninsula until the 1990s. Once introduced, ASF is especially difficult to eradicate due to the presence of wildlife reservoirs and competent soft tick vectors, the lack of a vaccine, and insufficient laboratory support for rapid and accurate diagnosis (38). It is important to note that the role of wild boar in the maintenance and transmission of ASFV varies significantly based on disease epidemiology and ecology. Wild boars were involved to some extent in the epidemiology of ASF on the Iberian Peninsula, but they did not appear to complicate control measures, which is in strict contrast to the current scenario in Eastern Europe where ASF has become established in wild boar populations independent of domestic pigs (39). Between 1960 and 1986, the disease emerged in a variety of European countries, including France, Madeira, Italy, including the island of Sardinia, Belgium, the Netherlands, and Malta (37, 40, 41). Extensive control has led to eradication in these countries, except for Sardinia, where the disease has been endemic since 1978 (20).

In June 2007, ASFV was introduced to the Caucasus region of Georgia, presumably from catering waste containing infected meat from ships docked at the Black Sea Port of Poti (38). The virus spread quickly throughout the country and by July 2007, ASFV was found in 56 of the 61 districts in Georgia. By August 2007, ASF was found in neighboring Armenia and by November 2007 was found in Azerbaijan and Russia. In 2014, outbreaks were reported in parts of the European Union, including Poland, Lithuania, Latvia, and Estonia and the first detections in each of these countries were in wild boar found dead (42). Epidemiological investigations from Lithuania and Latvia suggest that fresh grass and seeds contaminated with ASFV from infectious wild boar served as the source of infection for pigs on backyard farms (43). The viral amplification in backyard pigs then served as a viral source for other backyard farms and commercial piggeries. In 2017, ASF was reported in the Czech Republic and Romania, and between January and September 2017, the Animal Disease Notification System received notifications of about 3,700 cases in wild boar and approximately 140 cases in domestic swine from the 6 EU member states, including Sardinia (44). Interestingly, models of the most current epidemiological situation suggest that the most important risk estimator for ASF spread into disease-free EU countries is wild boar habitat and the least significant estimator is wild boar density; thus, indicating that the presence of wild boar is more important than density (45). This model can be used to identify countries that are at higher risk for ASF introduction through wild boar.

Experimental inoculation of wild boar with an ASFV isolate from the Caucasus region found that the infection resulted in uniform lethality, and the authors concluded that this highly virulent strain would be unsuitable for viral endemicity within the native population of wild boar (46). Despite this assertion, field observations show that the virus can persist independently in wild boar despite high virulence. Importantly, a low-dose challenge of wild boar with Caucasus region isolates of ASFV were found to be sufficient to result in infection of weak or runted animals (47). Once infected, these poor-doing wild boar could then serve to amplify ASFV to levels that were capable of infecting apparently healthy herd mates. The exact mechanism with which highly virulent strains of ASFV are being maintained in wild boar populations is unknown; however, in several epidemiological scenarios, it has become clear that ASFV can persist independently in wild boar populations.

Competent vector species, namely *O. erraticus* ticks, found on the Iberian Peninsula also contribute to difficulty eradicating the virus once introduced. Portugal was declared free of ASFV in 1993, but the virus re-emerged on a single farm in 1999 and ASFV-infected *O. erraticus* ticks in 1993 are suggested as the route of introduction (48). Ticks were collected from farms that were depopulated due to ASF and evaluated for their capacity to maintain an ASFV infection and transmit to susceptible domestic swine. Cell culture was used to evaluate tick infection, and four adult ticks were found to be positive using cell culture alone and another six adult ticks were found to be positive using both cell culture and PCR or direct immunofluorescence. 8.8% of tested farms were found to have infected ticks, and this infection could lead to virus isolation 2.5–5.25 years following the last possible ASFV exposure. Transmission to susceptible domestic swine occurred 2.3 years after the last possible exposure to ASFV. These findings suggest that the current European Administration regulations on ASF, where an infected property can be restocked 40 days after an outbreak in the absence of soft tick vectors, and the requirement of a 6-year quarantine if soft tick vectors are present, are appropriate (49). Furthermore, it is a distinct possibility that long-lived ASFV-infected *O. erraticus* ticks caused the single farm outbreak of ASF in 1999 after the eradication in Portugal. However, it is important to note that this finding was related to a very old and traditional housing of pigs, using pig sties in which soft ticks could become established. By using modern pig production methods, a soft tick infestation is unlikely.

DOMESTIC SWINE IN THE UNITED STATES

The overwhelming majority of the 65 million pigs in the United States are managed indoors under high biosecurity conditions.

Figure 1 illustrates the distribution of pig production within the United States from 2012 (50), with Iowa, North Carolina, Minnesota, Illinois, and Indiana being the five top pork producing states annually. Commercial swine production is a closed system from farrowing through slaughter as a means to reduce the risk of pathogen introduction (Personal communication, 2016). Animal feed, transport vehicles, personnel, and other fomites are also closely managed to limit cross-contamination. However, it is important to note that despite the biosecurity measures in place in the commercial swine industry, porcine epidemic diarrhea virus (PEDV) entered the United States in 2013, and epidemiological analyses suggest that transport equipment contributed to viral spread (51). Moreover, in summer 2014 in northeast Lithuania, ASFV was introduced to an industrial pig farm that was intensively managed with a closed cycle and very strict biosecurity measures resulting in the death or euthanasia of >20,000 pigs (20). These examples demonstrate that despite stringent biosecurity protocols and a vertically integrated industry, it can still be difficult to control pathogens.

For ASF vector-borne transmission, however, it is unlikely that ticks would interact with domestic swine raised in commercial facilities. However, hobbyists and backyard farmers often have domestic swine that are not managed with intensive biosecurity and thus are likely to be exposed to environmental elements and other domestic livestock, wildlife species, or their feral counterparts (52). Exposure to potential soft tick vectors and other blood feeding arthropods is plausible depending on both the geographical region and the management practice. Given these conditions, an ASF introduction into the United States may put backyard farms more at risk compared to commercial facilities, as has been reported in much of Eastern Europe and some European Union member states (e.g., Latvia) (43).

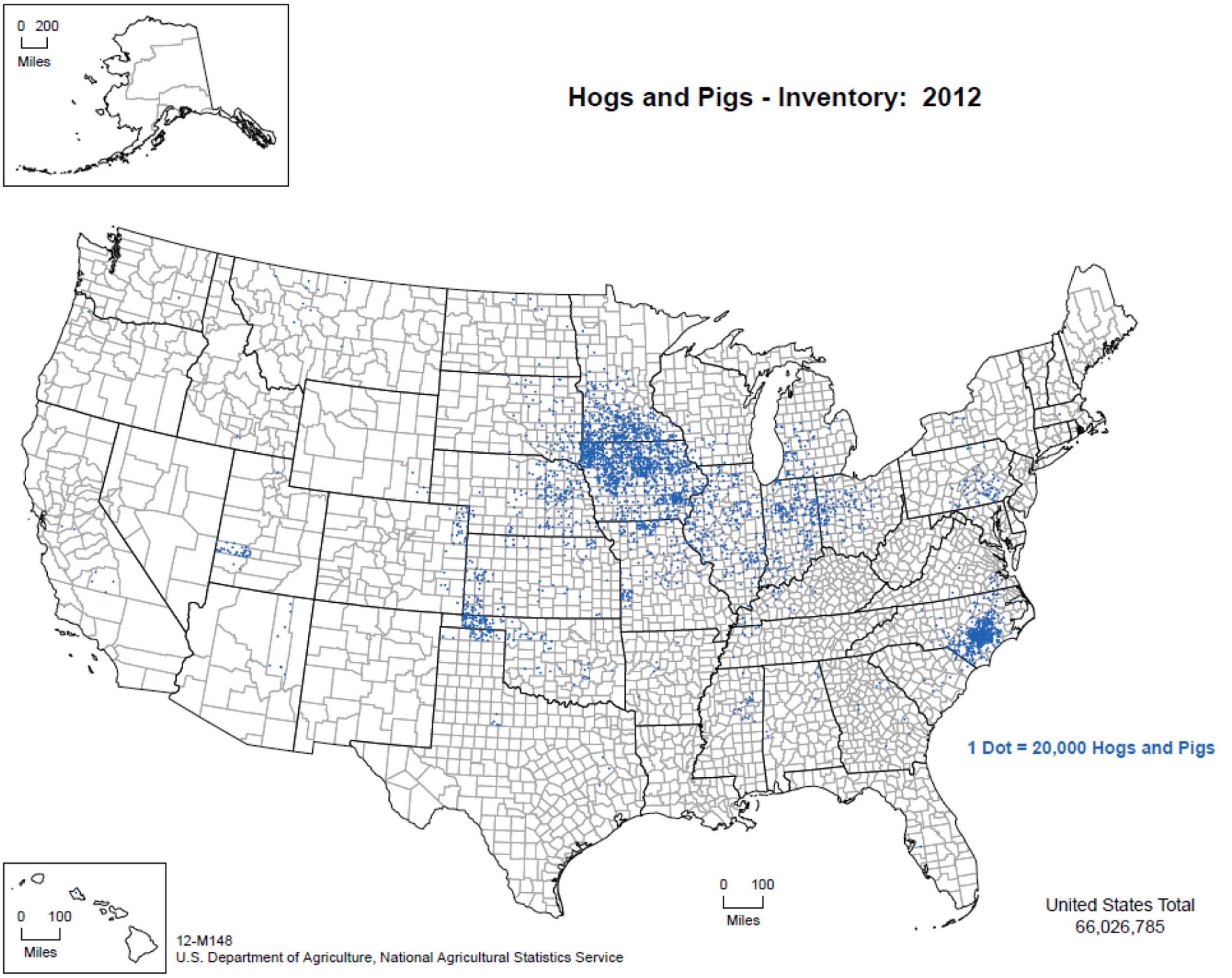

FIGURE 1 | Distribution of pig production within the United States, 2012. (Figure courtesy by United States Department of Agriculture, National Agricultural Statistics Service (2015), used with permission.)

DIAGNOSTICS

Virus isolation can be used for the diagnosis of ASF as live virus can be obtained from live animals or necropsy tissues although this method is typically only used in reference laboratories to confirm diagnosis (53). The spleen, kidney, tonsils, and lymph nodes are the best tissues for virus collection. Pig leukocyte cells, bone marrow cultures, porcine alveolar macrophages, and porcine blood monocytes can all be used in ASFV culture. Conventional and real-time PCR have been developed for the detection of ASFV, and multiple primer pairs have been developed to create a rapid diagnostic tool (53–56). A strong IgG response has been detected in domestic swine that survive infection with ASFV (57). As such, serological assays can also be very useful, especially in endemic regions. ELISA, immunoblotting, and indirect fluorescent antibody assays are the most common, and ELISA followed by immunoblotting is often used for international trade purposes.

VACCINES

It has been particularly challenging to develop an effective ASFV vaccine. To date, no vaccine is available because of a number of key factors, including the lack of identification of protective antigens, incomplete understanding of virus–host cell interactions, and inadequate knowledge relative to the diversity of viral strains currently circulating in natural reservoirs (58, 59). A number of vaccine options have been tried with varying levels of success, including using vaccines with naturally or experimentally deleted genes, subunit vaccines based on recombinant proteins, and DNA vaccines (23). However, none conferred complete protection. A live attenuated vaccine strain was developed and was shown to provide protection against a homologous strain challenge; however, use on the Iberian Peninsula is believed to have been the origin of some low virulence strains that induced a chronic disease form of ASF during the 1960–1995 outbreak (23). Despite this setback, live attenuated vaccines continue to be evaluated for their protective capacity (60, 61).

Knockout ASFV mutants have been evaluated for efficacy although findings have been inconsistent. Afonso et al. (62) describe a highly conserved gene, referred to as *NL*, and found that deletion of the gene from European pathogenic strains resulted in complete attenuation of the virus in domestic swine. *NL*-deleted mutants were created for two highly virulent African strains of ASFV, and inoculation in domestic swine found that these strains retained their virulence, irrespective of the absence of *NL*. These findings suggest that *NL* gene function is not required for these strains of ASFV and that *NL* gene deletion alone is insufficient to engineer live attenuated ASFV vaccines. Gene *9GL* is highly conserved, and *in vitro* evaluation determined that the protein encoded by this gene affects virion maturation and viral growth in macrophage culture (63). The deletion of *9GL* resulted in growth-defective mutants in culture and was found to be highly attenuated in domestic swine. Immunization with a *9GL* knockout virus followed by a challenge with a wild-type ASFV strain resulted in complete protection, and this mutant is being further evaluated as a vaccine candidate for ASFV. The *9GL* gene is also highly conserved and deletion was found to result in complete viral attenuation in swine (64). Vaccination with the mutant strain followed by infection with a wild-type homologous virus resulted in complete protection. Interestingly, however, evaluation of anti-ASFV specific antibodies, ASFV-specific IFNγ response, and circulating cytokine levels found that a complex immune scenario dictates whether infection is established.

Furthermore, A238L is an ASFV immunomodulatory protein that inhibits activation of the NFκB and NFAT pathways, which are responsible for regulating the synthesis of pro-inflammatory cytokines (65). This protein is believed to be a potent immunosuppressor that may contribute to viral evasion of the host immune response. Unsurprisingly, inoculation of pigs with A238L mutant viruses demonstrated an increase in TNFα, a potent pro-inflammatory cytokine. Much more work is needed to determine whether immunization with viruses with altered immunomodulatory proteins could be harnessed to assist the host immune response against virulent challenge.

Recombinant protein vaccines have also been characterized using a number of relevant viral proteins. p30 and p54 are externally located and involved in virus attachment and virus internalization, respectively (58). Immunization of domestic pigs with either recombinant p54 or p30 proteins induced neutralizing antibodies, but did not protect against lethal challenge and the disease course was unaltered. Combination p54 and p30 vaccines produced both neutralizing antibodies and modified the disease course resulting in a range of protection. Ivanov et al. (66) evaluated 46 peptides that mimic viral proteins for their ability to establish a protective immune response. Vaccination with some combinations of these peptides was found to delay mortality in domestic swine and warrants further investigation. A baculovirus vector expressing the ASFV hemagglutinin was used as a vaccine, and all pigs survived challenge with a virulent virus after immunization (67).

DNA vaccines have also been assessed as an option for ASF, and partial protection was afforded in domestic swine using p54 and p30 as antigens on the construct (68). The robust activation of $CD8^+$ cells appears to be extremely important for protection.

Exposure to a non-virulent strain in Portugal (OURT88/3 genotype 1) followed by a virulent strain (OURT88/1 genotype 1) conferred protection against challenge with virulent field isolates from Africa (69). This immunization strategy protected most pigs from both disease development and viremia. The cross-reactivity of the various strains of ASFV can be measured using IFNγ stimulatory assays and provide a strong correlation to the degree of protection conferred.

In addition to evaluating new vaccine preparations, Blome et al. (70) reassessed inactivated ASFV vaccination preparations using modern adjuvants, specifically Polygen and Emulsigen D, which are known to stimulate both humoral and cellular immune responses, including IFNγ. The efficacy of inactivated ASFV vaccines was not improved, and no protection was observed after vaccination followed by challenge with a homologous strain. In fact, vaccinated animals submitted to the disease more quickly, suggesting the possibility of antibody dependent enhancement.

Vaccine development for ASFV is ongoing and challenging due to the range of genetic and antigenic variability as well as the myriad of strategies utilized by the virus to evade the host's immune response. Further work is essential to develop a vaccine that is both biosafe and provides a high degree of protection across virulent ASFV strains. Subject matter experts believe that live attenuated vaccines are the most promising candidates in the short term due to their experimental successes; however, more studies are required to confirm vaccine safety, capacity to differentiate between naturally infected and vaccinated animals (DIVA), and long-term efficacy (60).

ASF AND THE UNITED STATES

The introduction of ASFV into the United States could negatively affect the domestic swine industry because of morbidity and mortality, the associated losses in production, and restrictions on interstate and international trade. The Foreign Animal Disease Preparedness and Response Plan (FAD PReP), Disease Response Strategy: African Swine Fever put together by USDA APHIS Veterinary Services (71) provides information relevant to all aspects of a disease response in the United States in the event of a viral incursion. The control and eradication strategies are based on four epidemiological principles: (1) prevent contact between ASFV and susceptible animals (primarily *via* quarantine and restricted movement), (2) stop the production of ASFV by infected and/or exposed animals, (3) stop vector transmission, and (4) increase the disease resistance of susceptible animals to ASFV. However, the primary control and eradication strategy are predicated on stamping out (depopulation of clinically affected and in-contact control susceptible swine). Currently, there is no active surveillance being conducted in the United States for ASF. The USDA FAD Prep Document provides information for responders and stakeholders such that they understand the disease agent. Furthermore, a stochastic risk assessment model created by Herrera-Ibata et al. (72) determined the months of highest risk, the origin of the imports of higher risk, and the US states most vulnerable to an ASF introduction. This information can be used to optimize surveillance plans and develop emergency response protocols to help reduce the impact of a potential ASF introduction into the United States.

SUMMARY OF UNITED STATES VULNERABILITIES FOR THE INTRODUCTION OR PERSISTENCE OF ASFV

Risk of Introduction into the United States

Vergne et al. (73) evaluate the pathways for the potential introduction of ASF in China, which maintains over half of the global pig population, and our risk assessment shows similar routes of concern for virus introduction. The legal or illegal movement of live animals or their products, byproducts, or animal feed, or an intentional viral release in an act of bioterrorism comprise the routes of highest concern for ASFV introduction into the United States. It is important to note that to result in an outbreak event, an imported ASFV would need to be released into a susceptible population. An initial outbreak event could occur in domestic or feral swine and then presumably spillover into the other population. Each of these possible routes of introduction is described.

Legal Movement of Live Animals

Domestic swine are imported annually from Canada, and **Table 1** summarizes the number of animals imported and their purpose. Currently, Canada is ASFV free and, as such, the importation of suids is unlikely to result in an ASFV introduction into the United States.

Legal Movement of Animal Products, Byproducts, and Animal Feed

Animal products and byproducts as well as animal feed that are imported into the United States all require permits upon entry. Products and byproducts that are coming from ASF-endemic regions must be treated in a manner that has previously demonstrated efficacy in destroying ASFV, typically involving heat, pH, or fixation processes. Products and byproducts derived from ASF-free countries can be imported in an unprocessed form. Animal feed from ASF-endemic regions is required to be cooked to a specific temperature and for a specified duration before importation. Products coming from the European Union, which is designated as a low-risk region, can be imported raw if desired; however, documentation is required to certify that the product is coming from an unaffected herd in an unrestricted region.

Illegal Movement of Live Animals and Their Products

The US Department of Homeland Security Customs and Border Protection (CBP) is primarily responsible for the confiscation of illegally imported products and specimens from domestic livestock species. Data provided by CBP depict products and specimens from domestic swine that were confiscated in the cargo or express courier environment, which includes companies such as FedEx and DHL, or *via* international mail facilities, including US postal service. Between calendar years 2012 and 2016, over 68,000 products and specimens derived from domestic swine were confiscated by CBP. The continents of origin for the majority of products and specimens confiscated by CBP are Asia and Europe, which comprise 49 and 44% of the confiscations, respectively. South America, Australia, Africa, and unknown account for ≤1% each, and products and specimens confiscated

TABLE 1 | Number and purpose of pigs that were imported into the United States from Canada between years 2012 and 2016.

Purpose	Number of animals imported				
	2012	2013	2014	2015	2016
Breeding swine	155,417	196,320	249,214	234,796	150,267
Feeding swine	4,706,866	4,177,805	3,936,987	4,314,664	4,626,477
Direct to slaughter	886,736	824,511	851,002	1,163,884	980,242
Total	5,749,019	5,198,636	5,037,203	5,713,344	5,756,986

from North America comprise 5%. These data are summarized in **Figure 2**.

A large number of products and specimens were derived from continents with regions that are enzootic for ASF. The exact number of products and specimens that are smuggled across the US border is difficult to ascertain, and it can be assumed that the products and/or specimens discovered represent a small subset of the types of goods that are illegally imported into the United States. Due to the types of products confiscated and the regions of the world from which they originate, the illegal importation of domestic livestock products and specimens certainly pose a risk for ASF introduction.

The US Fish and Wildlife Service (FWS) is responsible for the confiscation of illegally imported wildlife; however, a 1994 report from the Government Accountability Office estimated that 1–3% of illegal wildlife shipments carried by passengers, and 1–10% of illegally imported wildlife in declared cargo shipments are detected (74). This problem is believed to be primarily a result of a limited inspection workforce and budgetary restrictions on overtime, such that ports of entry are often without inspector coverage. FWS provided data relative to wild suid product confiscations in the United States between 2006 and 2016. The types of wild suids from which products were illegally imported and subsequently confiscated by US FWS agents can be found in **Figure 3**. Warthog products are responsible for more than 60% of confiscations followed by wild boar, bush pigs, unspecified swine products, and babirusa; however, the sample size is small because of the specific nature of the data (wild suids) and because not all illegal imports are likely detected.

The majority of products seized by FWS in the United States were those that originated on the continent of Africa (**Figure 4**). Approximately 25% of confiscations were of products derived from North and South America as well as unknown countries of origin. Asia, Australia, and Europe comprised 13% of confiscations. A large proportion of all confiscated products were derived from continents, which are endemic for ASFV (or unknown); hence, illegal animal/animal product transport presents a risk for ASFV introduction.

Bioterrorism

Bioterrorism is described as the intentional release or dissemination of bacteria, viruses, or toxins that cause morbidity or mortality events in humans, other animals, or plants. Due to the tremendous value of the domestic swine industry in the United States, the high morbidity and mortality associated with infection, the ease of viral spread due to the endemic status of many countries globally, the stability of the virus in chilled and frozen products, the safety for the individual(s) involved in the release as the pathogen is not zoonotic, and the crippling economic effects attendant with an introduction, ASFV is a potential candidate to be released in an act of bioterrorism. This route of introduction is difficult to prevent and as such spotlights the need for robust surveillance systems in both domestic and feral swine to ensure rapid detection and differential diagnosis.

Factors that Complicate Eradication Efforts following Introduction

The risk of ASFV introduction to the United States is low (72). Following a potential introduction, however, ASFV establishment, even short-term establishment, is an open question. ASF has never been found in the United States, but

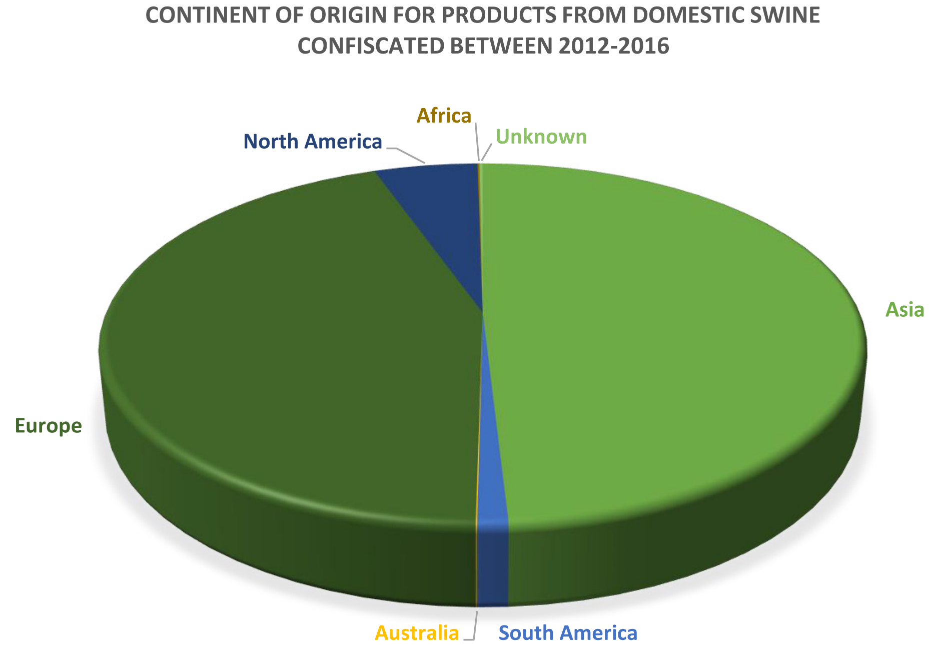

FIGURE 2 | A pie chart depicting the continent of origin for the products confiscated by US Customs and Border Protection between 2012 and 2016 (*n* = 68,594).

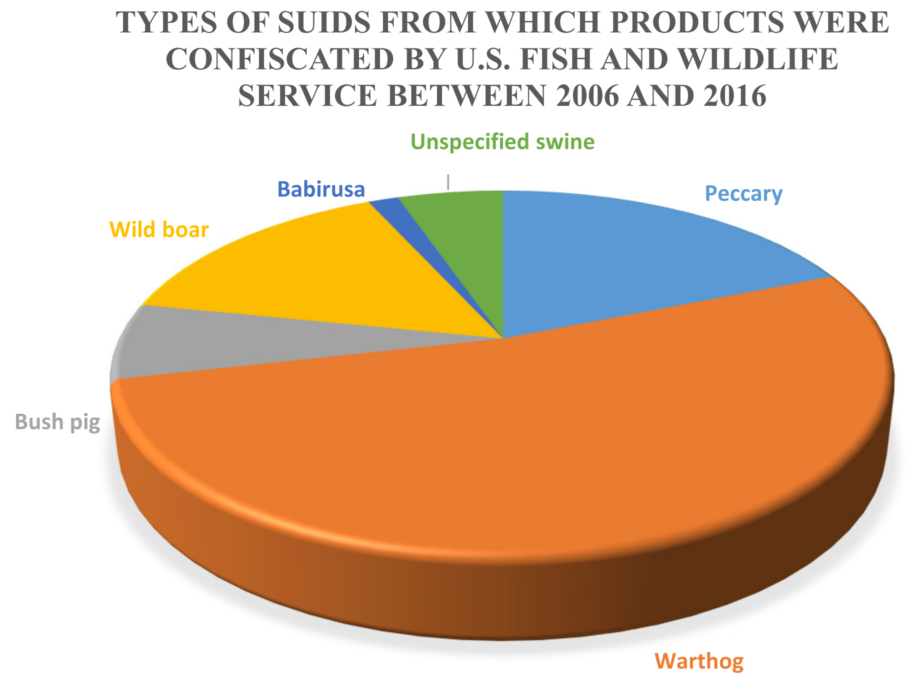

FIGURE 3 | Types of suids from which products were confiscated by the US Fish and Wildlife Services between 2006 and 2016 (*n* = 133).

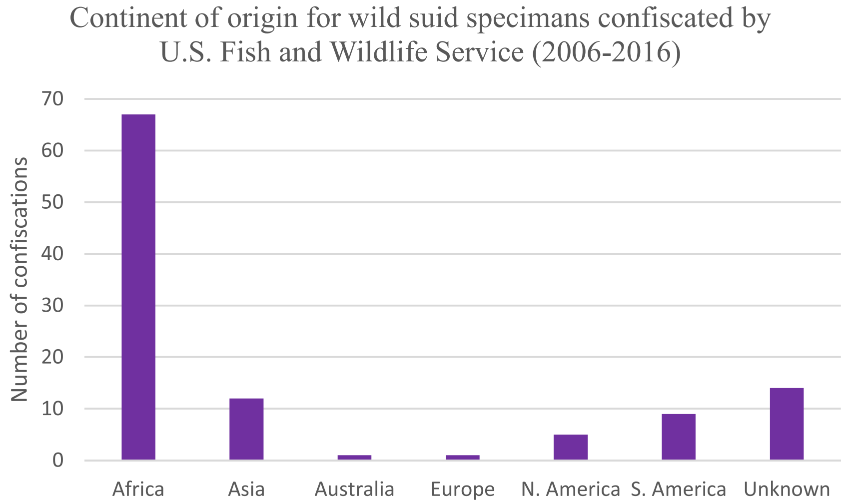

FIGURE 4 | Number of wild suid specimens seized by the US Fish and Wildlife Service between 2006 and 2016 based on continent of origin (*n* = 133).

it has successfully taken hold in areas of introduction around the world. Transmission cycles and viral ecology often differ in different locations, demonstrating at least some flexibility for the virus to persist in a range of climates, with or without tick vector involvement, and with or without a wild suid component (75). Climate would not limit ASFV establishment in the United States, and there are tick species that could potentially play a role in viral maintenance (76). The presence of backyard swine and feral swine could also aid in short-term establishment similar to what has been seen elsewhere (43). The biosecure nature of the US commercial swine industry would likely detect and limit ASF transmission without long-term establishment, but economic consequences could still be significant.

Feral swine, which are found in a large number of states, present a risk because of their free-roaming behavior and omnivorous diets and, in the event of a viral incursion, would likely contribute to amplification and transmission events to other feral swine or their domestic counterparts. Soft tick species in the *Ornithodoros* genus that are native to the United States also present an element of complexity as their competence in a field setting remains largely unknown but could substantially complicate viral persistence and disease eradication. These elements are described in detail below.

Feral Swine

The OIE defines feral animals as those that do not live under human supervision or control but have a phenotype that was selected by humans (77). Feral swine (*Sus scrofa*) include released and escaped domestic swine, truly wild Eurasian boars, and their hybrids and are believed to have been originally brought to the United States in the 1400s (78). APHIS experts estimate that over 6 million feral swine roam within at least 35 states in the United States with California, Florida, Oklahoma, and Texas having the largest populations (**Figure 5**). In addition to being an invasive species, feral swine can damage the environment and agricultural operations; alter ecosystems with their rooting behavior that can be detrimental to threatened and endangered species; and pose a health threat to humans, domestic livestock, wildlife, and companion animals as a result of the type of pathogens that they are capable of carrying and transmitting (79, 80). Studies involving global positioning system (GPS) collared feral swine demonstrated that they contacted domestic swine, and digital images indicated that feral swine attempted to enter pens containing domestic female pigs (52). These types of interactions, which are unsurprising because of the gregarious nature of both domestic pigs and feral swine, increase the risk of pathogen transmission events (81).

Typically male feral swine live a solitary life, while reproductively active females live in small groups with their young, referred to as sounders. Contact rates within and between sounders have been studied using GPS devices, and not surprisingly, contact rates are much higher amongst members of the same sounder compared to those between animals of different sounders (82). However, it has further been shown that sounder home ranges often overlap extensively (83). Sounder interaction is reduced when sounders are separated at distances >2 km, and as such, disease transmission is expected to be reduced between sounders at this distance and nearly negligible between sounders separated by >6 km. Based on these data, the quarantine radius surrounding a positive premise is likely to be at least 2 km, although feral swine activity would be but one factor to consider when determining quarantine size. Certainly, other factors may exist that lead to clustering, such as water availability or baiting activities, among others. Lone boars have been shown to have much larger home ranges compared to sounders and are far more likely to move great distances (84). Furthermore, feral swine densities should also be accounted for as movement may be influenced by density.

Bait stations have been considered as an alternative to fencing for containing feral pigs during culling activities; however, once evaluated empirically, it was found that baiting is not a suitable alternative as only 62% of feral swine trapped within proximity of the bait station used it (85). Baiting can be effectively used to describe patterns of swine movement, facilitate observations, and improve the outcome of removal programs. Interestingly, culling activities did not appear to greatly impact feral swine movements.

In the event of a disease outbreak that affects swine (either exclusively or in conjunction with other livestock species), feral

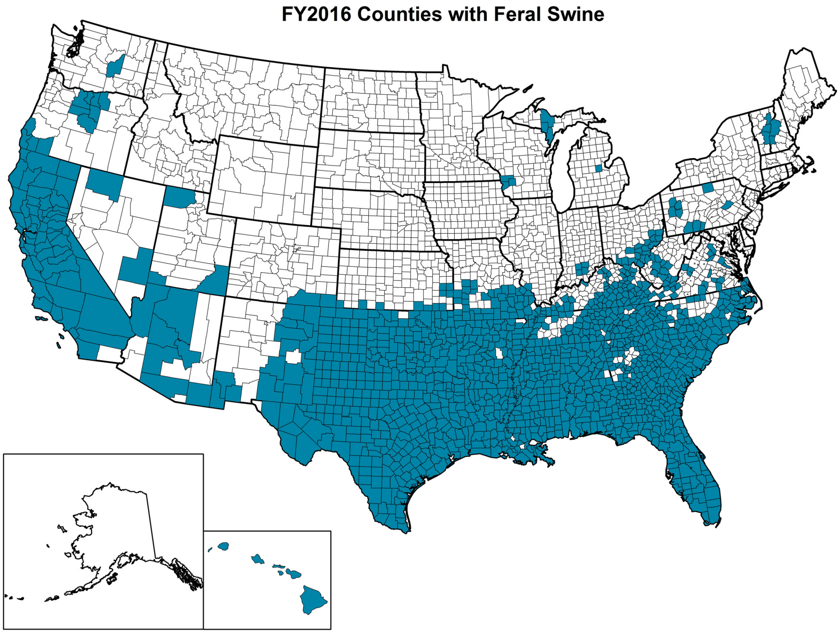

FIGURE 5 | Counties highlighted in blue within each state of the United States where feral swine have been found. (Figure courtesy by APHIS National Feral Swine Damage Management Program.)

swine could be problematic. Fencing types that can effectively contain feral swine have been evaluated, and hog panels have been found to be highly effective (86). These panels have been found to be effective even when feral swine motivation to escape is increased due to human intervention. In addition, they are relatively quick and cheap to erect—both of which are crucial components in the event of a disease outbreak. While fencing shows promise, it is an option typically reserved for a small, localized scale, such as the area surrounding a single positive farm.

Knowledge derived from ecological and behavioral experiments would be employed, and information specific to the infected premise would be utilized to make an informed decision regarding the frequency and nature of visitation between domestic livestock and feral swine. This information would be used in conjunction with data on other factors such as other nutrient accessibility and feral swine densities (if density data are available) to determine the appropriate spatial scale of fencing or surveillance. Feral swine home ranges can vary dramatically based on the habitat complexity and the availability of food, water, and shelter. For example, a study on feral swine movement in multiple regions of Texas found that the area used by GPS collared individuals could range from 4.5 to 22.23 km^2 depending on location and season (87). Fencing has been successfully used on large scales to exclude feral swine from a national park in Hawaii [>75 km (88)] and from a national monument in California [42 km (89)], although these were erected over a time frame that was longer than required for a typical outbreak situation. Fencing can also be used to control movement. It is likely that a perimeter fence would be erected around the infected premise with the aim to enclose all feral swine that may have direct or indirect contact with animals from the infected premise before targeted removal of all feral swine within the fenced region. Culling activities would likely be initiated immediately in an attempt to contain disease transmission. Sounders and lone boars that live outside, but near, the quarantine region would likely be closely monitored to evaluate disease transmission and may be subject to prophylactic culling. Outbreak specific characteristics would be important to include, such as the amount of time that has elapsed since the first case, the virulence of the ASFV strain, and the density of both domestic and feral swine, among many other components.

An ecological model developed in Europe showed that conventional wild boar management approaches such as banning feeding and targeted hunting of reproductively active females became slowly effective over multiple generations (90). As such, a buffer of 100–200 km was necessary to compensate for the forward spread of disease until the measures became effective. However, massive population destruction (>80% of the population in the control region within 4 months) or immediate removal of infectious carcasses reduced the buffer zone to <50 km. A hybrid approach of the control methods would result in an intermediate buffer zone width. Of note, hunting as a means to control population and reduce the spread of ASF is very effective, but all efforts should be made to reduce dispersal during this period as the gains made in ASF control *via* population reduction can be quickly offset by wild boar movement and subsequent introduction of ASFV into naive populations (42).

Controlling and/or eradicating disease outbreaks in feral or wild populations is extremely difficult for a number of reasons. Informed decision-making in the absence of knowledge or facts is often required in these types of settings, and as such a systems approach can be used to inform resource allocation and a systematic perspective (91). The publication by Delgado et al. (91) was written with classical swine fever (CSF) in mind; however, many of the components would likely be similar for ASF.

The United States is neighbored by two other countries, and feral swine populations move back and forth between countries on both the northern and southern borders. For example, it is not known if a detailed census of feral swine populations throughout Mexico has been done, but there are populations along the United States–Mexico border that are contiguous with the US feral swine population. **Figure 6** shows the distribution of feral swine in Mexico based on the subjective reports from the agriculture department of each municipality. Feral swine have been seen moving back and forth across the border along some stretches, depending on the landscape. While both Mexico and Canada are considered ASFV free, it still presents a concern that the borders are porous allowing for movement of feral swine between the countries along both borders. In the event of viral incursion in the United States, Mexico, or Canada that spills over into the feral swine population, this movement will present challenges related to disease control and eradication. Semiquantitative risk assessments have been developed to evaluate the risk of ASF introduction into the EU by wild boar movements as ASF is now considered endemic in much of Eastern Europe (92). In the event of an ASFV introduction in either Canada or Mexico, this type of modeling approach could be used to evaluate the risk of virus introduction into the United States by feral swine.

Ornithodoros Ticks in North America

Tick families of veterinary and medical importance include the Ixodidae, which are commonly known as hard ticks, and Argasidae, which are commonly referred to as soft ticks. Several soft tick species in the genus *Ornithodoros* are known vectors of ASFV, and have a nidiculous lifestyle, which indicates their preference to reside in the nest or burrow inhabited by their vertebrate hosts (93, 94). Their lifecycle involves immature and adult male and female stages that take short, repeated blood meals (95). Mated female soft ticks use the blood meal to produce eggs that are laid in a suitable habitat. Adult *Ornithodoros* ticks can live for several years without feeding, and their distribution tends to overlap the geographic range of their hosts (76).

Five species of *Ornithodoros* ticks are found in the United States. *O. coriaceus, Ornithodoros hermsi,* and *O. parkeri* occur in the western and Midwestern regions of the United States and *O. turicata* and *Ornithodoros talaje* are found in the arid regions of the southern United States (10, 96). Laboratory investigations reviewed by Kleiboeker and Scoles (33) demonstrated that *O. coriaceus, O. parkeri,* and *O. turicata* were capable of becoming infected with ASFV and *O. coriaceus* was competent in transmitting the virus to naive domestic swine. Of particular concern is *O. turicata*, found in Arizona, California, Colorado, Florida, Kansas, New Mexico, Oklahoma, Texas, and Utah. These states also provide suitable habitat for large numbers of feral swine,

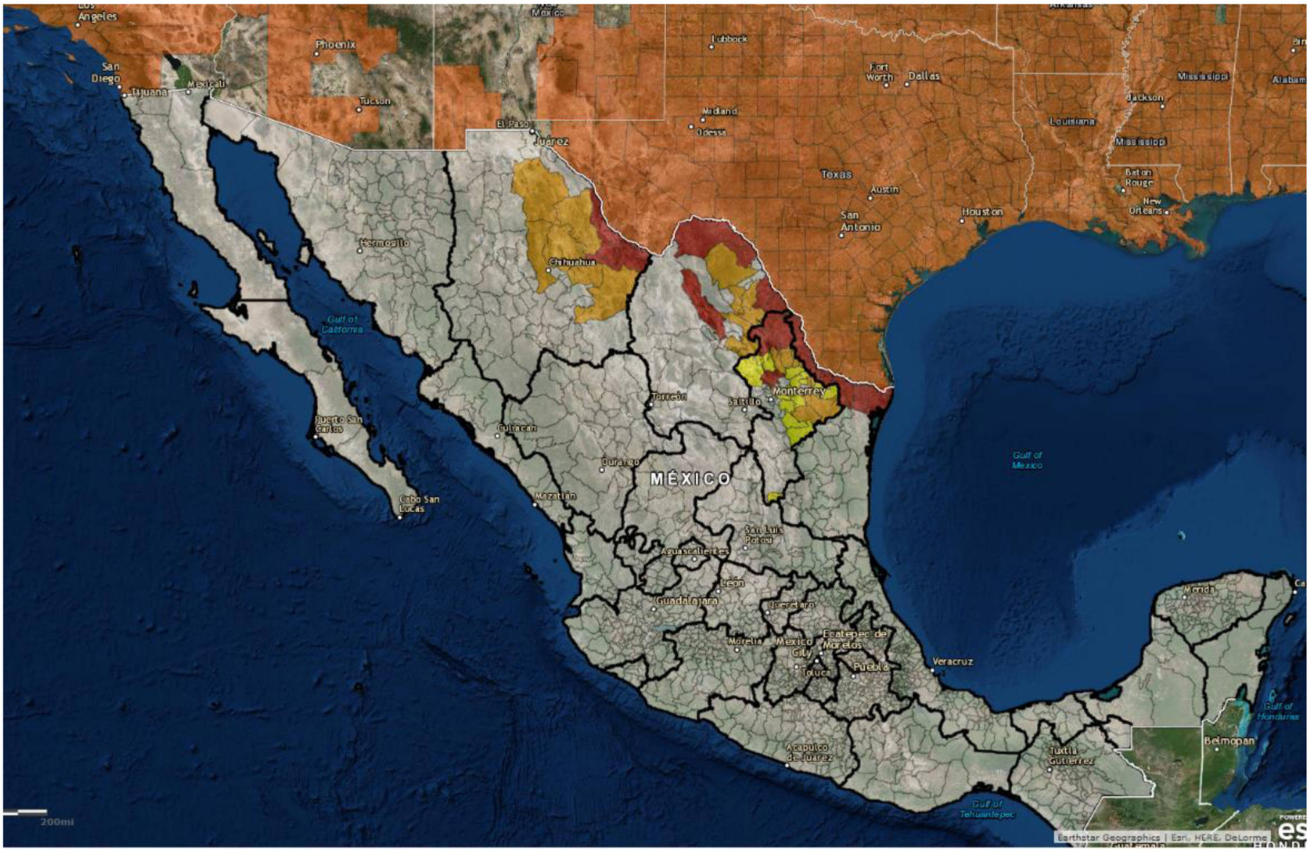

FIGURE 6 | Geographical distribution of feral swine in Mexico (2012): red = high density (>2,000 pigs/county), orange = moderate density (500–2,000 pigs/county), and yellow = low density (<500 pigs/county). (Figure courtesy by APHIS National Feral Swine Damage Management Program.)

which presents an opportunity for the maintenance of ASFV by *O. turicata* in the event of a viral incursion with the involvement of feral swine (97).

Because of their short-feeding duration and nidiculous lifestyle, the global distributions of soft ticks can be challenging to determine; however, their capacity to transmit pathogens makes this information of the utmost importance. A regional model using spatial multicriteria decision analysis to identify geographical areas that are suitable for specific species of *Ornithodoros spp.* was created by Vial et al. (98). This model was developed for the Western Palearctic region; although in the event of ASFV introduction into the United States, this methodology could be applied to native *Ornithodoros* ticks to determine species and regions of concern.

In addition to the competent *Ornithodoros* species found in the United States, *O. puertoricensis* is found in the Caribbean, specifically Jamaica, Haiti, the Dominican Republic, and in Panama (99) and can be infected with, and transmit, ASFV to susceptible domestic swine (33). The porous border between the United States and Mexico provides further complexity in the event of an introduction of ASFV in either country, and soft tick vectors could play a role as an epidemiological bridge as they might be transported to disease-free regions. It is important to note that although soft ticks engorge rapidly and tend to drop off their host after completion of the blood meal, reports of host infestation as "stowaways," including feral swine, captured outside of their nest or burrow has occurred (100, 101). Thus, this potential route of viral introduction or spread is worth mentioning. Furthermore, they are often promiscuous in their host preferences and have occasionally been recovered from birds, which also pose a risk for disease translocation (10).

CONCLUDING REMARKS

African swine fever virus introduction (either accidental or purposeful) to the United States could cause severe morbidity and mortality in domestic swine. Furthermore, the trade implications associated with ASFV in domestic swine are substantial and could severely affect the pork industry. The current regulatory systems in place for the importation of live animals, animal products, byproducts, and feed are comprehensive, involving considerable Federal oversight and encompassing information relevant to the country of origin, the product to be imported, and the species involved must conform to research methods that effectively demonstrate the deactivation of ASFV. Despite the robust regulatory framework, the illegal importation of animals and their products is in its very nature difficult to control, manage, or regulate. Semiquantitative approaches can be used to evaluate the risk of disease introduction *via* the illegal importation of pork and pork products, and modeling in the European Union suggests that this channel certainly serves as a risk for ASFV importation and subsequent introduction (102). Bioterrorism is another potential route of introduction. Given the complexities of preventing accidental or purposeful ASFV introduction into the United States, vigilant observance of domestic livestock and rapid reporting and differential diagnosis are necessary in the event of a disease detection in pigs. Channels for rapid communication and diagnostics already exist through state and national veterinarians and laboratories as evidenced by a pilot study in which samples were collected from culled feral swine and evaluated for the presence of ASFV. The evaluation of samples for ASFV suggests that labs are proficient in diagnostic techniques necessary for viral detection.

African swine fever virus introduction, or spillover, into feral swine populations would heavily complicate eradication. Furthermore, unrestricted movement of feral swine across porous borders presents a challenge in the event of an ASFV incursion into any countries that share borders. The presence of competent biological vectors, *Ornithodoros* ticks, further complicates the control and eradication of ASFV upon introduction to a new region. These ticks are often long lived and are believed to play an important role in viral maintenance and may contribute to the development of endemicity in a specific region. *O. coriaceus*, *O. parkeri*, and *O. turicata* are present in the United States and have been found to be capable of ASFV infection and in the case of *O. coriaceus*, ticks are capable of transmitting the virus to susceptible swine (33, 96). *Ornithodoros dugesi* are found in Texas and northern Mexico, and *O. talaje* are found in the southern United States; however, neither species has been evaluated for its competence as a biological vector for ASFV. Further laboratory studies should be designed to evaluate the ability for *O. dugesi* and *O. talaje* to become infected with various strains of ASFV and to characterize the ability for *O. parkeri* and *O. turicata* as well as *O. dugesi* and *O. talaje,* pending their capacity to become infected, to transmit infection to susceptible domestic swine. In addition, expanded analyses to explore the distribution of competent *Ornithodoros* ticks in relation to dense commercial pig production regions as well as high populations of feral swine are needed. Moreover, determining host preferences for competent vector species is important to characterize risk. The lack of a vaccine for ASFV makes disease control and eradication substantially more difficult, and as such, efficacious vaccine development is a high priority. Characterization of antigenic viral proteins that contribute to a host immune response and a determination of immune mechanisms that lead to protection are extremely important and foundational for the quest of a vaccine.

Currently, the United States does not have any active surveillance protocols for ASF in domestic or feral swine. The risk of introduction is believed to be low because the disease is not currently present in the western hemisphere. In addition, the introduction of ASFV into a naive population is typically accompanied by severe mortality such that passive surveillance, sampling of dead pigs, would likely be sufficient to detect an ASFV incursion (103, 104). Importantly, during calendar year 2017, the Foreign Animal Disease Diagnostic Laboratory (FADDL) at Plum Island Animal Disease Center performed only two cases requesting ASF testing and both were negative (Personal communication). Of note, however, a stochastic model used to evaluate transmission of ASFV within a population found that the virus may be circulating in a herd for several weeks before a marked increase in mortality is observed, which limits the usefulness of mortality data as a means of early detection in an outbreak scenario (105). It may also be useful to compare the conditions in the United States to those in Europe to determine whether the buffer zones necessary to quell an ASF outbreak in Europe (90) would be similar to those required for an outbreak in the United States.

USDA Veterinary Services have outlined a surveillance program for CSFV in domestic swine that could be harnessed to evaluate ASFV in the event the risk of introduction increases. The objectives are as follows: (1) surveillance for rapid detection of CSFV in US swine, (2) monitor the risk of introduction of CSF into US swine, (3) surveillance of international CSF status, and (4) surveillance to document freedom of CSF (71). Unthrifty pigs, considered to be those that gain weight poorly or are otherwise somewhat sickly, are often sold to off-market vendors. APHIS Veterinary Services field staff or other cooperating personnel collect tonsil samples in these markets as a way to survey for infectious agents, including CSFV. This method is deemed to be an effective surveillance strategy as poor-doing pigs from surrounding regions are often consolidated in these markets, which makes for an efficient means of sampling sickly pigs from a wider geographical area. Furthermore, high-risk areas, designated by APHIS as regions with garbage feeding operations, backyard swine operations, feral swine hunting clubs, military bases, international air or sea ports, farming operations utilizing an international labor force, and/or corporations engaging in international swine movement, are subject to active surveillance protocols *via* tonsil collection; 25 states are considered high risk. All garbage feeder operations in the United States are licensed and regularly inspected, and heat treatment of all feed is mandatory. Texas and Florida are considered particularly high risk, and as such, two swine slaughter establishments in Florida and three in Texas randomly collect blood, which is sent to the FADDL for further testing, especially from pigs in the southern portion of each state, light-weight pigs, or those in transition. This active surveillance for CSFV in domestic swine could readily be extended to include surveillance for ASFV as samples are already being collected and transported to FADDL for screening purposes.

Moreover, feral swine are also surveyed as a preventative and early sentinel in the event of a CSFV intrusion. For fiscal year 2017, USDA APHIS National Feral Swine Damage Management Program is rolling out a targeted surveillance plan in which existing feral swine populations, domestic hog production areas, and landfills are used as criteria for determining priority of feral swine samples collected for disease surveillance. Counties are weighted based on the presence or absence of each of the aforementioned criteria. This type of targeted surveillance is crucial to allow for the efficient use of time and resources and to increase the probability of detecting an outbreak early (106, 107). Samples are collected *via* culling operations as well as from hunter-killed pigs, and serology is performed to evaluate the presence of CSFV antibodies. Again, expanding this program to include ASFV screening in feral swine may be beneficial, especially if the perceived risk of ASFV entering the United States increases, as it would likely contribute to early detection in the event of a viral incursion and would be far less costly than an ASFV-exclusive active surveillance protocol.

Importantly, several strains of *Ornithodoros* soft ticks are found in regions with high feral swine populations, especially Texas, Florida, and Oklahoma (*O. turicata*) and California (*O. coriaceus*). Both of these tick species have been shown to become infected with ASFV and *O. coriaceus* is capable of transmitting the virus to susceptible domestic swine >500 days after infection (33). *Ornithodoros* ticks are permissive to ASFV infection with varying capacities for infection; thus, it is hypothesized that other *Ornithodoros* species ticks found in the United States are competent ASFV vectors. The high density of feral and domestic swine in these regions and a strong likelihood for overlapping distribution with potential soft tick vectors further the notion that an active surveillance protocol may be useful and contribute to early detection in the event that ASFV emerges in the United States.

It is important to note that much of the spread of ASF through Eastern Europe and the Caucuses region is likely driven by anthropogenic factors, such as the movement of infected pigs and their products as well as *via* swill feeding (108). However, ticks cannot be overlooked as they are believed to have maintained ASFV in Portugal over a 6-year period during which time the country was declared ASF free prior to the re-emergence of the disease on a single farm (48). The role of vectors in pathogen maintenance and transmission events is often poorly understood, and these long-lived ticks may play a crucial role in conjunction with human activities which likely facilitate ASFV spread.

Sampling of illegally imported and subsequently confiscated, suids, and their products would also provide meaningful data relevant to the types of pathogens being imported. The General Accounting Office (74) estimated that 1–3% of illegally imported wildlife carried by passengers was detected and 1–10% of illegally imported wildlife in declared cargo shipments. Smith et al. (109) performed a pilot study evaluating zoonotic agents in confiscated animal products from John F. Kennedy airport in New York, New York, and found that multiple strains of retroviruses and herpesviruses were present in several non-human primate specimens. Knowledge relative to the types of pathogens entering the United States in illegally imported swine products would be useful in understanding risk of both swine-specific pathogens and zoonotic organisms.

African swine fever presents a substantial threat to both domestic and wild suid species. The concern of viral introduction in the United States has contributed to the implementation of a series of preventive measures designed for importation of live animals and their products. Despite extensive research, knowledge gaps exist, and they have been highlighted as areas for future evaluation.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

VB was involved in the development of the idea, collection of the data, data analysis and interpretation, and preparation of the document. SB was involved in idea development and document preparation.

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REVIEW
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A Review of Classical Swine Fever Virus and Routes of Introduction into the United States and the Potential for Virus Establishment

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Classical swine fever (CSF) is caused by CSF virus (CSFV) which can be the source of substantial morbidity and mortality events in affected swine. The disease can take one of several forms (acute, chronic, or prenatal) and depending on the virulence of the inoculating strain may result in a lethal infection irrespective of the form acquired. Because of the disease-free status of the United States and the high cost of a viral incursion, a summary of US vulnerabilities for viral introduction and persistence is provided. The legal importation of live animals as well as animal products, byproducts, and animal feed serve as a potential route of viral introduction. Current import regulations are described as are mitigation strategies that are commonly utilized to prevent pathogens, including CSFV, from entering the US. The illegal movement of suids and their products as well as an event of bioterrorism are both feasible routes of viral introduction but are difficult to restrict or regulate. Ultimately, recommendations are made for data that would be useful in the event of a viral incursion. Population and density mapping for feral swine across the United States would be valuable in the event of a viral introduction or spillover; density data could further contribute to understanding the risk of infection in domestic swine. Additionally, ecological and behavioral studies, including those that evaluate the effects of anthropogenic food sources that support feral swine densities far above the carrying capacity would provide invaluable insight to our understanding of how human interventions affect feral swine populations. Further analyses to determine the sampling strategies necessary to detect low levels of antibody prevalence in feral swine would also be valuable.

Keywords: classical swine fever, viral introduction, domestic swine, feral swine, emergency preparedness

KEY POINTS

- Classical swine fever (CSF) is currently a foreign animal disease in the United States and the economic consequences associated with an introduction could be severe.
- The virus is endemic in many parts of the world, including Central America, Africa, Asia, and parts of South America.
- Classical swine fever virus is most likely to be introduced to the US *via* the legal or illegal importation of animals or their products.

- Feral swine involvement would significantly challenge disease control and eradication methods.
- Effective live attenuated vaccines are available for use; however, antibodies generated against the current vaccines cannot be differentiated from those generated during a natural infection which complicates control and eradication methods.
- Future research should involve (1) density mapping of feral swine populations in the US as well as long-term studies on a fine spatial scale to evaluate contact dynamics, and movement ecology based on habitat and seasonality, of feral swine as these components are important for contact and subsequently, disease transmission, (2) studies to evaluate the effect of anthropogenic food sources on home range and density of feral swine, and (3) expanded analyses to explore sampling strategies needed to detect low levels of CSF antibody prevalence.

INTRODUCTION

Classical swine fever (CSF), historically called hog cholera, is caused by CSF virus (CSFV) and can result in high morbidity and mortality in swine. This disease is reportable to the World Organization for Animal Health (OIE) and viral detection can severely diminish pork exports. The United States is currently free of CSFV, with the last reported case in 1978 (1). This manuscript outlines what is known about CSFV and aims to describe existing gaps in knowledge. Additionally, a summary of vulnerable sites of CSFV introduction into the United States and persistence within is provided.

Virus Description

Classical swine fever virus is a small, enveloped RNA virus that belongs to the *Flaviviridae* family and as such, is closely related to bovine viral diarrhea virus (BVDV) in cattle and border disease virus (BDV) in sheep (2). The genome contains 12,300 base pairs and comprises four structural and seven non-structural proteins (3, 4).

Transmission and Clinical Disease

Both domestic swine (and their feral counterparts) and wild suids, including javelina, bush pigs, and warthogs are susceptible to CSF. Natural and experimental infections have shown that suids are also capable of transmitting the virus (5, 6). Transmission routes include oronasal transmission through direct or indirect contact with infected pigs, the consumption of feed contaminated with virus, or *via* vertical transmission from infected sow to her offspring (7, 8). The virus is shed from all mucosal surfaces making sexual transmission a possibility. Pork and other pig products are a reservoir for CSFV and survival may be prolonged in heavily proteinaceous environments, especially that of cooled or frozen meat products (9–11). The infection can cause a range of clinical signs from an inapparent, subclinical infection to a hemorrhagic fever-like illness with high mortality (6). The incubation period is typically 7–10 days following infection; however, under field settings it is likely that a herd infection may not be detected for 2–4 weeks, primarily because of limited clinical signs and infrequent monitoring (8).

Classical swine fever strain differences have been observed and attempts have been made to categorize strains as highly virulent (those that kill nearly all pigs irrespective of other factors), moderately virulent (those that cause a sub-acute illness in postnatally infected piglets and sometimes cause abnormalities in fetuses), or avirulent (those that are attenuated and apathogenic in fetuses) (12). However, this classification system is incongruent with other findings where the degree of pathogenicity varies from one pig to another and is believed to be a response to host age (and immune status), viral strain, and inoculating dose (13, 14).

Very little is known about molecular or antigenic properties of the virus that are involved in determining virulence despite numerous sequencing and phylogenetic studies; however, characteristics have been described *in vitro* that allow for some viral virulence determination (12, 15). Virulent strains grow optimally at 39–40°C, moderately virulent strains grow optimally at 35–38°C, and low virulent strains grow optimally at 33–34°C. Highly virulent strains have also been found to grow faster and to higher titers compared to the other CSFV strains in cell culture and are more resistant to heat treatment (12). Furthermore, viral virulence can be artificially abrogated using laboratory techniques and specific proteins and post-translational modifications have been found to play an important role in viral virulence. The recoding of the structural glycoprotein E2 using codon usage deoptimization has been found to result in complete virus attenuation and is capable of protecting against a virulent CSFV challenge (16). p7 is a non-structural, hydrophobic polypeptide that, through the use of reverse genetics, has been found to be pore-forming and is involved in viral virulence (17). Finally, the three glycoproteins E^{RNS}, E1, and E2 were evaluated for the effects of post-translational modifications and those that were not glycosylated failed to induce a detectable virus neutralizing antibody response and did not protect against virulent CSFV (18). Despite our capacity to make targeted mutations that result in complete viral attenuation, the exact properties that contribute to viral virulence remain unknown.

Infection with CSFV typically takes one of three forms: acute, chronic, or prenatal (8) and age, clinical signs, and disease outcome are listed in **Table 1**. Piglets, less than 12 weeks of age, often develop an acute infection characterized by fever, anorexia, lethargy, conjunctivitis, respiratory signs, and constipation followed by diarrhea as well as neurological signs that often include a staggering gait, hind end paresis, ataxia, and convulsions. Death follows 1–3 weeks after the onset of clinical disease (19). With increasing age the clinical signs of an acute infection are less specific and recovery is possible (8). The chronic form develops when pigs are unable to develop an effective immune response. The initial signs are similar to those observed in the acute phase, but as the infection persists the clinical signs become nonspecific, often including intermittent fever, chronic enteritis, and wasting. Pigs may survive 2–3 months before succumbing to the infection and shed virus consistently from viral incursion to death. The pre-natal form occurs when the virus crosses the placenta and infects the fetus during any stage of pregnancy. Abortion, stillbirths, mummification, and malformations are common when

TABLE 1 | Description of each disease form of CSFV.

Age	Virulence of strain	Infection form	Clinical signs	Disease outcome	Reference
<12 weeks >12 weeks	High High to moderate	Acute	Fever, anorexia, lethargy, conjunctivitis, respiratory signs, constipation followed by diarrhea, and neurological signs Less specific and less severe signs when compared with those in younger animals	Typically death Recovery is possible	(8, 19)
Any age	Low	Chronic	Similar to those in the acute phase but as infection persists, signs become non-specific and include intermittent fever, chronic enteritis, and wasting	Typically death	(8)
Neonatal piglets Newborn piglets	Moderate to low	Prenatal (early gestation) Prenatal (days 50–70 gestation)	Abortion, stillbirth, fetal mummification, and malformations Normal at birth then begin to show poor growth, wasting, and/or congenital tremors	Death	(8, 22)

the virus crosses the placenta during early pregnancy; however, if infected 50–70 days into gestation the piglets may become persistently infected. They often appear clinically normal at birth and may survive for several months (called late-onset CSF) prior to showing poor growth, wasting, and/or congenital tremors. These piglets are believed to be the most important cause of viral perpetuation within a population as they constantly shed large amounts of virus (20). This persistently infected phenotype can also be generated by an early postnatal infection with either a lowly or moderately virulent strain of CSFV. While the chronic and prenatal forms of CSFV are always lethal infections, acute infections with CSFV are not always lethal and outcome is dependent upon a myriad of factors, including host age and immune status, and virulence of the acquired strain, among other factors (21). The age component seems to be an important factor that heavily impacts disease outcome, with the same virus and dose potentially resulting in a nearly asymptomatic infection in adult or breeding animals but may cause nearly 70% mortality in young animals (Volker Moennig, Personal communication, 2016). To date, neither beneficial nor detrimental host reaction patterns have been defined, suggesting that the outcome is largely dependent on the immune response of the host, with age as a strong factor. Additionally, differences in pig breed have been evaluated relative to infection with CSFV and it was not found to be a strong predictor of disease course; further suggesting that individual differences are the main driver for the clinical course of infection (6).

Experimental infections using highly virulent, moderately virulent, and lowly virulent strains, classified as described above by van Oirschot (12), demonstrated that the quantity of highly virulent virus shed is far greater when compared with either moderately or lowly virulent strains and is shed from an earlier point of infection (23). Interestingly, a difference is not only observable in the timing and quantity of virus excreted but also in the type of excretions that contain virus. Highly virulent strains are shed *via* all secretions and excretions while lowly virulent strains are restricted to oronasal secretion routes. This variation is thought to be due to viral tropism. Highly virulent strains spread rapidly throughout the body whereas lowly virulent strains are restricted to specific target organs. Mittelholzer et al. (15) developed a clinical score scheme for CSFV infections in pigs that allows for the quantification of observable clinical signs which includes 10 signs that are ranked between 0 (normal) and 3 (severe clinical sign) with a maximal score of 30. Using this clinical scoring format in conjunction with pyrexia it was found that highly virulent strains have clinical signs >15 and a fever ≥ 41°C, moderately virulent strains have clinical signs between 5 and 15 and a fever between 40 and 41°C, and lowly virulent strains have clinical signs below 2 and a fever ≤ 40°C.

While limited data is available for infection of wild boar with CSFV, it is widely assumed that there are no substantial differences between domestic pigs and wild boar in terms of susceptibility and clinical manifestations (21) and the reports that exist concur with this assertion. An experimental inoculation using Eurasian wild boar of various ages and sexes found that the acute course of the disease was independent on the origin of the isolate and that clinical signs varied strongly, both of which have been found in domestic swine (24). Chronically infected suids, those which shed copious volumes of virus for 2–3 months prior to succumbing to infection, serve as a reservoir in domestic swine; however, it is unknown if chronically affected wild boar could survive in their environment, and as such, how much of a role they may play in transmission of CSFV (21). Furthermore, pregnant wild boar sows infected during gestation were found to yield persistently infected piglets (25); although, the role congenitally infected piglets play in CSFV transmission in wild populations is likely limited due to their short survival time (26).

Geographical Distribution

Classical swine fever is endemic in many parts of the world in both domestic swine and wild boar. As a reportable disease, information on specific countries and their annual CSF case load can be found at the OIE website (27). Canada and the United States are disease free and have been for 50+ and 30+ years, respectively (28). Mexico is recently disease free; however, Central America (excluding Panama and Belize which are disease free) is endemically infected, with control maintained through vaccination. Much of South America is endemically infected; however, countries are implementing control strategies such as vaccination, laboratory testing, stamping out, quarantine, control of transit, and import regulations which appear to be facilitating progress toward disease eradication. CSFV is present in Cuba, Haiti, and the Dominican Republic and control practices have been tried and, to date, have failed due in large part to a lack of funding and institutional support. Excluding Japan, CSF outbreaks occur with frequency in Asia and Southeast Asia, with the largest viral diversity found in these regions (1). Africa is believed to be CSF free; however, Madagascar has historically reported cases. Western Europe, specifically European Union member states, have sought progressive eradication throughout the twentieth century and vaccination was banned in 1990; however, the region is not CSFV free due to endemic infection in wild boar, especially in the Baltic states (Latvia and Lithuania), which is transmitted to domestic pigs through direct or indirect contact or swill feeding (1, 28, 29). In Eastern Europe, CSF remains a problem and vaccination in conjunction with stamping out is used to curb outbreak events (1, 28).

In 1997, there was an outbreak of CSFV in the Netherlands which resulted in direct economic losses of $2.3 billion and the death of approximately 9 million pigs (30). The virus is believed to have entered in mid-late December 1996, although the first case of CSFV was not detected until the middle of January and was not confirmed by laboratory diagnosis until the beginning of February. The primary case was at a mixed sow and finishing herd with nearly 1,500 pigs of varying ages in a very pig dense region of the country. A contaminated transport lorry from Germany is believed to have initiated the outbreak but the disease quickly spread between farms in the Netherlands and was exported to Italy, Spain, and Belgium. Routes of transmission that were believed to play an important role in the outbreak were the purchase of infected animals, transport vehicles, personnel, rendering plant cadaver collection service, artificial insemination (contaminated semen), pig slurry, neighborhood transmission, and other unknown factors; the disease was re-eradicated in March 1998. It has since been shown that neighborhood transmission (transmission between herds located within several kilometers of one another) presents a tremendous problem and modeling tools can be used to determine the risk of local transmission patterns (31).

Immune Response to CSFV

Classical swine fever virus targets endothelial cells, lymphoreticular cells, macrophages, and some types of epithelial cells (2). Severe leukopenia is a characteristic finding associated with the early stages of CSFV infection, especially affecting lymphocytes (32). Reduced numbers of circulating B cells (33) and $CD4^+$ and $CD8^+$ T cells (34) have been observed prior to the onset of viremia and the function of T cells isolated during a CSFV infection were found to have compromised function, which is believed to be driven by apoptotic events (35–37). *In vitro* experiments have demonstrated that CSFV readily replicates within endothelial cells where it promotes a strong pro-inflammatory and procoagulatory response (38). If a similar process occurs *in vivo* it is suspected that the host immune response plays an important role in the hemorrhagic pathogenesis of the disease. Granulocytopenia has also been observed within several days of infection with CSFV in both peripheral and bone marrow-derived neutrophils which is thought to be a result of hematopoietic cell death likely due to indirect virus-host mediated mechanisms (39). Microarray analysis following infection with CSFV in swine macrophages and found 79 genes that had altered patterns of expression within 48 h of infection (40). Most of the expression patterns that were changed were found to be involved in the development of the innate immune response.

In young pigs there is a strong correlation between serum IFN-α and the acute disease process, which also directly correlates to the degree of lymphopenia; thus suggesting that high levels of IFN-α do not control the virus but, in fact, may mediate immunopathology (37). The release of pro-inflammatory and vasoactive mediators by macrophages following infection is an important contributor for CSFV pathogenesis. Dendritic cells are likely to release pro-inflammatory cytokines as well as large quantities of IFN-α and IL-12 which promotes T_H1 activation.

DOMESTIC SWINE IN THE US

The commercial production of swine involves high biosecurity in a vertically integrated industry from farrowing through slaughter (National Pork Producers Council, Personal communication, 2016) A majority of the 65 million pigs in the United States are managed indoors under these conditions with Iowa, North Carolina, Minnesota, Illinois, and Indiana boasting the top pork production annually [**Figure 1**; (41)]. Strict rules exist relative to the management of animal feed, transport vehicles, personnel, and other fomites as a means of preventing cross-contamination. Despite regimented biosecurity practices, porcine epidemic diarrhea virus entered the United States in 2013 and has since been traced back to contaminated feed bags, suggesting that the commercial swine industry may not be as refractory to pathogens as previously thought (42). This viral introduction and subsequent spread, suggest that viral stability plays a crucial role in the effectiveness of the biosecurity practices and highly stable pathogens may not be adequately safeguarded against (43).

Hobbyists and backyard farmers are likely to have domestic swine that are not managed under intensive conditions and may be exposed to environmental elements and a number of other species (45). Furthermore, it is possible that their feed may be more diverse and likely involves less oversight when compared with commercial operations. The map of domestic pig production in the US depicts high densities of domestic swine and indicates where a CSF introduction would likely be most problematic to the swine industry (**Figure 1**).

EURASIAN WILD BOAR AND CSFV

Native to much of Europe and Asia, Eurasian wild boar pose a challenge for the control and eradication of CSFV in that region. Experimental inoculations have demonstrated that they are acutely susceptible to the virus but their exact role in maintenance or transmission events to domestic swine remains unknown (6). Large scale culling events of domestic swine due to CSF occurred in Austria, Belgium, the Czech Republic, Germany, Italy, Spain, and the Netherlands between 1991 and 2001. It has been suggested that CSF in wild boar is associated with the persistence of the disease in domestic swine, although viral persistence in either population can readily be transmitted to the other (46). Interestingly, serum samples collected from 259 wild boar in Croatia in 2003 found that 47% of the pigs sampled were positive for the presence of CSFV antibodies using a highly specific ELISA

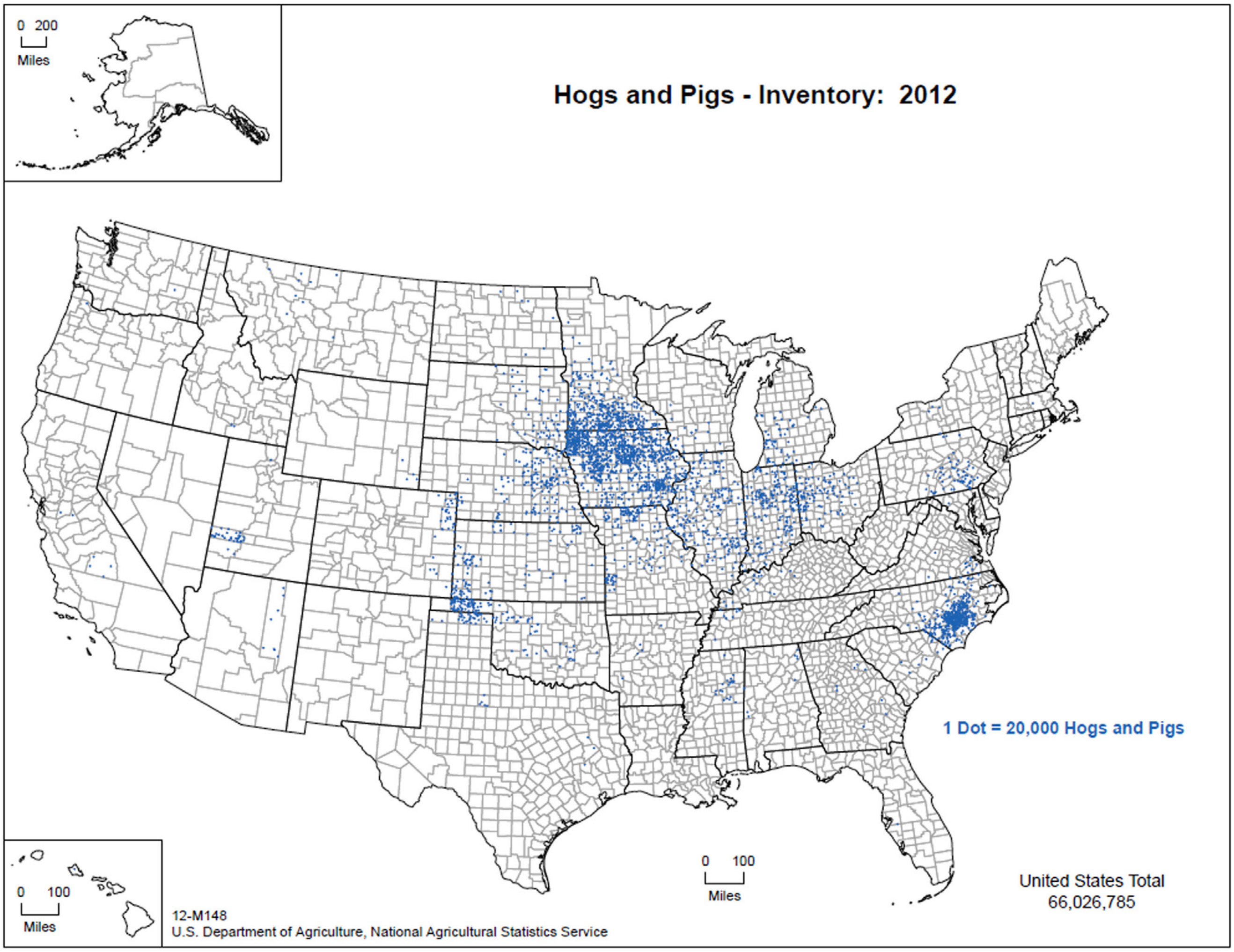

FIGURE 1 | Distribution of pig production within the US, 2012 (figure courtesy of: United States Department of Agriculture, National Agricultural Statistics Service (44)—used with permission).

(47). There was a statistically significant difference in CSFV seropositivity recorded between age groups with pigs less than 1 year having the highest likelihood of being seropositive, which may be reflective of the presence of maternal antibodies. These data suggest that the virus is either being maintained within the population and infects the young after maternal antibodies wane or that the virus is being constantly reintroduced from domestic swine infected with CSFV ((47); Volker Moennig, Personal communication, 2016).

In France, wild boar were sampled between 1992 and 2002 as part of a compulsory monitoring project to track CSF infection in the native suid fauna (48). Originally viroprevalence was used to estimate incidence; however, low incidence makes finding a viremic animal very rare as virus can only be detected for a few weeks following infection. Conversely, antibodies can be detected for extended time periods following infection and recovery. Antibody prevalence was monitored over the ten year period with overall seroprevalence used to estimate the proportion of immune wild boar, seroprevalence in juveniles used to estimate the incidence, and seroprevalence in different age classes used to estimate incidence evolution in a given cohort. Spatial and temporal trends found that after 2000, no seropositive juveniles were detected and that the epizootic was regionally extinct. Using seroprevalence in juveniles to estimate incidence is likely an underestimation of the true incidence as CSFV is acutely virulent in juveniles such that most would die prior to antibody development; however, it is believed to be a useful metric given the limitations associated with sampling wild species and the short duration of a viremia. A capture-mark-recapture study confirmed the above findings, such that most (80%) wild boar piglets that become infected with CSFV succumb to infection within two weeks and those that survive infection (20%) recover quickly (49). Host innate immunological factors were found to be associated with fitness in wild boar, with high complement activity associated with the highest probability of survival (49). No chronic CSFV infection was observed in wild boar piglets (49).

Historically, the viral kinetics of CSFV entering a naive wild boar population appear to proceed to extinction within a few years of viral incursion; however, recent outbreaks observed in France, Germany, and Italy were characterized by a mortality peak succeeding the initial infection followed by a slow progressive decrease in the infection rate over a long time period (46). Laddomada (50) articulated that there are two main factors that contribute to either an epizootic event or a persistent, endemic infection: (1) the availability of susceptible animals (which is influenced by population size, herd immunity, and age structure and dynamics) and (2) the frequency of infectious contacts (which is influenced by density and animal movements). Artois et al. (46) articulate that wild boar are unlikely to serve as a true reservoir species because, (1) eradication of CSFV from domestic populations typically results in disease disappearance from wild suids, (2) intentional release of CSFV was performed in wild, free ranging boars and was not found to persist, and (3) when appropriate epidemiological data was collected regarding the outbreak among wild boars, human errors (feeding, burying of contaminated carcasses, among others) were found to be involved in nearly every case. However, wild boar density is important and factors into the role that these native suids play in viral maintenance and transmission. In high density regions the disease tends to become endemic, whereas in lowly dense regions it often dies out over time. Young wild boar whose maternal antibodies have waned are believed to be responsible for the majority of transmission as older animals are already immune, either as a result of vaccination or having survived a natural infection. Hunting targeted at reducing the young wild boar population can be used to diminish the number of susceptible hosts which can also be useful in curbing an outbreak event (Volker Moennig, Personal communication, 2016).

Feeding of wild boar in Europe has risen in popularity which results in both more interaction among the wild boar as well as population sizes that may exceed the natural carrying capacity. Factors particular to a specific epidemiological scenario play an important role in the capacity for wild boar to become endemically infected: population density, frequency of interaction, and social structure, among others (48). Furthermore, frequent reintroductions from infected domestic swine may give an impression that the disease has become endemic within the wild boar population (Volker Moennig, Personal communication, 2016).

The control of disease in wildlife is often very challenging, however, vaccines can be used to combat infectious disease by decreasing the proportion of susceptible animals below a threshold necessary for disease maintenance within the population (49). The C-strain live attenuated vaccine (discussed in detail below) has been found to be highly efficacious and palatable baits have been developed for oral delivery in wild boar. In order to curb an outbreak of CSFV in wild boar in Germany in 2009, a vaccine regimen was developed which involved three double campaigns in spring, summer, and autumn (51). The protocol was designed to maximize both antibody titers and the proportion of vaccinated juvenile wild boar, as such, an initial bait was dropped followed by a booster 28 days later. The C-strain vaccine is derived from a genotype 1 strain whereas the circulating field virus was a genotype 2 strain; thus, using a multiplex real-time RT-PCR assay with partial sequencing assay vaccinated animals could be differentiated from those naturally infected. This strategy depends on the epidemiological setting as regions with genotype 1 viruses circulating would not be able to use this multiplex assay to differentiate.

Developing an oral bait that is detectable (odor, color), palatable (odor, taste), and that is effectively ingested are all crucial components for a successful mass oral vaccination program and quite difficult (49). Despite their omnivorous diet, wild boar were found to prefer baits containing plant derived compounds when compared with animal derived compounds. To further complicate this program, it is necessary that the vaccine be released in the oral cavity such that the tonsils can initiate the immune response. The vaccine must be perforated by pig teeth prior to swallowing; bait size is crucial as too small will likely be swallowed prior to perforation and too big will limit the number of animals that uptake the vaccine. Field trials were first performed in Germany in the 1990s and were then deployed to other European countries during the 2000s. Prebaiting, the practice of accustoming wild boar to the bait prior to vaccine distribution, was found to be necessary and the vaccine bait was delivered by hunters to account for wild boar foraging which occurs in groups, such that concentrating baits in feeding places was far more effective when compared with random distribution by aircraft.

DIAGNOSTICS

The diagnosis of CSF typically consists of four complementary elements (although all four are not always detectable) which include field clinical signs, gross pathology findings, indirect detection (serology), and direct detection (virus isolation or antigen or nucleic acid detection) (52). Live virus, as well as RNA, can be detected from blood, tonsil swabs, or tissues upon necropsy (19). Samples collected from live animals should be taken when the animal is pyrexic as it substantially increases the probability of viral detection. The OIE manual recognizes a myriad of assays as acceptable means for detecting CSFV, such as a fluorescent antibody test, immuno-peroxidase staining, antigen capture ELISA, virus isolation in cell culture, and RT-PCR; some assays are designed to evaluate live virus or viral particles while others detect CSFV-specific antibodies (19, 53–55).

Antibodies are first detectable 2–3 weeks following initial infection and often persist for the duration of the life of the pig (52). Serological assays are highly useful for both diagnostics and surveillance and the OIE recommends a fluorescent antibody neutralization test, a neutralizing peroxidase linked assay, or an antibody ELISA (55). Viral neutralization assays are regarded as the "gold standard" but they are labor intensive and require cell culture capabilities, which are not always available. ELISAs for CSFV diagnosis are typically designed for the E2 glycoprotein and this assay type is heavily used as a screening tool for antibodies during and after outbreaks, monitoring CSFV infection in wild boar populations, and to evaluate vaccine coverage following an oral administration in wild pigs (52). Despite widespread use, the majority of well-established traditional assays used to detect CSF virus or antibodies are not validated by OIE standards as the first chapter outlining methods of validation for diagnostic assays was not published until 1996 (55). It is important to note that antibodies for both BVDV and BVD can cross react with CSFV-specific antibodies and in some cases, serological assays may provide an inaccurate read unless specifically guarded against (56).

VACCINES

Currently, multiple live attenuated vaccines are commercially available and have been found to be safe and highly efficacious against infection with CSFV (57). As an example, the lapinized Chinese vaccine (C-strain) was developed in the 1950s by Chinese researchers and has been used extensively to control CSF in China and many other countries (58). Despite providing sterilizing immunity to nearly all vaccinees within one week of vaccine administration, current live attenuated vaccines for CSF have a major disadvantage: it is impossible to differentiate between naturally infected and vaccinated animals using serological methods (55, 59). This is concerning as vaccines are being used to supplement other control methods and viral transmission cannot be effectively modeled due to the lack of assays capable of differentiating between vaccinated and infected animals. However, Zhao et al. (58) describe a multiplex real-time RT-PCR assay that is both rapid and sensitive for differentiating between wild-type viruses and the C-strain vaccine for CSFV in China. This assay is only applicable for C-strain based vaccines, and is not capable of distinguishing between other exotic vaccines; with further work, it remains a possibility that differentiating assays may become available for more live attenuated CSFV vaccines that work on a global scale.

As a means of circumventing the primary concern associated with live attenuated vaccines, several other vaccine strategies have been employed, such as the generation of immunogenic CSFV particles, DNA vaccines, viral vectors expressing CSFV proteins, chimeric pestiviruses, and trans-complemented deleted CSFV genomes (57). These novel vaccines require a myriad of doses and dosages to attain various levels of protection against CSFV challenge. E2 has been found to be a highly antigenic envelope glycoprotein that can be expressed using a baculovirus expression system and has been found to induce a neutralizing antibody response in pigs (60, 61). Importantly, animals vaccinated with the recombinant E2 vaccine, also referred to as a subunit vaccine, can be readily differentiated from those naturally infected as the latter will generate a polyclonal response that involves E2 in addition to NS3 and E^{RNS} whereas vaccinated animals will develop a monoclonal response against E2 exclusively (62). An ELISA has been developed that detects E^{RNS} and can effectively be used to differentiate samples from vaccinated and infected animals (55, 63). Two E2 vaccines are currently licensed by the European Agency for the Evaluation of Medicinal Products and commercially available despite the higher risk of persistently infected animals and the need for increased caution with pregnant sows (63). In addition to the subunit E2 vaccines, chimeric pestivirus vaccines have been evaluated and promising results were found for CP7_E2 alf (which is a BVDV backbone expressing the CSFV E2 glycoprotein) and flc11 (a CSFV backbone with the E^{RNS} gene replaced by the corresponding BVDV gene) (64). Both of the aforementioned pestivirus candidates were comparable to the C-strain vaccine and upon early challenge, CP7_E2 alf was found to be better for safety and efficacy following oral administration. The marker concept has been demonstrated but the discriminatory assays require further optimization.

Field efficacy of the CP7_E2 alf vaccine was evaluated using experimental infections of both Eurasian wild boar and domestic swine (65). Following vaccination, experimental domestic swine or wild boar are typically challenged with a CSFV genotype 1.1 strain, which represents a homologous virus to the vaccine strain but is unlikely to be reflective of circulating field isolates. Vaccination with CP7_E2 alf followed by challenge with virulent CSFV genotypes 2.1 and 2.3 led to complete protection which affirms the field applicability of the chimeric pestivirus vaccine. Furthermore, longevity of immunity studies were undertaken to evaluate the duration of protection following vaccination with CP7_E2 alf followed by infection with a virulent CSFV strain (66). Domestic swine were vaccinated orally or *via* intramuscular injection with CP7_E2 alf and challenged with a virulent CSFV strain six months following vaccination. Antibody titers were stable for the duration of the 6 months irrespective of the route of vaccination and high antibody titers lead to full virus neutralization and full protection following lethal challenge was observed. One non-responder was observed following oral vaccination, suggesting that the oral route of vaccination leads to a more variable response. The CP7_E2 alf vaccine is a very promising prophylactic and its capacity to be differentiated from a natural infection provides a tremendous advantage. This vaccine is currently licensed by the European Medicines Agency.

CSF AND THE UNITED STATES

The domestic swine industry in the United States would likely be very negatively impacted in the event of CSFV introduction. Passive and active surveillance programs, defined as using reports and testing of animals found dead and developing a program to capture animals in some way (live-capture, hunter harvest, euthanasia) for testing, respectively (67), exist for both domestic and feral swine. USDA Veterinary Services have outlined a surveillance program for domestic swine and the objectives are as follows: (1) surveillance for rapid detection of CSFV in US swine, (2) monitor the risk of introduction of CSF into US swine, (3) surveillance of international CSF status, and (4) surveillance to document freedom of CSF (68). Passive surveillance is performed in all states and requires involvement by producers, diagnosticians, and slaughterhouse inspectors, among others to report and sample any suspect cases. Unthrifty pigs, considered to be those that gain weight poorly or are otherwise somewhat sickly, are often sold to off market vendors and APHIS field staff or other cooperating personnel collect tonsil samples in these markets as a way to survey for infectious agents, including CSFV. This method is deemed to be an effective surveillance strategy as pigs from surrounding regions are often consolidated in these markets which makes for an efficient means of sampling sickly pigs from a wider geographical area. Furthermore, high risk areas, which include regions with garbage feeding operations, backyard swine operations, feral swine hunting clubs, military bases, international air or sea ports, farming operations utilizing an international labor force, and/or corporations engaging in international swine movement, are subject to active surveillance protocols; 25 states are considered high-risk. All garbage feeder operations in the United States are licensed and regularly inspected and heat treatment of all feed is mandatory. Texas and Florida are considered particularly high risk and as such, two swine slaughter establishments in Florida and three in Texas randomly collect blood which is sent to the Foreign Animal Disease Diagnostics Laboratory for further testing, especially from pigs in the southern portion of each state, light weight pigs, or those in transition.

Feral swine are also surveyed as a preventative and early sentinel in the event of a CSFV intrusion. Feral animals are typically referred to those who have been phenotypically selected by humans but do not live under human supervision or control (69). Feral swine (*Sus scrofa*) describe a genotypically diverse composite of suids that include escaped domestic swine, truly wild Eurasian boars, and their hybrids (70, 71). There are ongoing surveillance programs for CSFV antibodies in feral swine and targeted areas typically include domestic hog production areas and landfills. Counties are weighted based on the presence or absence of each of the aforementioned criteria (72). This type of targeted surveillance increases the probability of early detection in the event of virus introduction (73, 74). Samples are collected *via* culling operations as well as from hunter-killed pigs and serology is performed to evaluate the presence of CSFV antibodies. Serological assays are used in series, beginning with an ELISA, followed by an immunoperoxidase test, and finally virus neutralization. As soon as a sample tests negative, it is no longer assayed (e.g., if a sample is negative at the ELISA it is not tested by immunoperoxidase or virus neutralization). This series of diagnostic assays exists as antibodies against BVDV and BDV cross react with CSFV which may result in a false-positive reading; thus, the downstream diagnostics are increasingly specific.

In addition to domestic and feral swine surveillance efforts, plans have been developed in the event of viral incursion (75). The primary document outlines the four key outbreak strategies: (1) stamping out, which involves depopulating clinically affected animals and in-contact susceptible animals; (2) stamping out with emergency vaccination to kill, in which clinically affected and in-contact susceptible animals are depopulated and at-risk animals are vaccinated and subsequently depopulated and disposed of; (3) stamping out with emergency vaccination to slaughter, in which clinically affected and in-contact susceptible animals are depopulated and at-risk animals are vaccinated and slaughtered and processed; or (4) stamping out with emergency vaccination to live, which involves depopulating clinically affected animals and in-contact susceptible animals and vaccinating at-risk animals whence they are not depopulated. Multiple factors influence the response strategy, including scale of the outbreak, outbreak consequences, acceptance, available veterinary countermeasures, and available resources for implementing response strategies. A detailed approach is also needed on how to provide relevant information to responders and stakeholders during an outbreak (76).

The United States also harbors a supply of CSFV vaccines at the National Veterinary Stockpile. The US maintains both the C-strain live attenuated vaccine as well as the CP7_E2 alf vaccine (Personal communication, 2016). The C-strain vaccine has been used extensively in control and eradication programs in many countries throughout the world and its efficacy, safety, onset of immunity, duration of immunity, and many other factors are well characterized in the field. The CP7_E2 alf vaccine has been used to a much lesser extent in field applications and performance is less well characterized. Each product requires one vaccination to result in sterilizing immunity; however, as discussed above in the vaccine section, antibodies generated in response to vaccination with the C-strain vaccine cannot be differentiated from those developed by animals who are naturally infected whereas the CP7_E2 alf vaccine allows for differentiation.

SUMMARY OF VULNERABILITIES FOR THE INTRODUCTION OR PERSISTENCE OF CSFV IN THE US

Risk of Introduction into the United States

The legal movement of live animals or their products, byproducts, or animal feed, the illegal movement of live animals and their products, or an intentional viral release in an act of bioterrorism are all channels through which a disease outbreak of CSFV is likely to occur. These routes are the most probable means of introduction due to patterns of virus transmission, viral stability, and current global instability. Each of these possible routes of introduction are displayed in **Figure 2** and described in detail in this section.

Legal Movement of Live Animals

In 2015, the only live swine imports into the United States (domestic or exotic/wild species) came from Canada in which just under 6 million pigs were imported for breeding, feeding, or direct to slaughter. Canada is CSFV free so it is unlikely that a live pig imported from the northern border would be responsible for a viral incursion.

Legal Movement of Animal Products, Byproducts, and Animal Feed

A number of animal products, byproducts, and feed are imported into the United States annually and permits are required. Treatments typically involving heat, pH, or fixation processes are required for all animal products and byproducts that are being imported from CSF-endemic regions. Unprocessed products are permitted for entry from CSF-free countries. Specific temperature and duration cooking is required for animal feed imported from countries endemic for CSF. The European Union is designated as a low-risk region and as such, products can be imported raw; although documentation is required to certify that the product is coming from an unaffected herd in an unaffected region.

Illegal Movement of Live Animals and Their Products

The risk of virus introduction through illegal transport of live animals and their products is related to the types of products being moved, and their country of origin and final destination.

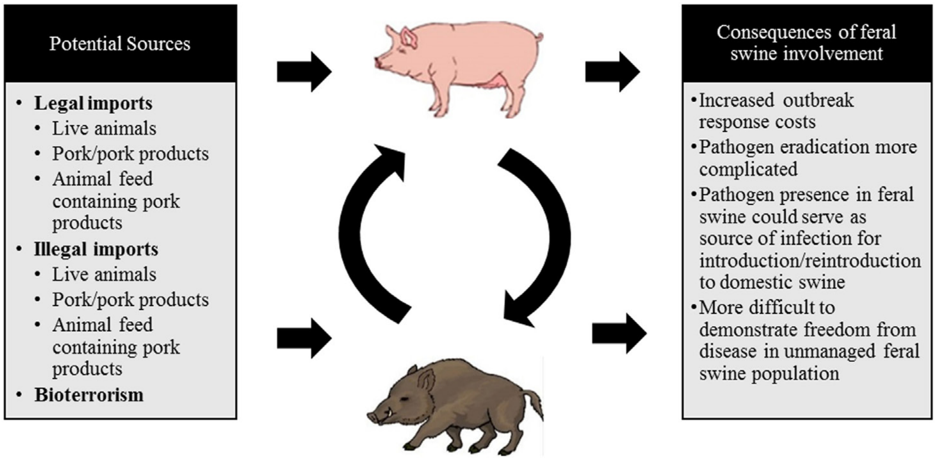

FIGURE 2 | A schematic diagram depicting the potential sources of a classical swine fever virus (CSFV) incursion into the US and the consequences of feral swine involvement.

The US Department of Homeland Security Customs and Border Protection (CBP) confiscates products and specimens from domestic animals in the cargo and express courier environments. More than 68,000 products and specimens were confiscated by CBP between 2012 and 2016 and over 90% of confiscations were of products originating in Asia and Europe. These findings confirm that illegally imported products and specimens serve as a route for CSFV introduction as Asia and parts of Europe are endemic for the virus.

The confiscation of illegally imported wildlife and their products fall under the jurisdiction of the US Fish and Wildlife Service. Between 2006 and 2016, 133 wild suid products were confiscated and the majority of the products seized were from Africa. It is important to note that a tiny fraction of all illegally imported wildlife shipments are believed to be detected (77); thus, these numbers likely represent a gross underestimation.

Bioterrorism

Classical swine fever virus is a bioterrorism candidate because of the tremendous economic value of the domestic swine industry in the United States, the clinical disease associated with infection, the endemic status of many countries globally making viral acquisition a ready option, the robust stability of the virus in a proteinaceous environment and the crippling implications for international trade. As such, domestic and feral swine must be surveyed for disease frequently and systems for rapid diagnosis must be readily available.

Factors That Complicate Eradication Efforts following Introduction

Feral swine are found in large numbers across much of the US and their highly flexible diet, interaction with domestic pigs, and unmanaged lifestyle make them an opportune vector for further disease transmission following an introduction event. In the event of a viral incursion feral swine could contribute to amplification and transmission events to other feral swine or their domestic counterparts and would serve to significantly complicate disease control measures (78).

Feral Swine

Feral swine include released domestic swine, truly wild Eurasian boar, and their hybrids (79). Nearly 6 million feral swine roam the US and are believed to be found in at least 35 states. They have been shown to carry a wide variety of pathogens capable of infecting domestic livestock (80) and GPS data has shown feral swine interacting with domestic swine (45). These types of interactions increase the risk of pathogen transmission from feral to domestic swine. Alternatively, the evaluation of interspecies interactions using GPS and proximity loggers between cattle, domestic pigs, Eurasian wild boar, and red deer found very limited interactions between wildlife and livestock (81), although feral swine may not behave as Eurasian wild boar relative to their social behavior. Peccaries are not included as a risk as they are not believed to be important for virus maintenance and transmission (82).

While domestic swine are likely the higher risk group for acquiring CSF, it is important to note that feral swine could participate in a CSFV outbreak in the US in one of the several ways. Feral swine regularly are found scavenging in landfills and consumption of CSFV-contaminated garbage (e.g., airport waste) could lead to an introduction event directly into feral swine. The index feral swine could then infect other feral swine as well as domestic pigs, particularly those housed in a backyard setting. Alternatively, some domestic pigs are fed swill and illegally imported or improperly treated swill could result in a CSFV introduction directly into domestic swine which could then spillover into feral swine.

As described previously, feral swine are routinely culled and samples are collected as part of an active surveillance program to ensure rapid detection and diagnosis in the event of a CSFV incursion. Swafford et al. (83) published antibody data against CSFV in feral swine and found that no antibodies were detected in collection years 2007 and 2008. In fact, no CSFV-specific antibodies have been detected in feral swine since the inception of the program through the current day (Kerri Pedersen, Personal communication, 2016). In the event of a CSFV introduction, large sample sizes are required to detect low prevalence pathogens and sampling on a country-wide scale, with a feral swine population exceeding 6 million animals, would necessitate an extremely large sample size and subsequently, a substantial and sustained economic investment. Simulation models conducted in Germany demonstrated that the financial resources and personnel necessary for reliable testing are substantial and difficult to sustain over time. In addition, sufficient sample sizes to detect low virus prevalence are difficult to obtain (84). Large and costly efforts would be needed to test a statistically significant portion of the population, which would require significant funding and a continuous effort over time.

Disease-emergence dynamics modeled in feral swine, a highly gregarious and social species (85), demonstrated that under realistic demographics and contact structure, a CSFV-like disease could persist for long periods of time resulting in many more cases (78). These data are in agreement with a model that demonstrated that CSFV in wild pigs in Australia was able to both persist and spread across the landscape (86). These findings support the assertion that feral swine present a concern for viral incursion because of their behavior—omnivorous and opportunistic diets, close social interaction, and unmanaged movement—and that despite active surveillance efforts, infection may remain silent until a substantial portion of the population displayed antibody presence or morbidity or mortality events. However, introduction of CSFV into a naive population is often accompanied by high morbidity and mortality as observed by the detection of an increase in the number of dead animals on the landscape, which would presumably be detected and investigated by field biologists (87).

Disease transmission modeling in feral swine must account for their social activities and lifestyles. Sounders are comprised of reproductively active females and their young while males typically live a solitary life. GPS data has shown that contact rates are much higher for animals within the same sounder when compared with those animals in different sounders (88). Sounder interaction is reduced at distances >2 km and disease transmission is also expected to be reduced (89). These findings suggest that the quarantine surrounding a positive premise should be at least 2 km. Importantly, lone boars tend to have much larger home ranges (90) which could complicate quarantine and surveillance efforts in the event of a disease outbreak. It is important to note that under the assumption that transmission of CSFV among wild boar occurs primarily through direct contact, CSF incidence should increase with increasing host density and should go extinct under a threshold density of susceptible hosts. Interestingly, however, analysis of the incidence and viral persistence of CSF in the French Vosges Forest demonstrated that infection depressed density but did not support the hypothesis of density dependence of incidence (91). This suggests that the presence of circulating CSFV reduced the population of wild boar but that viral transmission was not strictly density dependent. However, these findings may not be representative of all ecological settings as density has appeared to be a crucial factor for CSFV transmission in a multitude of other studies (2, 8, 21).

Feral swine are of tremendous concern in the event of disease outbreaks that affect domestic livestock. Hog panels have been evaluated and can effectively contain feral swine (92). Although relatively cheap and quick to erect, fencing is only a viable option to control movement of feral swine on a small scale.

Targeted culling of young wild boar would likely not be efficacious in the United States to curb an outbreak as the entire US feral swine population is susceptible to infection with CSFV when compared with the scenario in Western Europe where older animals are typically immune as a result of vaccination or a survived infection with CSF.

In many instances in the United States, vaccination of susceptible animals following the introduction of a foreign animal disease is not a viable option for a myriad of reasons; however, it could be an option in the event of a widespread outbreak. Vaccination using oral baits would be a potential strategy to curb an outbreak in feral swine as they have been used very effectively in Eurasian wild boar in the European Union.

Outbreak specific characteristics would be important to include, such as the amount of time that has elapsed since the first case, the virulence of the CSFV strain, the density of both domestic and feral swine, among many other components. Multiple control and mitigation strategies could be employed in the event of a foreign animal disease introduction that may have spilled over into feral swine populations (**Figure 3**).

Sounders that are in direct contact with swine from the infected premise ("direct contact sounders") are included as well as "distant sounders" which are likely to interact with "direct sounders." A lone boar is also included in this depiction and often have a

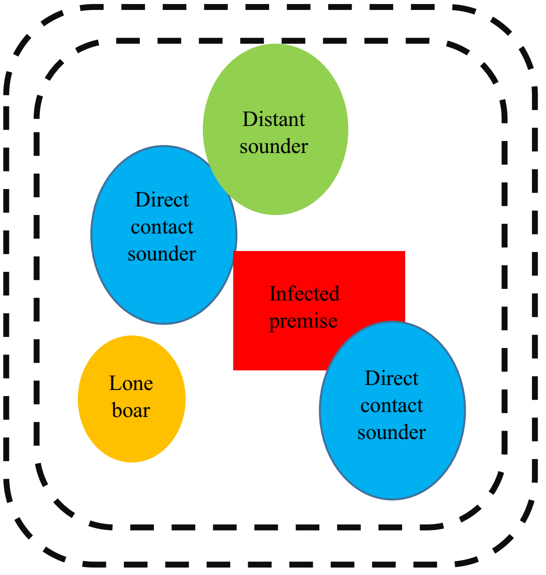

FIGURE 3 | Example illustration of how disease management may be approached in a feral population. It is important to note that this graphic is not drawn to scale (e.g. sounders contain more pigs but have smaller ranges when compared with lone boars) and that all of the groups depicted in this graphic are dynamic and in constant movement.

much larger home range when compared with sounders; they are likely to be interacting with domestic swine on the infected premise, "direct contact sounders," and "distant sounders," as well as sounders that exist outside of this graphic and other boars. The exact distance of the perimeter fencing (demarked as black dotted lines) would be determined based on factors related to sounder home range, the environment, and pathogen specific components; however, the below diagram provides insight into how disease management may be approached in a feral population. A double perimeter fence would be employed as CSFV can be readily transmitted by direct contact; thus, it would be essential to prevent feral swine within the quarantine region from interacting with feral swine outside the quarantine region. The graphic has been simplified to include only one infected premise within the quarantine area; however, this is unlikely to be accurate in rural regions of the United States where hobby farms are abundant.

Disease surveillance in feral and wild animals is challenging for a number of reasons and control and eradication measures are abundantly complicated. Resource allocation and a systemic perspective is useful when making important decisions in the absence of information (93). Currently both the northern and southern borders in the United States are porous, which in the event of a CSFV introduction into either Canada or Mexico, the fluidity of feral swine moving across the border region will be challenging.

CONCLUDING REMARKS

Due to the severe morbidity and mortality caused by CSFV in domestic swine, introduction (either accidental or purposeful) presents a risk to the United States. Federal oversight is provided for the importation of live animals and their products and comprehensive mitigation strategies are mandated for products originating in CSFV-endemic countries. Despite these safeguards, the illegal importation of animals and their products is an avenue that is inherently unrestricted; thus, serves as a viable route for viral introduction. An act of bioterrorism is also a potential route of viral introduction. Active surveillance of domestic swine and efficient channels for disease reporting are imperative to allow for the rapid diagnosis of infectious disease in swine.

Classical swine fever virus introduction in feral swine would complicate management and eradication. Unrestricted movement of feral swine along the US, Canada, and Mexico borders could be an issue in the event of a virus introduction into any of the three countries. Simulation models have demonstrated that CSFV-like pathogens are likely to persist and spread across the landscape in feral swine populations (78, 86) and despite active antibody surveillance it is likely infeasible to sample the appropriate number of feral pigs in order to reliably determine that CSFV is not present in the population (84); although the sampling is likely sufficient for a morbidity or mortality event. However, developing detailed population and density maps for the feral swine population in the United States would be useful in the event of a viral incursion. This knowledge could be harnessed to anticipate risk of transmission to domestic livestock or wildlife species and to determine strategies for preventing spread and eradicating the virus in the specific region of concern. In conjunction with population density mapping, long-term studies on a fine spatial scale should be conducted to evaluate contact dynamics, movement ecology based on habitat, and seasonality of feral swine. Kramer-Schadt et al. (94) articulate that knowledge of social structure, dispersal, and population densities are key to understanding epidemics. Furthermore, studies evaluating anthropogenic causes of clustering in feral swine (e.g. landfills, bait stations, etc.) would be very useful in determining the distance of attraction for these food sources and how their presence alters both the natural carrying capacity for feral swine as well as their behavior and any related changes in home range. Finally, further analyses should be performed to determine the sampling protocols necessary to detect low levels of CSF antibody prevalence in feral swine.

AUTHOR CONTRIBUTIONS

VB was involved in the development of the idea, collection of the data, data analysis and interpretation, and preparation of the document. SB was involved in idea development and document preparation.

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Trends and Predictors of Large Tuberculosis Episodes in Cattle Herds in Ireland

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Persistence of bovine tuberculosis (bTB) in cattle is an important feature of *Mycobacterium bovis* infection, presenting either as herd recurrence or local persistence. One risk factor associated with the risk of recurrent episodes is the severity of a previous bTB episode (severity reflecting the number of bTB reactors identified during the episode). In this study, we have sought to identify predictors that can distinguish between small (less severe) and large (more severe) bTB episodes, and to describe nationally the severity of bTB episodes over time. The study included descriptive statistics of the proportion of episodes by severity from 2004 to 2015 and a case-control study. The case-control study population included all herds with at least one episode beginning in 2014 or 2015, with at least two full herd tests during the episode and a minimum herd-size of 60 animals. Case herds included study herds with at least 13 reactors whereas control herds had between 2 to 4 (inclusive) reactors during the first 2 tests of the episode. A logistic regression model was developed to identify risk factors associated with a large episode. Although there has been a general trend towards less severe herd bTB episodes in Ireland over time (2004–2015), the proportion of large episodes has remained relatively consistent. From the case-control study, the main predictors of a large episode were the year the episode started, increasing herd-size, previous exposure to bTB, increasing bTB incidence in the local area, an animal with a bTB lesion and a bTB episode in an associated herd. Herds that introduced more animals were more likely to have a smaller bTB episode, reflecting the reduced risk of within-herd transmission when an episode was due to an introduced infected bTB animal. Some of the risk factors identified in this study such as reactors in previous bTB episodes, herds with an associated herd undergoing a bTB episode, herds in high incidence areas etc. may help to target future policy measures to specific herds or animals for additional surveillance measures. This information has important policy implications.

Keywords: bovine tuberculosis, *Mycobacterium bovis*, large episodes, Ireland, cattle

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INTRODUCTION

Bovine tuberculosis (bTB) is a zoonotic disease caused predominantly by infection with *Mycobacterium bovis*. In Ireland, bTB is endemic in cattle and a test and slaughter eradication programme has been in place since 1954. The programme consists of testing every bovine in all herds annually using the Single Intradermal Comparative Tuberculin Test (SICTT) using Bovine Tuberculin PPD at 30,000 I.U./ml and Avian Tuberculin PPD at 25,000 I.U./ml, along with abattoir surveillance. The latter involves inspection of all bovine carcases at slaughter for tuberculous lesions by veterinary practitioners. When one or more positive animal is identified at the SICTT or at slaughter the herd is then "restricted" i.e., outward or inward movement of cattle is permitted only in accordance with EU Directive 64/432/EEC (1). The herd remains restricted until two consecutive negative herd tests, at 60 day intervals, are achieved. A bTB episode is defined as the full period when movement restrictions are imposed; that is, from initial detection of infected animals through to the final clearance test (generally the second consecutive negative herd test). Following "de-restriction" the herd is again free to trade, is then tested at 6 month intervals for two years and thereafter it returns to annual test intervals.

When the national programme first began, animal incidence was 17% (2) but has since declined to 0.26% in 2015 (More et al., submitted). In the latter year there were 3,823 new herd restrictions, giving a herd incidence rate of 3.37% (3). McGrath et al. (4) have highlighted an improving trend in many areas of Ireland. However, the improvements were highly heterogeneous and the overall decreasing trend was not uniform across the country. During 2003–12, the majority of herds had none or only one movement restriction due to bTB (hereafter referred to as a restriction), while 3.7% underwent two or more high risk restrictions and 0.9% had three or more, with a high risk restriction defined as at least 2 positive (reactors i.e., an animal removed under the bTB programme or lesion in a non-reactor) animals (5). Similar figures, in terms of positive animals, were found in Northern Ireland where 27% of herds contributed 56% of reactors between 2001 and 2003 (6). There are few published statistics, using Irish data, available that describe the severity of a restriction. A recent study (More et al., submitted), has looked at various measures of severity, duration and frequency of restrictions within the UK and Ireland, however, the herds included in the study had to meet certain criteria in order to make national comparisons. One aim of the current study will be to describe the severity of bTB restrictions within Ireland over time.

Persistence of bTB in cattle herds is an important feature of *M. bovis* infection, presenting either as herd recurrence or local persistence, and can be attributed to several sources such as residual infection, environmental infection (including wildlife), farm to farm transmission and the introduction of new infection following cattle movement (5). One risk factor identified as being associated with the risk of recurrent restrictions is the severity of a previous episode (7–12). Olea-Popelka et al. (7) found that herds with more than 8 reactors to the SICTT were nearly 3 times more likely to have another episode within 5 years compared with herds not previously infected. Similarly Wolfe et al. (8) found that cattle moved from a herd that had just had a bTB episode with at least 8 reactors were 1.8 times more likely to be bTB positive in the next 2 years when compared to animals moved from a non-infected herd. They also found that cattle moving from herds with 1 to 7 reactors had a non-significant increase (1.2 times) in the future risk of being positive. Wolfe et al. (9) looked at the future risk of a restriction for herds restricted in 2001 and found those with 1–5 standard reactors had a hazard ratio (HR) of a future restriction of 1.3 (95% CI: 1.1–1.4); those with more than 5 standard reactors had a HR of 1.6 (95% CI: 1.4–1.8) compared to herds with 0–1 standard reactors. Clegg et al. (12) found that herds with a more severe bTB episode in the past had higher odds of a future episode and that persistence continued for many years. In this study, herds with 2 or more reactors had a significantly increased risk of a future episode compared to those with 0 or 1 standard reactor. The risk decreased as time since the previous restriction increased but not significantly until at least 2 years prior to the current restriction.

Several studies have looked at the risk factors for predicting chronic episodes by considering either the length of the restriction period (6, 13, 14) or both restriction length and repeated episodes within herds (15). Griffin et al. (15) carried out a case-control study of chronic episodes identified as herds with recurrent episodes (≥2) or long duration episodes (>12 months) compared to herds free from bTB. The risk factors they identified for chronic episodes were: presence of badgers, nutritional factors, purchasing of cattle, and spreading of slurry. Doyle et al. (6) looked at longer duration episodes (lasting >1 year) and identified the following risk factors: location, previous history of bTB within the herd, severity of the index episode and presence of an animal with a lesion. Karolemeas et al. (14) compared prolonged (≥240 days) with non-prolonged episodes (<240 days). The main predictors that they identified were the confirmation status of an episode (i.e., an animal with visible lesion(s) at slaughter), cattle kept in covered yards, contact with non-contiguous domestic species on other farms, herd-size and movements during the episode into the herd. These were all associated with increased odds whereas salt lick use and movements in the previous year were associated with decreased odds. Reilly and Courtney (13) also compared transient (<6 months) and persistent (>6 months) episodes and found persistent episodes to be associated with herd type, silage storage, location and density of badgers.

The first aim of this study is to describe trends in the severity of bTB episodes in Ireland in terms of the number of infected animals that were detected per herd. A second aim is to identify predictors that can distinguish between small and large bTB episodes. Previous studies (6, 13–15) have concentrated on chronic herds by considering the duration of an episode. To the authors' knowledge there are no other studies that have looked at predictors of large episodes in terms of the number of infected animals. Therefore, the objectives of this study were to identify risk factors associated with large bTB episodes in herds in comparison with smaller episodes, and to describe nationally the severity of bTB episodes occurring in Ireland.

MATERIAL AND METHODS

bTB Surveillance in Ireland

In Ireland, all cattle, aged over 6 weeks at the time of the test, or younger if introduced or in an infected herd, are tested annually for bovine tuberculosis using the SICTT in accordance with Annex B of Directive 64/432/EEC as amended section 2.2 (1). The SICTT involves the injection of bovine (potency 30,000 I.U./ml) and avian (potency 25,000 I.U./ml) tuberculin PPDs in the mid-third of the neck; the skin thickness at the site of the test is recorded at the time of injection and 72 h [±4 h] later. Any animal that displays clinical signs at the bovine injection site, such as oedema, exudative necrosis, heat and/or pain is positive and therefore a reactor. An animal with "a positive bovine reaction which is more than 4 mm greater than the avian reaction" is positive as per section 2.2.5.3.2 of the Directive (1) and deemed a "standard reactor". When a standard reactor or an animal with clinical signs is identified, all animal movements are restricted until two clear consecutive SICTT tests are achieved on all animals within the herd, with at least a 60 day interval, the second of which must be carried out at a minimum of 4 months post removal of the last positive animal from the herd. An episode may also be triggered when an animal with a bTB lesion is detected at slaughter and movement restrictions and testing requirements are imposed in the same way as when a SICTT reactor is identified. In addition, "non-standard reactors" may also be identified during an episode, these are defined as all other animals removed under the bTB eradication programme during an episode with 2 or more standard reactors or bTB lesion animals cumulative, that have been defined as higher risk herds (12, 16). These "non-standard reactors" will include animals with "a positive or inconclusive bovine reaction which is from 1 to 4 mm greater than the avian reaction" i.e., standard inconclusive reactors and may include animals with a positive or inconclusive bovine reaction which is 0 to 2 mm less than the avian reaction i.e., severe interpretation inconclusive reactors, animals with a bovine reaction of 4 mm or more regardless of any avian reaction i.e., positive to the SIT (Single Intradermal Test), animals removed for epidemiological reasons by a Veterinary Inspector (VI) regardless of reaction at the bovine site or animals removed following the results of ancillary blood test(s), such as the interferon gamma (IFN-γ) assay (1, 16). In 2015, national policy in relation to strategic application of the IFN-γ assay in restricted herds was enhanced, with VIs instructed to sample cohorts of positive animals immediately after the first test of the episode in all herds with 4 or more animals already identified as reactors following the SICTT (16). It is acknowledged that the inclusion of non-standard reactors and particularly IFN- γ positive animals as reactor will have served to increase the number of reactor animals in episodes, however, in the Irish bTB eradication programme, such animals have a high probability of being bTB infected, of showing visible bTB lesions at slaughter and/or failing tests at a future date and thus their removal as reactors at the earliest possible stage under the programme is justified (17–21). Further details describing the protocol of managing bTB infected herds are described in the "Veterinary handbook for herd management in the bovine TB eradication programme" (16).

Descriptive Analysis

The following descriptive statistics of herd-size and episode severity/duration were calculated from 2004 to 2015 inclusive:

- *Average herd-size over time*: Average size of the herd on the 31st December each year was taken from statistics published by the Department of Agriculture, Food and Marine (DAFM; https://www.agriculture.gov.ie/animalhealthwelfare/animalidentificationmovement/cattle). The average herd-size at the start of each episode beginning within the respective year was estimated, based on the first full herd-test during the episode.
- *Number of bTB reactors during an episode by year the episode ended*: The number of SICTT reactors or animals with a bTB lesion at slaughter, for restrictions ending during the year of interest was calculated. For episodes starting after an animal with a lesion was detected at slaughter, it is assumed that a single animal with a bTB lesion triggered the episode.
- *Number of standard reactors/non-standard reactors at the start of an episode*: The number of standard/non-standard reactors detected on the first test during the episode (a full-herd test or if the first test was a part-herd test then the reactors identified on the part-herd test and at the next first full-herd test). Note a part-herd test may occur when only part of the herd is tested such as when conducting pre-movement testing or re-testing one or more animal(s) that were inconclusive at the previous test.

Case-Control Study Population

The following criteria were used to identify herds eligible for consideration as either case or control herds: all herds with at least one episode beginning in either 2014 or 2015, with at least two full herd tests whilst restricted and before the end of 2015, and a minimum herd-size of 60 animals (this was the average herd-size in Ireland in 2015).

Case herds included all of the eligible herds with at least 13 reactors during the first 2 tests of the episode (unless the initial test was a part herd test, in which case the first 3 tests were used). A threshold of 13 reactors was chosen to represent a large episode, this being the top 5% of the distribution of the total number of reactors per herd within the first 2 tests of the episode during 2014/2015.

Control herds representing a small episode, included all of the eligible herds with between 2 to 4 (inclusive) reactors during the first 2 tests of the episode.

The study herds include both the case and control herds. For herds with more than one eligible episode, only the first episode was include in the study.

Estimated Sample Size Needed for a Case-Control Study

The assumed exposure was whether the herd ever had a previous episode. An estimated sample size was based on 60% exposure in control herds (12), 95% CI, 80% power and an odds ratio (OR) of 1.9 for a future episode for a herd restricted in the last 5 years compared to those not restricted. The estimated sample size per group was 173.

Risk Factors

The outcome measure was whether the herd was a case or a control herd. The following risk factors were considered in the analysis:

- Year the episode started (*epiyear*)
- Herd-size at the initial test of the episode (*herd_size*)
- Herd type (*herd_type*)
- Breeding herd (or non-breeding) (*breeding*)
- Test type (i.e., reason for testing the herd) at the initial episode test (annual test/(voluntary) pre-movement tests; re-test of an inconclusive reactor; forward trace of high risk animal(s) test; next test post de-restriction (i.e., the test 6 months following de-restriction); test of a herd contiguous to a restricted herd; test of a herd with an animal with a lesion found at slaughter) (*test_type*)

- Lesion present in one or more reactors, or the episode began with the detection of a lesion in an animal at slaughter (*lesion_present*)
- Previous history of bTB:
    - No. of previous episodes in last 10 years *(num_epi10yrs)*
    - Interval since last episode *(timesincelastepi)*
    - Number of reactors/standard reactors, reactors with a lesion or non-reactors with a lesion at the previous episodes *(prev_rct, prev_sdrct, prev_rxles, prev_facles)*
- Introductions: number of animals introduced into the herd in the current year/previous 3 years (*broughtin_currentyr, broughtin_3 years*)
- Ratio of introduced animals to herd-size at the initial episode test *(broughtin_hs_ratio)*
- Was the tester at the episode test the same as the tester at the previous test? *(same_prev_vet)*
- Was the tester at the episode test the same as the tester at the start of the previous episode? (*samebdvet*: 0 = not same tester, 1 = same tester, 2 = no previous episode)
- Average age of reactors and max age of reactors. Note for 9 animals born prior to 1996 the date of birth was not recorded and these animals were assigned an age of 19 (*mean_age, max_age*).
- Any current reactors present during the last previous episode in the same herd (*present_prebdown*)
- Any current reactors that were in the same age category (i.e., calves, heifers, cows, steers, bulls) as reactors at the previous episode in the same herd (*present_samegp_prebdown*)
- Any current reactors present during any previous episode in the same herd (*ever_prebdown*)
- Herd expanding? (% change in herd-size since previous year?) (*herd_expansion*)
- No. of fragments of land assigned to the herd (*fragment*)
- No. of neighbouring farms within 25, 150 or 500 m (*num_contigherds25, num_contigherds150, num_contigherds500*)
- Badgers: No. of badgers captured per year for previous 10, 5, 3 or 1 years (up to the year the restriction started but excluding the year the restriction started) within 25 m, 500 m or 1 km of the land fragment including area within the fragment *(bad25_10y, bad25_5y, bad25_3y, bad25_1y, bad500_10y, bad500_5y, bad500_3y, bad500_1y, bad1km_10y, bad1km_5y, bad1km_3y, bad1km_1y)*
- Geographical risk: Standard reactors per km^2 in the previous year or 1–3 years (*rr_1 year, rr_3* years)
- Associated herd with an episode in the same year/previous year (*ass_epi*).

Logistic Regression Model

Initially each of the risk factors listed above were tested in a univariable logistic regression model developed to model the probability of a herd being a case or a control herd. Risk factors that were significant in the univariable model ($p \leq 0.20$) were considered for inclusion in a multivariable model. A backward selection procedure was used to eliminate risk factors from the multivariable model based on a likelihood ratio test ($p \geq 0.050$). All variables with a p-value ≤ 0.20 in the univariable analysis were tested for collinearity to ensure a variance inflation factor (VIF) of <10 before being offered to the multivariable model. For risk factors with more than one measurement (e.g., contiguous herds within 25, 150 or 500 m), the appropriate measurement included in the multivariable model was based on the lowest AIC (Akaike Information Criteria). A plot of continuous variables against the log odds of the outcome and the variable in question was used to determine whether to include the variable as continuous or whether to transform the variable or include as a categorical variable (based on the quintiles of the variable). Interactions that were considered in the multivariable model were 2-way interactions between herd-type and herd-size; herd-type and reactor age. An assessment of the goodness-of-fit of the model was based on the Hosmer-Lemeshow test, the discriminatory ability of the model was assessed using the Area Under the ROC Curve (AUC) (22) and outliers were examined using influence statistics.

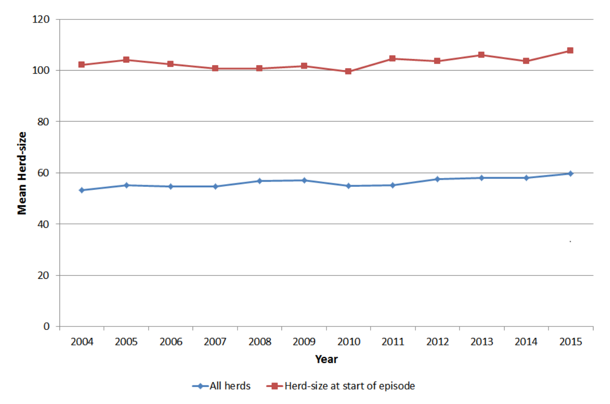

FIGURE 1 | Mean herd-size of all herds in Ireland and for herds with a bTB episode starting in the year of interest, during 2004 to 2015.

RESULTS

Descriptive Analysis

The average size of herds in Ireland has been steadily increasing over time (**Figure 1**) from a herd-size of 53 animals in 2004 to 60 animals in 2015. For herds that had a bTB episode, the average herd-size at the start of the episode was considerably higher than for all herds nationally, increasing from 102 animals in 2004 to 107 animals in 2015, reflecting the higher risk of bTB in larger herds.

Among herds with episodes ending during each year of interest, the mean size of episodes has stayed relatively constant over time at around 4 reactors (**Figure 2**). The 95th percentile of episode sizes increased to 17 reactors in 2008/2009 then decreased to 13 reactors in 2013/2014 with a slight increase in 2015 to 15 reactors (**Figure 2**). There was a significant (chi-square test $p < 0.001$) change in the proportion of restricted herds by severity of the episode over time (**Table 1**). The proportion of episodes that only involved 1 reactor/lesion has increased from 48.6% in 2004 to 57.8% in 2015 (**Table 1**). The proportion of herds having large episodes (≥13 reactors) peaked in 2008 at 7.4% and was the lowest in 2013 at 5.0%. There were more standard reactors compared to non-standard reactors, on average, at the start of an episode with between 1.81 and 2.11

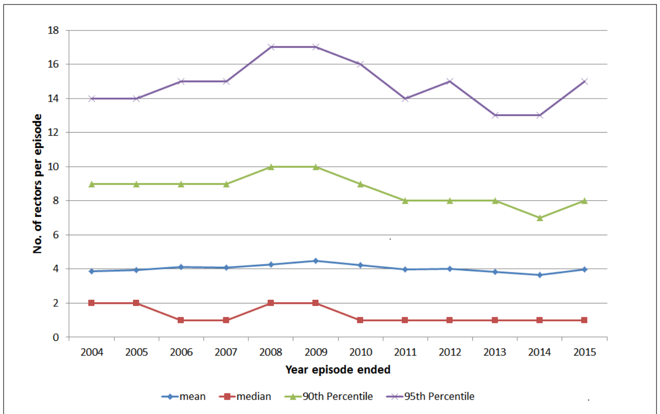

FIGURE 2 | The number (mean, median, 90th and 95th percentile) of reactors (standard/non-standard and animals with a bTB lesion at slaughter) in herds with a bTB episode ending in the year of interest, during 2004 to 2015 in Ireland.

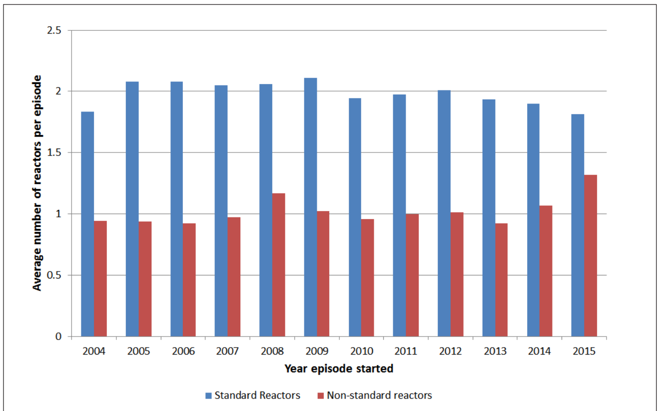

FIGURE 3 | Mean number of standard and non-standard reactors at the first test during a bTB episode, for episodes in Ireland starting each year.

standard reactors compared to between 0.92 and 1.3 non-standard reactors (**Figure 3**). Between 2014 and 2015, there was a decrease in the mean number of standard reactors at the start of an episode, but an increase in the mean number of non-standard reactors.

Case-Control Study Population

A total of 321 herds met the case definition criteria and 996 herds met the definition of a control herd, giving a total of 1317 study herds. Of these, 722 study herds had episodes that began in 2014 (of which 164 (22.7%) were case herds) and 595 study herds had episodes that began in 2015 (of which 157 (26.4%) were case herds) (**Table 2**). Of the case herds, 57% were dairy, 34% suckler and 6% beef compared to 55%, 30% and 12% among control herds respectively. Control herds had a median number of 2 reactors per episode (range from 2 to 4) and case herds had a median of 22 reactors (range from 13 to 294). Of the control herds, 6.6% had been tested using the IFN-γ assay compared to 57.9% of case herds. In 2015 a higher proportion (70.1%) of case herds were tested with the IFN-γ assay compared to case herds in 2014 (46.3%). Of the case herds, 54 (16.8%) had <13 SICTT reactors, the remainder were positive to the IFN-γ assay. These 54 herds had a median of 9 SICTT reactors and 43 of them were in episodes starting in 2015.

TABLE 1 | Percentage of bTB episodes in Ireland by number of reactors detected during the episode (episode severity) and year that the episode ended.

Year episode ended	Number of reactors*				No. of episodes	No. of herds
	1	2–4	5–12	≥13		
2004	48.6	30.5	15.0	5.9	6474	6397
2005	49.5	28.7	15.9	5.8	6031	5960
2006	50.9	26.5	16.0	6.6	6025	5921
2007	52.9	26.2	14.0	6.9	6083	5964
2008	49.1	28.9	14.7	7.4	6204	6102
2009	48.0	28.4	16.3	7.3	5749	5640
2010	51.1	28.1	13.8	7.0	5061	4990
2011	52.3	29.3	12.8	5.6	4318	4261
2012	50.2	29.2	14.3	6.2	4247	4188
2013	52.8	28.4	13.8	5.0	4016	3939
2014	57.0	26.3	11.4	5.3	4057	3981
2015	57.8	25.2	11.0	6.0	3577	3500

**Number of reactors includes standard reactors, non-standard reactors and animals with a lesion at slaughter (assume 1 animal with a lesion identified at slaughter).*

Logistic Regression Model

Risk factors that had a p-value < 0.2 in the univariable analysis are presented in **Table 2**. When there was more than one variable used to measure the same risk factor, the one with the lowest AIC in the univariable model was included in **Table 2**. At the univariable level, there were significant differences ($p < 0.05$) in the number of case herds by: herd-size, herd-type, breeding herd, episode test type, lesion present in one or more reactors, number of previous episodes in the last 10 years, maximum age of reactors, any reactors present at previous episodes, badger density, geographical risk and episodes in associated herds.

Variables representing a previous episode i.e., *present_prebdown, present_samegp_prebdown, ever_prebdown* were all correlated. The variable: "reactors ever in a previous episode" (*ever_prebdown*) had the lowest AIC and was included in the multivariable model.

Herd-size as a log transformed continuous variable gave the best fit at the univariable level based on the AIC and a plot against the log odds of being a case and was included in the multivariable model. Similarly the log of the area relative risk gave the best fit at the univariable level. Herd-type and breeding herds were correlated, herd-type had the lower AIC and was considered for inclusion in the multivariable model. Out of all the measures of badger density, the number of badgers within 1 km of the farm over 10 years had the lowest AIC.

The final multivariable model included the variables: year the episode started; log of herd-size; episode test type; log of the area relative risk in the previous year; whether any reactor(s) had ever been in a previous episode, whether an associated herd had an episode in the same/previous year, the ratio of the number of animals brought-in: herd-size and whether there was a lesion present in a reactor or an animal at slaughter (**Table 3**). For herd-size and area relative risk, the log of the variable was included in the final model. The inter-quartile range for herd-size in this

TABLE 2 | Percentage of herds in Ireland with a large bTB episode (≥13 reactors in the first 2 full herd tests) commencing in 2014–15, by significant ($p < 0.2$) risk factors.

Risk factor	Categories	No. of herds with an episode	Large episodes		p-value
			Number	%	
Year episode started (*epiyear*)	2014	722	164	22.7	0.123
	2015	595	157	26.4	
Herd size (*herd_size*)	60–84	267	47	17.6	0.002
	85–113	262	61	23.3	
	114–154	259	65	25.1	
	155–223	266	62	23.3	
	>223	263	86	32.7	
Herd type (*herd_type*)	Beef	138	21	15.2	0.034
	Dairy	729	183	25.1	
	Suckler	410	109	26.6	
	Other	40	8	20.0	
Breeding herd (*breeding*)	Yes	1167	301	25.8	<0.001
	No	150	20	13.3	
Episode test type (*test_type*)	Annual/premovement test	566	113	20.0	<0.001
	Inconclusive reactor re-test	73	6	8.2	
	Forward trace of a high risk animal(s) test	179	62	34.6	
	Post de-restriction test	119	26	21.8	
	Contiguous herd test	244	66	27.0	
	Lesion at slaughter: herd test	136	48	35.3	
Lesion present in one or more reactors/animal at slaughter (*lesion_present*)	None	465	30	6.5	<0.001
	One or more	852	291	34.2	
Number of previous episodes in last 10 years (*num_epi10yrs*)	0	409	100	24.4	0.010
	1	391	84	21.5	
	2	265	81	30.6	
	3	136	38	27.9	
	>3	116	18	15.5	
Ratio of introduced animals to herd-size at the initial episode test (*broughtin_hs_ratio*)	0	277	75	27.1	0.079
	0.001–0.0167	249	71	28.5	
	0.0168–0.0706	264	63	23.9	
	0.0707–0.2194	264	63	23.9	
	>0.2194	263	49	18.6	
Same tester at most-recent previous episode (*samebdvet*)	Different tester	777	175	22.5	0.151
	Same tester	401	106	26.4	
	No previous episode	139	40	28.8	
Max. age of reactors (years) (*max_age*)	0–3.5	263	22	8.4	<0.001
	3.6–6.0	264	17	6.4	
	6.1–8.4	263	45	17.1	
	8.5–11.2	263	76	28.9	
	>11.2	264	161	61.0	
Any reactor present at the most-recent previous episode	None	676	119	17.6	<0.001
	One or more	641	202	31.5	
Any reactor present at the most-recent previous episode in same age class (*present_samegp_prebdown*)	None	868	180	20.7	<0.001
	One or more	449	141	31.4	
Any reactor ever present during any previous episode (*ever_prebdown*)	None	535	73	13.6	<0.001
	One or more	782	248	31.7	

Continued

TABLE 2 | **Continued**

Risk factor	Categories	No. of herds with an episode	Large episodes		p-value
			Number	%	
No. of farm fragments (*fragment*)	1–2	196	41	20.9	0.126
	3–4	351	95	27.1	
	5–6	287	57	19.9	
	7–8	200	52	26.0	
	>8	279	75	26.9	
No. of badgers captured over previous 10 years within 1 km of the farm (*bad1km_10y*)	0–2	249	51	20.5	0.011
	3–9	270	50	18.5	
	10–17	283	85	30.0	
	18–28	254	66	26.0	
	>28	257	68	26.5	
Geographical risk in the previous year : Reactors per km^2 (*rr_lyr*)	0–0.001	263	25	9.5	<0.001
	0.001–0.002	263	46	17.5	
	0.002–0.004	263	71	27.0	
	0.004–0.007	263	79	30.0	
	>0.007	263	100	38.0	
Associated herd with an episode in current/previous year (*ass_epi*)	None	1270	297	23.4	<0.001
	One or more	47	24	51.1	

study was approximately 100 animals, an increase in herd-size by 100 would mean a 40 times increase in the odds of having a large episode (OR: 40.3, 95% CI: 11.4–145.0). Similarly the inter-quartile range for the area relative risk was approximately 0.004 reactors per km^2. An increase in the area relative risk by 0.004 reactors per km^2 would more than double the odds of having a large episode (OR: 2.7, 95% CI: 2.1–3.4).

Herds had significantly larger episodes (case herds) when they began in 2015, were larger herds, involved an animal with a bTB lesion, were in an area with a high relative bTB risk, had one or more reactor(s) present during a previous episode and/or had an associated herd with an episode in the current/previous year. Herds that introduced more animals relative to herd-size were significantly less likely to have a large episode and episodes that began with a post-derestriction test (i.e., at 6 months following a previous de-restriction) also had lower odds of a large episode compared to herds starting an episode at the annual test. The Hosmer-Lemeshow test ($p = 0.290$) and the residual analysis indicated no significant

TABLE 3 | Parameter estimates from the logistic regression model of the probability of a large bTB episode (≥13 reactors) in Irish herds during 2014–15.

		OR	Lower	Upper	P-value
Year episode started (*epiyear*)	2014	Referent			.
	2015	1.48	1.11	1.99	0.009
Log Herd-size (*herd_size*)		2.23	1.70	2.95	<0.001
Log geographical risk in the previous year: Reactors per km^2 (*rr_lyr*)		2.03	1.70	2.43	<0.001
Associated herd with an episode in current/previous year (*ass_epi*)	No	Referent			.
	Yes	2.06	1.05	4.08	0.037
Any reactor ever present during any previous episode (*ever_prebdown*)	None	Referent			.
	One or more	2.64	1.90	3.69	<0.001
Ratio of introduced animals to herd-size at the initial episode test (*broughtin_hs_ratio*)	0	Referent			.
	0.001–0.0167	0.88	0.56	1.37	0.564
	0.0168–0.0706	0.67	0.42	1.04	0.076
	0.0707–0.2194	0.59	0.38	0.93	0.023
	>0.2194	0.51	0.32	0.81	0.005
Lesion present in one or more reactors or an animal at slaughter (*lesion_present*)	None	Referent			
	One or more	6.63	4.41	10.30	<0.001
Episode test type (*test_type*)	Annual/premovement test	Referent			.
	Inconclusive reactor re-test	0.45	0.16	1.07	0.095
	Forward trace of a high risk animal(s) test	1.31	0.85	2.02	0.217
	Post de-restriction test	0.56	0.32	0.97	0.044
	Contiguous herd test	0.90	0.59	1.35	0.601
	Lesion at slaughter: herd test	1.02	0.64	1.61	0.945

lack of fit, the AUC of 0.817 indicated an adequate discriminatory ability of the model.

The median age of reactors was also a significant variable and gave a better fitting model (Supplementary material, Table S1) than that in **Table 3** (AIC: 1136.8 versus 1170.8). However, in control herds the median age was only based on a small number of reactors (2 to 4) and was very variable (see Figure S1) in these herds therefore this variable was excluded due to uncertainty regarding whether any observed differences were mainly due to small number of animals. Similarly, the maximum and minimum age of reactors was considered; however, due to the large variation in range (see Figures S2, S3) among the larger episodes, it was decided to exclude any age variables from the models.

A model using the variable: "number of episodes in the previous 10 years" was created by introducing this variable instead of the variable: "any reactor present in a previous episode" (Table S1). This model was not as good a "fit" to the data as the model in **Table 3** (AIC 1193.5 versus 1170.8), however, this variable is informative regarding the previous history of herds with larger episodes. The odds of a large episode decreased once the number of episodes in the previous 10 years increased to more than 3.

DISCUSSION

National Trends

In Ireland, the average size of an episode has remained relatively constant over the last 10 years at approx. 4 reactors per episode (**Figure 2**). The proportion of episodes with only 1 reactor has been increasing over time, whereas there has been a decrease in the proportion of most other sizes of episode over the last 10 years (**Table 1**). It is probable that a small proportion of the episodes with a single reactor are due to false positive reactions to the SICTT given the imperfect specificity of the test (23–25). As the national prevalence of bTB decreases, we would expect to see a higher proportion of singleton restrictions as the relative percentage of restrictions due to the decrease in true infection over time. However, the proportion of episodes with ≥13 reactors has remained fairly constant at 5.0 to 6.2% of episodes over the last 5 years (**Table 1**). The overall size of herds and the size of herds with a bTB episode have remained relatively constant reflecting that any improvements are unlikely to be due to changes in herd-size. Given the consistent proportion of larger episodes over time, it is important to identify any underlying risk factors.

Residual Infection

This study identified a number of significant predictors of a large episode compared to small episodes with limited within-herd transmission. Some of these predictors are indicative of residual infection [that is infected but undetected cattle (5)] within the herd resulting in within-herd transmission prior to disclosure. One such predictor is whether an animal with a lesion was present within the episode. Episodes that included an animal with a lesion were more than six times as likely to result in a large bTB episode compared to episodes with no animal with a lesion. Karolemeas et al. (14) also found that an episode that was confirmed (following detection of a visible lesion or culture of *M. bovis* in one or more reactors) was a significant predictor for a prolonged episode. Similarly, Reilly and Courtenay (13) found 92% of persistent episodes (>6 months) were confirmed compared to only 63% of transient (<6 months) episodes. Episodes without any animals with a lesion may be a consequence of latent infection (26), or a less advanced stage of disease, each of which may not be detected by examination at slaughter. In the case of latent infection, within-herd transmission may follow subsequent to the reactivated infection in an animal. Evidence of reactivation in cattle comes from the Australian bTB eradication programme where infected cattle were detected in the absence of an external infection source [Cousins et al., (27) as cited in Karolemeas et al. (10)]. Within-herd transmission in herds where a lesioned animal had been detected at slaughter has been examined by Olea-Popelka et al. (28). They found that one risk factor for disclosure of additional animals was whether the animal with a lesion had been present in a previous bTB episode and the time the animal had spent in the study herd. In this study, a herd with a reactor that had been in a previous bTB episode had 2.6 times the risk of having a large episode compared to herds with no reactors in a previous bTB episode. Doyle et al. (6) also found previous history, measured as the total time restricted in the previous 5 years, was the best predictor of both long and recurrent episodes. Many studies have also found previous bTB history to be a predictor of bTB within a herd (2, 12) and for recurrence within a herd (7, 10, 11, 29). Animals that have been in a previous bTB episode were possibly missed at a previous SICTT, which may partly reflect the imperfect sensitivity of the SICTT, with a median value of 80% (range 52 to 100%) based on several studies (20, 25, 30, 31) and between 64.5 and 73.0% based on a Bayesian latent-class analysis of Irish data (32). The imperfect sensitivity will result in infected animals being missed by the SICTT and left in the herd with the possibility of subsequent within-herd transmission.

Post-Derestriction Test and Number of Previous Episodes

Episodes that began at a post-derestriction test had significantly lower odds (OR: 0.56, 95% CI: 0.32–0.97) of being a large episode compared to episodes that began with an annual test. The post de-restriction test takes place 6 months following de-restriction of the herd and non-standard reactors are removed even if no standard reactor is present on this test i.e., these tests have the severe interpretation of the SICTT applied. The proportion of herds positive at the post-derestriction test in Ireland has been reasonably constant over time at around 12% between 1995 and 2009 (33) falling to 9.4% in 2015 (More et al., submitted). Infected animals detected at this test may plausibly reflect animals that have been missed in the previous episode.

Herds that previously had more than three bTB episodes in the previous 10 years (Table S1) also had lower odds of a large episode. It is likely that these herds have had more severe controls imposed such as an increased number of tests following previous episodes and a more severe interpretation level (16). Infected animals identified in the current episode are animals either previously missed or bought-in following previous episodes, therefore with limited within-herd transmission.

Geographical Risk

Herds in areas with a high incidence of bTB were more likely to have a large episode reflecting the increased infection pressure within the locality. This has been found in many other studies that looked at both the occurrence (2) and recurrence (7, 9, 11) of bTB within herds. Doyle et al. (6) also found an increased risk of chronic episodes due to infection in the neighbourhood. One source of neighbourhood infection is infected wildlife, which in Ireland is mostly considered to be badgers (15, 34–36). White et al. (2) found an increased risk of bTB associated with herds at a distance of between 25 m and 1 km, the authors concluded that infected wildlife was the most likely explanation of this locality risk. Badger density in the vicinity of the study herds was examined in several different ways, including varying the distance from the farm and the number of years of culling. The best fitting predictor at the univariable level was the number of badgers culled within 1 km of a farm over 10 years; however, this was not significant within the final model. Farms that had culled 10–17 badgers had the highest proportion of large episodes, possibly reflecting an ongoing problem in the area.

Herd-Size

Only herds that were above the national average herd-size of 60 animals were included in the study. However, the odds of a large episode still increased with increasing herd-size. The mean herd-size of restricted herds was larger than the mean of the national population of herds (**Figure 1**), reflecting the higher risk of these herds having an episode. In this study, the risk of a large episode increased with the log of the herd-size (**Table 3**). Of the largest herds (>223 animals), 32.7% experienced a large episode (≥13 reactors) compared to 17.6% of the smallest herds in the study (60–84 animals) (**Table 2**). Many studies [summarised by Skuce et al. (37)] have found an association between herd-size and the risk of bTB occurrence, others (6, 7, 9, 11) found an association with recurrence and two others (14, 38) with prolonged episodes. These higher risks to larger herds may be due to a number of factors such as the larger area of the farm which increases the risk of exposure to infected wildlife and infected neighbouring herds. In addition, as the herd-size increases there is an increasing risk that an infected animal may not be detected by the SICTT due to the imperfect test sensitivity, which therefore prolongs the episode allowing the potential for additional transmission of infection. In addition, intensive management of larger herds such as less attention to individual animals, has also been associated with an increased risk of a chronic episode (15).

Year the Episode Started

The odds of a herd having a large episode were 1.5 times higher in 2015 compared to 2014. This could, at least partially, be attributed to the increased and more targeted use of IFN-γ in 2015 in episodes with at least 4 reactors. In 2015 a higher proportion of case herds were tested with the IFN-γ assay compared with 2014 case herds (70.1% versus 46.3%). In addition of the 54 herds that qualified as a case herd due to additional IFN-γ positives 80% of the episodes began in 2015. This is also reflected by the increase in non-standard reactors in 2015 (**Figure 3**). Prior to the enhanced policy instruction, VIs were recommended to sample animals from all episodes with at least 4 SICTT reactors, however, not all such episodes were subjected to sampling. The application of the IFN-γ test will have had the potential to remove infected animals, particularly those in the earlier stages of infection sooner. Gormley et al. (20) found animals that were SICTT negative/IFN-γ positive, were up to 9 times more likely to become SICTT positive when followed up for two more SICTT tests compared to SICTT negative/IFN-γ negative animals. Clegg et al. (17) looked at post-mortem results of animal that were negative to the SICTT and IFN-γ tested and slaughtered in the same year. In this study, the odds of an IFN-γ positive animal being positive at post-mortem was nearly five times higher compared to IFN-γ negative animals. Therefore, the increased use of the IFN-γ will initially be expected to give rise to larger episodes but should potentially reduce the risk of missing infected animals that could cause future recurrence and within-herd transmission.

Introduced Cattle

The odds of a large episode decreased as the ratio of animals introduced: herd-size increased. This plausibly reflects episodes due to introduced animals tending to involve very little within-herd transmission. Reilly and Courtenay (13) looked at transient (<6 months) and persistent (>6 months) episodes in Great Britain and found variables associated with cattle purchase were important risk factors for transient episodes but not for persistent episodes. Karolemeas et al. (14) also found decreased odds of a prolonged episode associated with increasing number of cattle bought-in during the 12 months prior to the episode.

Associated Herds

An associated herd is a herd that is linked to another for management or epidemiological reasons e.g., due to a family or partnership relationship with individuals managing different aspects of the farming livestock business/enterprise on separate holdings. Many larger herds tend to split animals into different production and epidemiological groups e.g., calf/heifer rearing/breeding separated from milking cows often with more than one herd number. Thus a large herd that has its animals spread between two herd numbers may therefore have split infected animals between herds prior to the commencement of an episode. The increased risk from an associated herd may also be representative of a contiguous risk since the animals in the associated herd may remain within the immediate neighbourhood and are often in much closer contact compared to contiguous herds due to shared management and risk factors. Associated herds are subjected to the same controls and restrictions when positive animals are detected in one or other which necessarily results in restriction and testing of associated herds in cases where the index herd had an episode.

Methodological Issues

This study looked at restricted herds only i.e., the difference between a large and small episode as opposed to having/not having bTB. All herds had two full-herd tests within the study period to be included in the study. This rule was included so that herds with an ongoing episode towards the end of 2015 were excluded unless they had 2 full herd tests. The study results were, therefore, based on the number of reactors found at the beginning of an outbreak, reflecting risk factors for more "explosive" episodes with considerable within-herd transmission prior to detection. In GB, Karolemeas et al. (14) found that episodes with more reactors at the start were associated with longer episodes, although this was confounded with confirmation status. This is at odds with work from Northern Ireland where Doyle et al. (6) found that an increased number of reactors at the breakdown test were associated with reduced odds of a prolonged episode. Doyle et al. (6) speculated that the more severe the initial intervention, and therefore the more reactors identified, the faster the infection was cleared.

The significant effect of some variables such as the age variables may be an artefact of the number of reactors in the case and control herds. In the supplementary material, Figures S1–S3 show how the median, min and max age of reactors vary with the number of reactors within the episode. The same may also be true for the presence of a reactor with a lesion, since the sensitivity of the post-mortem test is thought to be lower than the SICTT (25) and the probability of detecting an animal with a lesion can vary by slaughterhouse (39); therefore there is a higher probability of finding a lesion when the sample size is larger. However, even if these variables are artefacts of the sample size the remaining variables were consistent across all of the models.

It was not possible to look at some of the risk factors identified in other studies such as silage storage, salt licks, nutrition etc. as such data are not available. More detailed case-control studies may be able to identify other risk factors that may be associated with larger episodes.

Policy Implications

In Ireland, herds with more severe episodes (2 or more standard reactors or bTB lesion animals, cumulative) are designated as higher risk status and accordingly undergo more rigorous testing post de-restriction and must pass three tests at 6 month intervals before returning to default risk status. In Australia, during the bTB eradication programme, herds were placed under longer restriction controls and herds were not entirely free to trade until 8 years after the last infected animal was detected (40). Herds that have had a large episode have been shown to pose a risk of having another episode in the future (7, 9, 11, 12, 29). Future controls on these herds will need to be continually reassessed to look at whether additional measures are appropriate, such as maintaining the higher risk classification and rigorous testing of herds following a severe episode for longer periods after the episode has ended.

Some of the risk factors identified in this study such as reactors in previous episodes, herds with an associated herd undergoing an episode, herds in high incidence areas etc. may help to target future policy measures to specific herds or animals that could be targeted for additional surveillance measures. Additionally, further work is needed to assess whether the increased and focused use of the IFN-γ assay in herds experiencing a severe episode during 2015 has shortened the duration of the episode and/or reduced the risk of repeat episodes of bTB in these herds.

CONCLUSIONS

Although there has been a general trend towards less severe herd bTB episode in Ireland over time, the proportion of large episodes has remained relatively consistent. An understanding of the risk factors that influence these large episodes is important, to improve national controls. Based on the results from this study, the main predictors of a large episode were the year the episode started, increasing herd-size, previous exposure to bTB, increasing bTB incidence in the local area, an animal with a bTB lesion and a bTB episode in an associated herd. Herds that introduced more animals were more likely to have a smaller bTB episode, reflecting the reduced risk of within-herd transmission when an episode was due to a purchased infected bTB animal. This information has important policy implications.

AUTHOR CONTRIBUTION

MG and MH formulated the idea for the study. SM, TC, MG, MH and AD developed the study design. TC carried out the statistical analysis. GM prepared the geographical information. TC wrote the first draft. All authors contributed to the final draft.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fvets.2018.00086/full#supplementary-material

TABLE S1 | Parameter estimates from two additional logistic regression models of the probability of a large (≥ 13 reactors) bTB episode in Ireland during 2014-15; (i) including the variables: median age of reactors and (ii) substituting the variable 'any reactor ever present in a previous breakdown' with 'number of previous episodes in the last 10 years'.

FIGURE S1 | Median age of reactors by the number of reactors in the breakdown.

FIGURE S2 | Minimum age of reactors by the number of reactors in the breakdown.

FIGURE S3 | Maximum age of reactors by the number of reactors in the breakdown.

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Characterization of the Temporal Trends in the Rate of Cattle Carcass Condemnations in the US and Dynamic Modeling of the Condemnation Reasons in California With a Seasonal Component

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Based on the 2016 National Cattlemen's Beef Association statistics, the cattle inventory in the US reached 93.5 million head, from which 30.5 million were commercial slaughter in 2016. California ranked fourth among all the US states that raise cattle and calves, with 5.15 million head and approximately 1.18 million slaughtered animals per year. Approximately 0.5% of cattle carcasses in the US are condemned each year, which has an important economic impact on cattle producers.In this study, we first described and compared the temporal trends of cattle carcass condemnations in all the US states from Jan-2005 to Dec-2014. Then, we focused on the condemnation reasons with a seasonal component in California and used dynamic harmonic regression (DHR) models both to model (from Jan-2005 to Dec-2011) and predict (from Jan-2012 to Dec-2014) the carcass condemnations rate in different time horizons (3 to 12 months).Data consisted of daily reports of 35 condemnation reasons per cattle type reported in 684 federally inspected slaughterhouses in the US from Jan-2005 to Dec-2014 and the monthly slaughtered animals per cattle type per states. Almost 1.5 million carcasses were condemned in the US during the 10 year study period (Jan 2005-Dec 2014), and around 40% were associated with three condemnation reasons: malignant lymphoma, septicemia and pneumonia. In California, emaciation, eosinophilic myositis and malignant lymphoma were the only condemnation reasons presenting seasonality and, therefore, the only ones selected to be modeled using DHRs. The DHR models for Jan-2005 to Dec-2011 were able to correctly model the dynamics of the emaciation, malignant lymphoma and eosinophilic myositis condemnation rates with coefficient of determination (R_t^2) of 0.98, 0.87 and 0.78, respectively. The DHR models for Jan-2012 to Dec-2014 were able to predict the rate of condemned carcasses 3 month ahead of time with mean relative prediction error of 33, 11, and 38%, respectively. The systematic analysis of carcass condemnations and slaughter data in a more real-time fashion could be used to identify changes in carcass condemnation trends and more timely support the implementation of prevention and mitigation strategies that reduce the number of carcass condemnations in the US.

Keywords: syndromic surveillance, dynamic harmonic regression, slaughterhouse, early detection, warning system

INTRODUCTION

The meat industry is the largest segment of US agriculture based on economic land use and environmental impact. The United States Department of Agriculture (USDA) reported that in 2015, more than 28,296,403 cattle were commercially slaughtered with a total carcass weight of 3,276,367,000 pounds (1). From those, 141,450 carcasses were condemned, representing approximately 0.5% of the total cattle carcasses produced in the US. Similar results were provided from 2003 through 2007 (i.e., 5 years), where more than 163 million cattle (excluding bob veal, veal, and heavy calves) arrived at USDA-inspected slaughter facilities and 769,339 (0.47%) were condemned at either antemortem or postmortem inspection (2).

Slaughter data are valuable sources of information because they include both demographic (age, type of cattle) and health related (reason for condemnation) factors. The systematic analysis of slaughter data can help identify the most important condemnation reasons, characterize seasonality and emerging spatio-temporal trends, and provide the foundations of syndromic surveillance systems and early-detection of outbreaks. Two European countries and Canada are currently using (e.g., Switzerland; 3) or plan to use [e.g., France, (4) and Canada (5)] data collected during meat inspection for syndromic surveillance of animal health. For example, the French Ministry of Agriculture started the Nergal-Abattoir project to collect data in real-time during the slaughtering process for timely surveillance and risk factor analysis (4). However, there are very few published studies using slaughterhouse data for outbreak investigation or syndromic surveillance in the US. The study by White and Moore (2) highlighted the need to understand condemnation reasons for producer education and early intervention by veterinarians. Studies by Kaneene et al. (6) and Humphrey et al. (7) used US slaughterhouse data to trace cattle to the herd of origin after detection and confirmation of bovine tuberculosis. Their main concern was the failure to trace back some bovine tuberculosis-positive animals. Other studies have shown the value of using slaughter data for example to early detect erysipelas outbreaks in the US swine industry (8) or predict increases in transport losses of swine in-transit and just prior to slaughter (9).

Real-time access to slaughter data and a better knowledge of temporal trends of the number of reported condemned carcasses in slaughterhouses in the US could be used to identify times, areas and cattle types with higher than expected numbers of carcass condemnations that may be associated with specific management practices, adverse climatic events, or emerging syndromes and new diseases (e.g., 10). This can help to target the allocation of risk based strategies or the implementation of education and preventive management practices in those high risk areas that are particularly affected by specific condemnation reasons.

However, before the interpretation of results suggesting an increase or decrease in the number of carcass condemnation cases it is important to understand and identify seasonal trends or components, which are considered to be normal variations of the time series, as without correcting for those trends the results may be biased (5). A dynamic harmonic regression (DHR) model can be regarded as a time-series component model, where the phases and amplitude of seasonal and cyclic components are represented by "dynamic" or "time-varying" parameters (TVP), reflecting relevant changes in the evolution of the patterns of different carcass condemnation reasons. This can be contrasted with conventional seasonal auto regression, where it is assumed that the amplitude and phase of the periodic components are time invariant (11). The DHR model structures can be defined by autoregressive spectrum analysis thus avoiding the subjective choices of frequencies in the seasonal and cyclic components (12).

In this study, our first objective was to describe and compare the temporal trends of reported condemnation rates in slaughterhouses in California with those in the US from Jan-2005 to Dec-2011. The second objective was to identify those condemnation reasons with a seasonal component using autoregressive spectrum analysis. A third objective was to evaluate if DHR models could predict the rate of carcass condemnations that showed seasonality in future time periods (i.e., from Jan-2012 to Dec-2014) using the seasonal component of the time series. All this information will be useful to increase awareness of producers about the main reasons of carcass condemnations in their specific state and can help them to prioritize the management practices and risk mitigation strategies that could be implemented to minimize future carcass condemnations.

MATERIALS AND METHODS

Data

Data on cattle condemnation reasons were obtained with the Freedom of Information Act from the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA), the agency responsible for slaughter conditions and meat inspection. The FSIS inspectors across the US followed similar practices/protocols and had similar training (13). Data consisted of daily reports of condemned carcasses per cattle type and condemnation reasons reported in 684 federally inspected slaughterhouses in US from Jan-2005 to Dec-2014, including 29 slaughterhouses located in California. For each animal, the database contained: condemned reason ($n = 35$; **Figure 1**), cattle ID number, slaughter date, slaughterhouse's name ($n = 684$) and state ($n = 49$). Slaughtered cattle types were beef cow, bob veal, bull/stag, dairy cow, formula-fed veal, heavy calf, heifer, non-formula-fed veal and steer (14). The monthly number of cattle sent to slaughterhouse per state and per cattle type from Jan-2005 to Dec-2014 was also provided by FSIS.

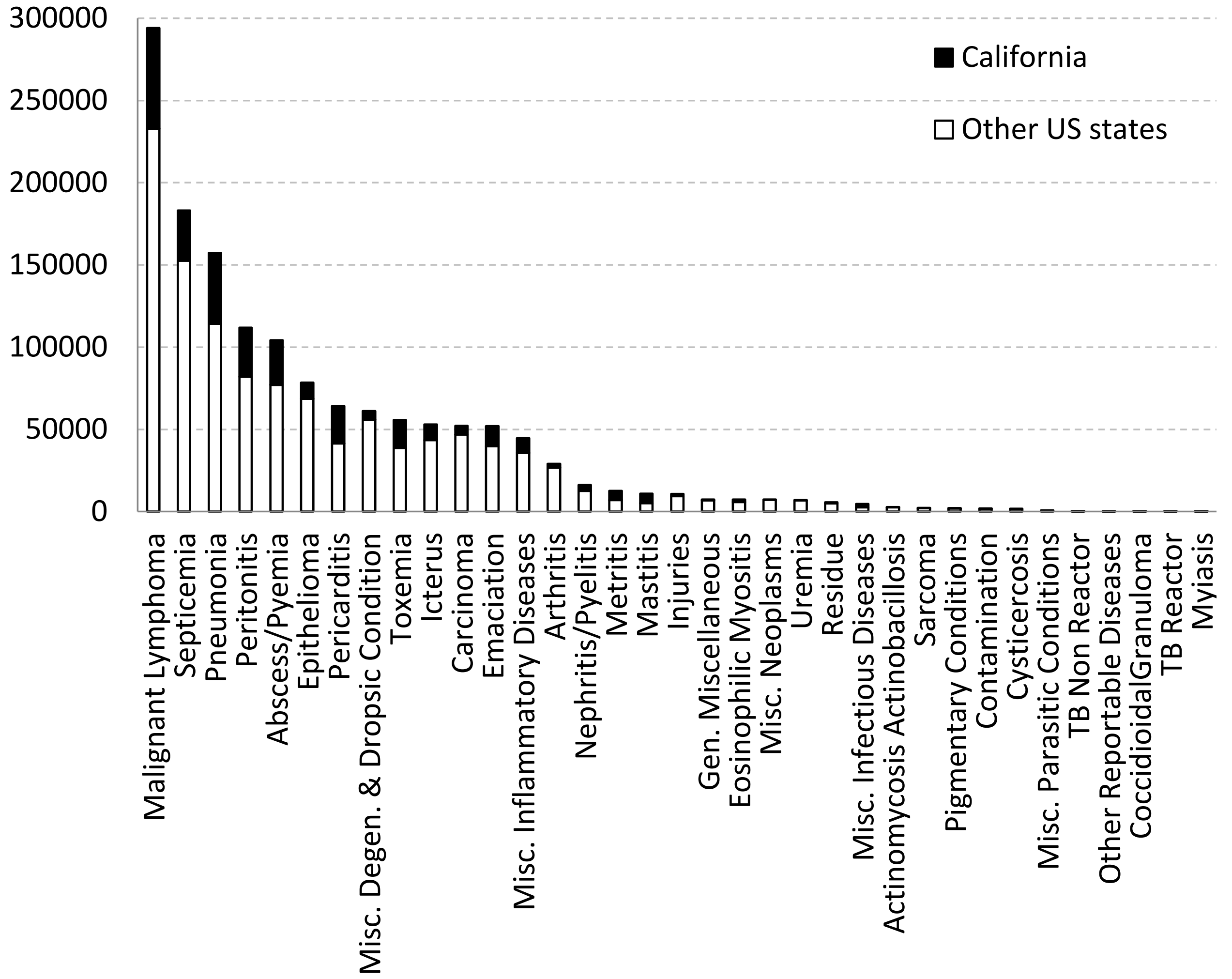

FIGURE 1 | Total number of carcass condemnations per reason in slaughterhouses in California (black) and in other US states (white) from Jan-2005 to Dec-2014.

Dynamic Harmonic Regression Model

The DHR model was conducted using the monthly rate of reported carcass condemnations for each condemnation reason (i.e., the number of the carcass condemned for a particular reason divided by the number of the slaughtered cattle per month and per type of cattle) in slaughterhouses in California from Jan-2005 to Dec-2014. The number of condemned carcasses in California slaughterhouses were reported daily but aggregated on a monthly basis to compute monthly rates because the number of slaughtered animals were only available per month. In general, the theoretical seasonal periodic components are 12, 6, 4, 3, 2.4 and 2 months per cycle (i.e., the seasonal periodicity is defined as T/j where $j = 1, 2, \ldots, 6$ and $T = 12$ is the duration of one year with 12 samples as suggested by Taylor et al. (12). However, rather than rely on this theoretical periodicity, we identified the most significant periods in each of the time series of carcass condemnations using an autoregressive spectrum (15). The order of the autoregressive spectrum was selected using the Akaike Information Criteria (16). We considered the rates of carcass condemnation by each specific reason to have a seasonal component if it has a pronounced peak value of equal or close to 12 months per cycle and has at least another dominant peak with the value close to one of the theoretical seasonal periodic component.

For those condemnations reasons that showed evidence of periodicity based on autoregressive spectrum a DHR model was used to quantify their seasonality (15). The DHR approach has been used to quantify periodic components of a time series and understand seasonal fluctuations and temporal trends of time series data in previous studies (e.g., 11, 17, 18). The DHR model is defined as follows (12):

$$Y(t) = L(t) + Q(t) + e(t) \tag{1}$$

where $Y(t)$ is the observed time series (i.e., rate of carcass condemnations in California slaughterhouses); $L(t)$ is a trend or low frequency component; $e(t)$ is a "residual" component, normally defined for analytical convenience as a normally distributed Gaussian sequence with zero mean value and variance σ^2 (i.e., discrete-time white noise); and $Q(t)$ is a cyclical term defined as follows:

$$Q(t) = \sum_{i=1}^{n=7} \left\{ \alpha_i(t) \cos\left(f_i 2\pi t\right) + \beta_i(t)\, sin\left(f_i 2\pi t\right) \right\} \tag{2}$$

In this model, $\alpha_i(t)$ and $\beta_i(t)$ are stochastic time variable parameters and, $f_i = \frac{1}{T/i}$, $i = 1,2,..., n$ are the fundamental and harmonic frequencies associated with the periodicity (T/i) in the series (as mentioned before, T is the duration of one year with 12 samples).

The trend components $L(t)$ in equation 1 and time variable parameters in equation 2 [$\alpha(t)$ and $\beta(t)$] were all represented using a general random walk model. The general random walk family of models includes the well-known Random Walk and Integrated Random Walk models (12). We limited analyses to these two types of models.

In particular, each of the time variable parameters in this analysis [$\alpha_i(t)$ and $\beta_i(t)$, $i = 1, 2, \ldots, n$] were represented as a Random Walk process of the form:

$$\alpha(t) = \alpha(t-1) + \eta_{\alpha i}(t) \tag{3}$$

$$\beta(t) = \beta(t-1) + \eta_{\beta i}(t) \tag{4}$$

where $\eta_i(t)$ is an error with zero mean for either α or β. In this manner, the estimation algorithm is instructed that the parameter in question$(\alpha$ and $\beta)$ is a stochastic variable that is likely to change by an unknown but small amount over each sampling interval (here, one month), within the stochastic limits imposed by the variance $\sigma_{\eta_i}(t)$ (12).

The trend component $L(t)$ in equation 1 was modelled as an Integrated Random Walk of the form:

$$\begin{aligned} L(t) &= L(t-1) + l(t-1) \\ l(t) &= l(t-1) + \eta_L(t) \end{aligned} \tag{5}$$

where $l(t)$ represents the "slope" of the trend, $\eta_L(t)$ is a zero mean, white noise input. Changes in trends [$L(t)$] of the reported reasons for carcass condemnation in cattle slaughterhouses in California and US were evaluated by calculating the slope of each of the time series. The trend is calculated using an integrated random walk model with noise-variance ratio (NVR) of 0 (equation 5). If the calculated slope is positive, the rate of condemned carcasses is increasing. A negative slope indicates a general decrease in the rate of condemned carcasses. The larger the absolute number of the slope, the steeper the changes in the rate of condemned carcasses.

The variances of the white noise, $\eta_i(t)$, in the Random Walk and Integrated Random Walk models (equation 3, 4 and 5) are critical in estimating the time variable parameters and need to be optimized against the data. The ratio of these variances to the variance of the residual $e(t)$ (equation 1) is called the noise-variance ratio (12).

The DHR algorithm estimates the time variable parameters using the Kalman Filter (19). First, we used DHR model to simulate the dynamics of the rates of carcass condemnations from Jan-2005 to Dec-2011. Then, we used the DHR model to predict the rates of carcass condemnations from Jan-2012 to Dec-2014. The DHR parameters obtained in the simulation (from Jan-2005 to Dec-2011) were used to predict the carcass condemnations for the time period Jan-2012 to Dec-2014. Analyses were conducted using the Captain toolbox® version 7.5 (12) in Matlab® (version 2016a, MathWorks Inc., Natick, MA).

The resulting models were evaluated by the coefficient of determination (R_t^2) (20), which indicates the goodness of fit between the regression line (simulated results) and the observed rate of carcass condemnations.

Evaluation of the Predicted Capability of the DHR Models

The goodness of fit of the forecasting models were calculated by using the mean relative prediction error (MRPE) defined as (21):

$$MRPE\,(\%) = \frac{1}{n} \sum_{t=1}^{n} \sqrt{\left[\frac{Y(t) - \hat{Y}(t)}{Y(t)}\right]^2} \times 100 \tag{6}$$

where $Y(t)$, $i = 1, 2, 3 \ldots n$ are the rate of cattle carcass condemnations reported in California slaughterhouses from Jan-2012 to Dec-2014, $\hat{Y}(t)$ are the last predicted rate of cattle carcass condemnations at the time to which forecasts were made (3 to 12 month) and represents discrete-time instants with a measurement interval of 1 month. For each sample of the data set (i.e., every month from Jan-2012 onwards), we estimated the model parameters and generated the 3-month-ahead prediction. To calculate the MRPE for each predictive horizon (3 up to 12 months ahead), we followed the same processes. MRPE values were calculated for all samples, and then averaged for the whole data set. The best model was considered to be the one with the lowest MRPE value. MRPE has been used by several authors and considered a good measure to quantify model prediction performance (22, 23).

RESULTS

Descriptive Analysis and Identification of Emerging Carcass Condemnation Reasons in California and the Rest of the US

In the US a total of 1,442,745 carcasses were condemned at slaughterhouses across the US over the 10 year study period (Jan-2005 to Dec-2014). The most common condemnation reasons were malignant lymphoma followed by septicemia and pneumonia, all of them representing 39.6% of the US and 37.6% of the CA condemned carcasses, respectively (**Figures 1, 2**). Among all condemned carcasses in the US during that time period, California contributed approximately 50% of the cases of mastitis and 40% metritis cases and accounted for more than 20% of the condemned carcasses due to pericarditis, toxemia,

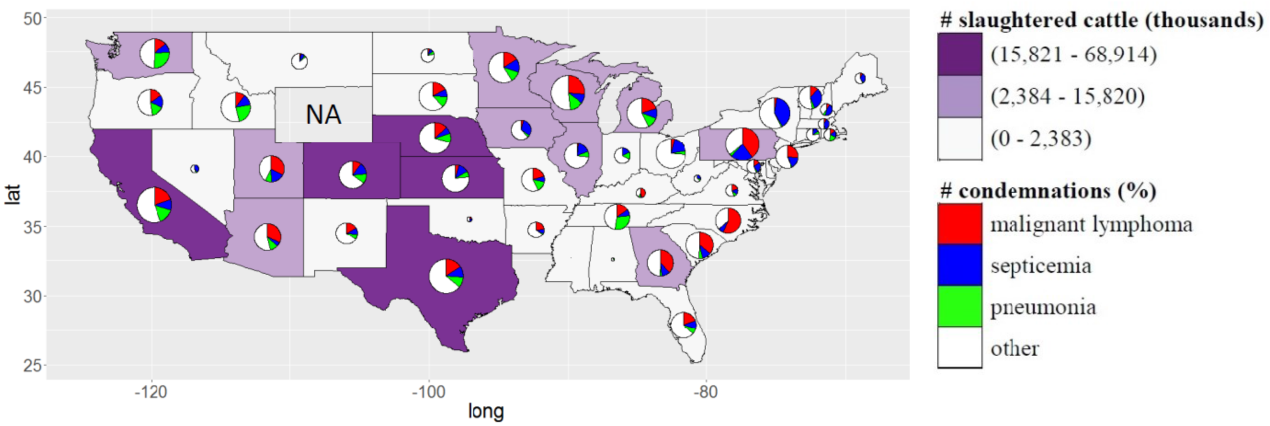

FIGURE 2 | Number of the slaughtered cattle per state from Jan-2005 to Dec-2014 based on the 70, 90 and 100% percentile. The pie chart represents the percentage of the condemnation due to malignant lymphoma, septicaemia and pneumonia and other reasons of condemnation (red, blue, green and white, respectively). The size of the pie chart is computed as: log(number of condemnation carcasses)/10. NA = data not available. The accessible and dynamic version of this map is available in Disease BioPortal (http://bioportal.ucdavis.edu/).

pneumonia, peritonitis, abscess/pyemia, emaciation, nephritis/pyelitis, eosinophilic myositis, malignant lymphoma and miscellaneous inflammatory diseases. Although during this period, California was the main contributing state with 21% of total number of carcass condemnations in the US followed by Wisconsin with 16%, the highest rates of carcass condemnations were observed in New York and Ohio (**Figure 3**).

Results from the Random walk model with NVR = 0 are shown in **Figure 4**. The rate of cattle carcass condemnations per month associated with most of the condemnation reasons did not change (slope ~0) over the 10 year study period in both California and the rest of the US. The rate of cattle carcass condemnations associated with peritonitis, miscellaneous inflammatory diseases, abscess/pyemia, pneumonia, metritis, toxemia, nephritis/pyelitis, mastitis and carcinoma increased in California (slope >0) but remained stable (slope ~0) in the rest of US during the study period. Cattle condemnation rates associated with septicemia and icterus increased in both California and in the other US states. Cattle condemnation rates associated with epithelioma decreased in both California and other US states (slope <0); although the decrease in California was steeper than in the other US states. Miscellaneous infectious diseases, emaciation, pericarditis, malignant lymphoma decreased in California (slope <0) but remained stable in the other US states (slope ~0). Detail time series of all the cattle carcass condemnations are provided in the supplementary Figure S1 and Figure S2.

There were 35 different reasons for carcass condemnations recorded in slaughterhouses in California from Jan-2005 to Dec-2014 but only one slaughterhouse reported all 35 reasons. The main condemnation reasons in California were malignant lymphoma (20%), pneumonia (14%), septicemia (10%) and peritonitis (10%; **Figure 1**).

There was an increase in the number of condemned carcasses in California from 2005 (27,556) to 2014 (39,101). Similarly, the number of slaughtered cattle in California increased from 2005 (1,356,302) to 2013 (1,734,792), but it decreased in 2014 (1,361,047), which lead to an increase in the rate of condemnations from 175 carcass per 10,000 in 2013 to 287 carcasses per 10,000 in 2014 (**Figure 3**). Interestingly, Tennessee was another state experiencing similar changes in the rate of carcass condemnations, with an increase from 174 carcass per 10,000 in 2013 to 285 carcass per 10,000 in 2014 (**Figure 3**). The main condemnation reasons contributing to the increased rate of carcass condemnations in California during 2014 were Pneumonia, Septicemia, Peritonitis, Abscess/Pyemia and Toxemia. Slaughtered dairy cows were more likely to be condemned due to malignant lymphoma, pneumonia, septicemia and peritonitis (99, 97, 70 and, 95% of the condemned carcasses, respectively) than beef cows in California (**Table 1**). Dairy cows and steers constitute 40 and 41%, respectively, of the total number of cattle sent to the slaughterhouses in California from Jan-2005 to Dec-2014, however, most (90%) of all cattle condemned in California from Jan-2005 to Dec-2014 were dairy cows (**Figure 5**, **Table 1**).

Modeling and Prediction of Temporal Dynamics of Cattle Carcass Condemnations in California From Jan 2005 to Dec 2014

Based on the autoregressive spectrum results in California, only the rate of cattle carcass condemnations associated with emaciation, eosinophilic myositis, and malignant lymphoma showed seasonal components (**Figure 6**). Therefore, three

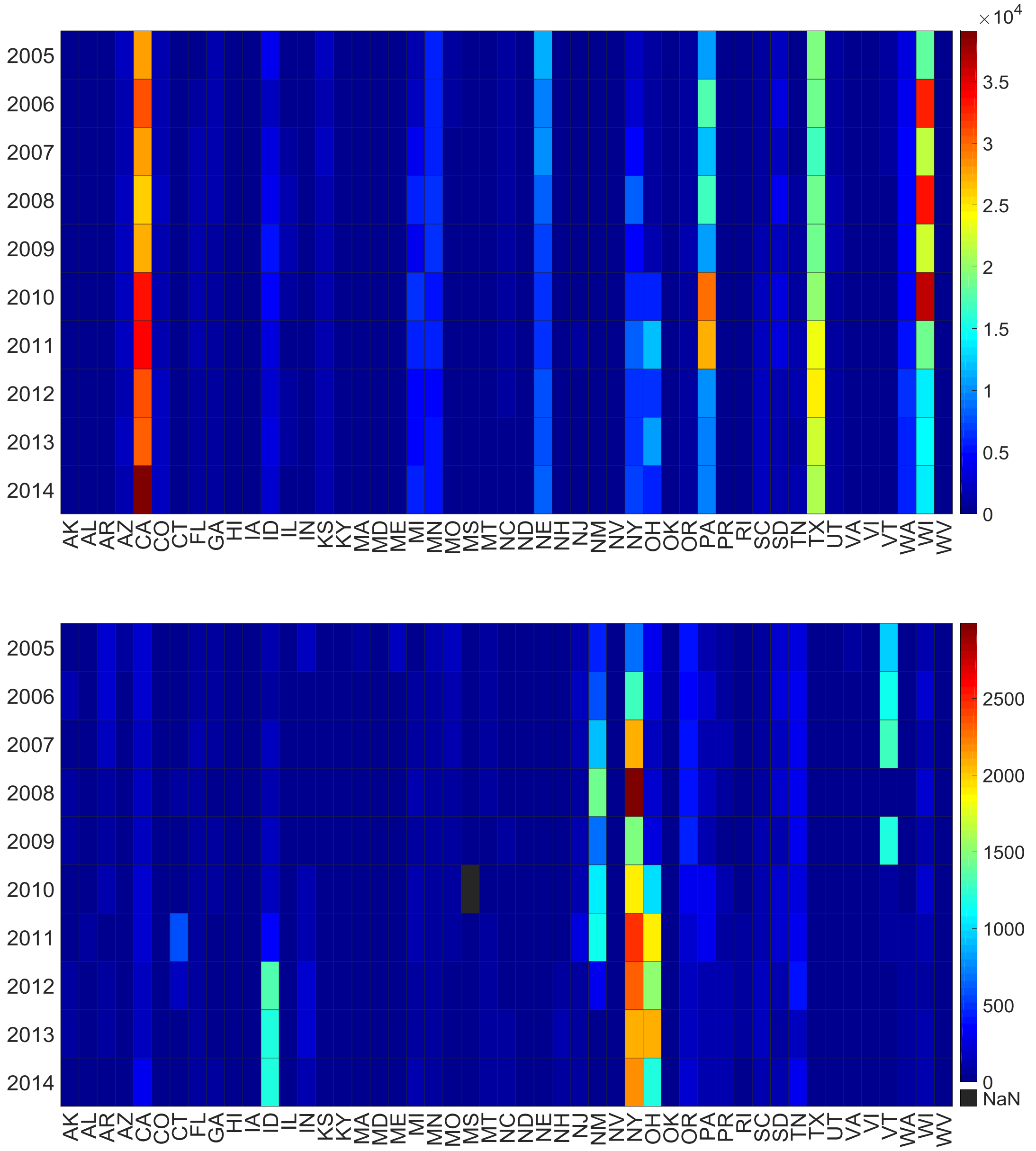

FIGURE 3 | Number **(top)** and rate **(bottom)** of cattle carcasses condemned per state and per year in the US. The rate is in 10,000 cattle heads.

predictive DHR models were built to detect the annual frequencies of emaciation, eosinophilic myositis, and malignant lymphoma. The trend estimate, forecasts of the series (with ± two times SE), seasonal component and the remaining residual components for the three condemnation reasons, are all illustrated in **Figure 7**.

The rate of condemnations associated with emaciation showed pronounced peaks at periods of 13.3, 6.1, 4.3, 2.5 and 2.1 (month/cycle), with clear increase in spring and fall and decrease in summer **Figures 6 top and 7**). The rates associated with eosinophilic myositis showed both multiannual and seasonal periodic component by the main pronounced peaks at 29.5, 11.8, 8.3, 6.8, 5.9, 2.6 and 2.4 (month/cycle) that declined in spring and peaked in summer and winter (**Figures 6 middle and 7**). The malignant lymphoma time series contained a 12 month periodicity and multiannual fluctuations with pronounced peaks at periods of 29.9, 12, 6.9, 5.6, 4.5, 3.4, 2.5 and 2.0 (month/cycle) with an increase observed during the winter (**Figures 6 bottom and 7**).

The DHR model simulated the dynamics of the reported rate of emaciation, malignant lymphoma and eosinophilic myositis from Jan-2005 to Dec-2011 with an R_T^2 of 0.98, 0.87, and 0.78, respectively, and could predict the rate of condemned carcasses

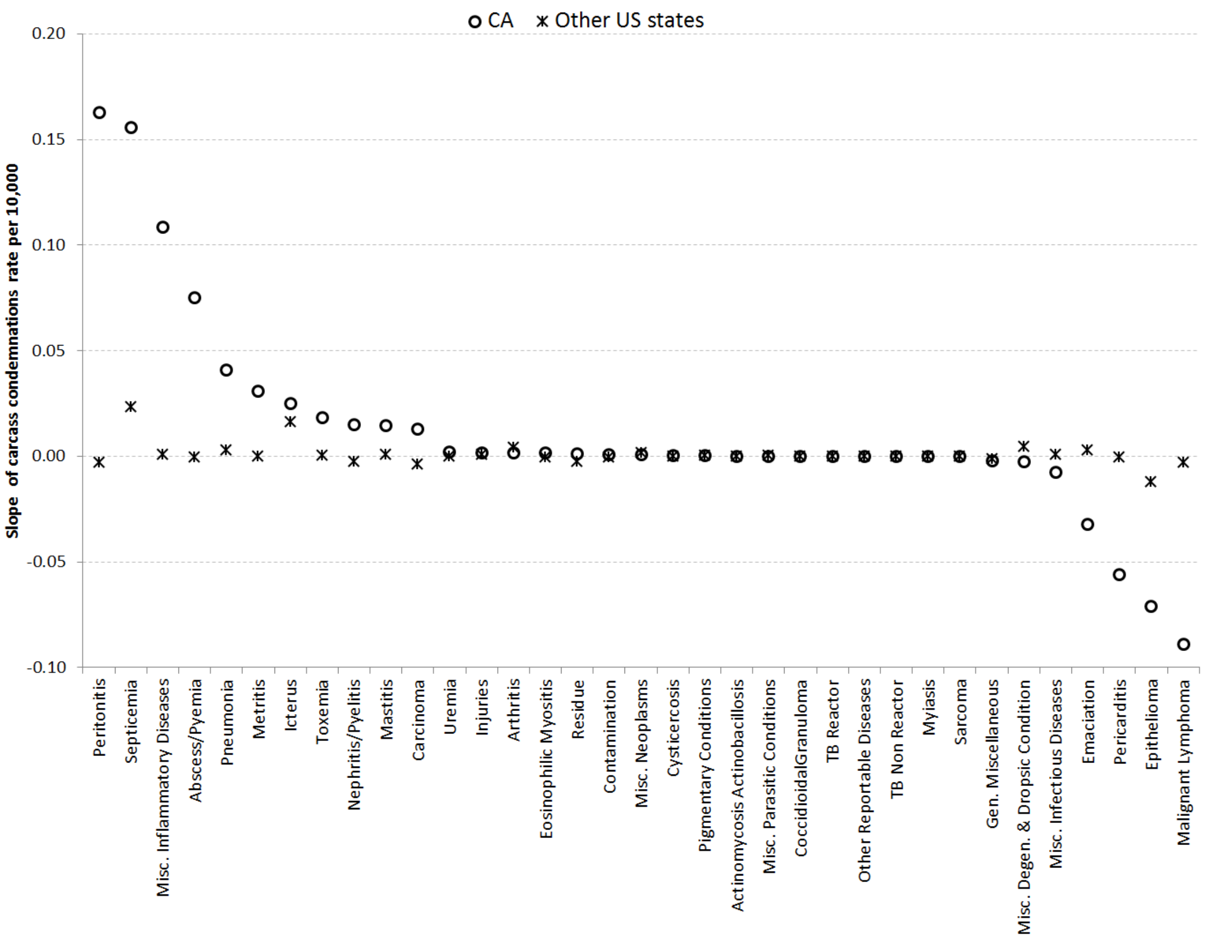

FIGURE 4 | Calculated slope (based on integrated random walk model; Equation 5) for the rate of carcass condemnations (number of the condemned carcasses per reason/number of the slaughtered cattle heads) associated with different reasons from Jan-2005 to Dec-2014 in slaughterhouses in California (circle) and other US states (star). A positive calculated slope indicates an increase in the reported rate of carcass condemnations whereas negative slope indicates a decrease in the rate of carcass condemnations in slaughterhouses.

three months ahead from Jan-2012 to Dec-2014 with MRPE of 33, 11, and 38% for the three carcass condemnation reasons, respectively (**Figure 8**). The MRPE was used to compare the model performance for a range of prediction horizons (from 3–12 month, **Figure 8**). The rate of the carcass condemnation can be predicted 12 month ahead with the MRPE of 38, 18 and 32%

TABLE 1 | Number of malignant lymphoma, abscess/pyemia, emaciation and, epithelioma carcasses condemned in California from Jan-2005 to Dec-2014 by type of cattle. The percentage is presented in parentheses.

Type of cattle	Malignant Lymphoma	Pneumonia	Septicemia	Peritonitis
Beef cow	316 (0.51)	262 (0.61)	481 (1.57)	97 (0.32)
Bob veal	18 (0.03)	146 (0.34)	7,982 (26.09)	914 (3.06)
Bull/Stag	72 (0.12)	64 (0.15)	104 (0.34)	58 (0.19)
Dairy cow	60,474 (98.71)	41,698 (96.68)	21,415 (70.00)	28,478 (95.38)
Formula-fed veal	0 (0)	4 (0)	0 (0)	1 (0)
Heavy calf	29 (0.05)	433 (1.00)	83 (0.27)	90 (0.30)
Heifer	88 (0.14)	181 (0.42)	129 (0.42)	83 (0.28)
Non formula fed veal	1 (0)	47 (0.10)	28 (0.09)	13 (0.04)
Steer	267 (0.44)	296 (0.69)	367 (1.20)	123 (0.41)
Total	**61,265**	**43,131**	**30,589**	**29,857**

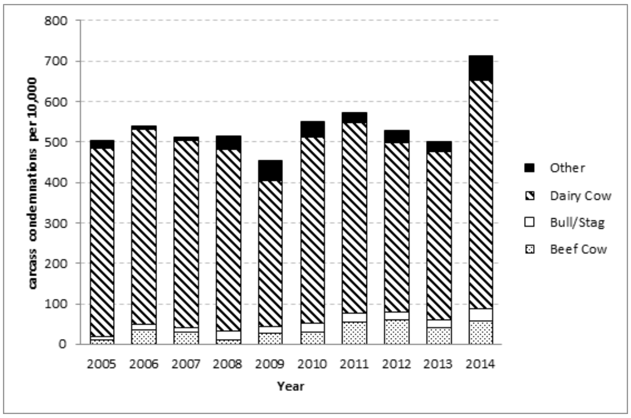

FIGURE 5 | The rate of carcass condemnations per 10,000 animals per year (number of condemned carcasses per type of cattle i.e., dairy cow, bull/stag, beef cow and other)/number of cattle slaughtered per type of cattle) in California. The category of "other" type of cattle includes bob veal, formula-fed veal, heavy calf, heifer, non formula-fed veal and steer cattle.

for emaciation, malignant lymphoma and eosinophilic myositis, respectively (**Figure 8**).

DISCUSSION

In this study we analyzed slaughterhouse data from the US to show the value of the systematic analysis of slaughter data and to describe the most important condemnation reasons in the US and California, identify emerging condemnation reasons, characterize seasonality and temporal patterns and predict the rate of carcass condemnations in different time horizons (i.e., from 3 to 12 months ahead). We believe that the continuous and systematic analysis of slaughter data as shown here can lead to establishment of a low-cost monitoring system for early detection of emerging problems and zoonotic diseases in food animals.

The rate of carcass condemnations associated with septicemia and icterus showed clear evidence of increase both in California and in the rest of the US. Peritonitis, misc. inflammatory diseases, abscess/pyemia, pneumonia and metritis were increasing only in California (**Figure 4**). The emergence of these condemnations could be the result of a number of impacts in all parts of the food chain, such as environmental and management conditions, stress, pathogens, etc. (24). Because the state of origin of the cattle may be different from the location of the slaughterhouse, a better understanding of the patterns of carcass condemnations together with the successful trace back of any diagnostic cases found in the slaughterhouse could help identify where an animal may have acquired infection, the location of any other animals that may have been exposed to the same pathogens, or find locations that share risk factors or management practices that may lead to carcass condemnation.

The USDA FSIS data from 2005 to 2015 showed that the cull dairy cows in CA were more likely to be condemned for malignant lymphoma, pneumonia, septicemia and peritonitis than the beef cows (**Table 1**). This disproportion of condemned carcasses among the type of cattle is due to the fact that the priorities in the dairy industry are milk production, herd reproduction, and prevention of health conditions such as mastitis (25) and dairy producers also have a number of other issues that might compete with their need to change culling policies (2). In general the production system in dairy cattle is intense and their lifespan is long (around 3–4 years) therefore there is more chance for dairy cattle to develop age related problems or chronic diseases such as Johne's disease or cancer that can cause emaciation and other deteriorating symptoms in the animals.

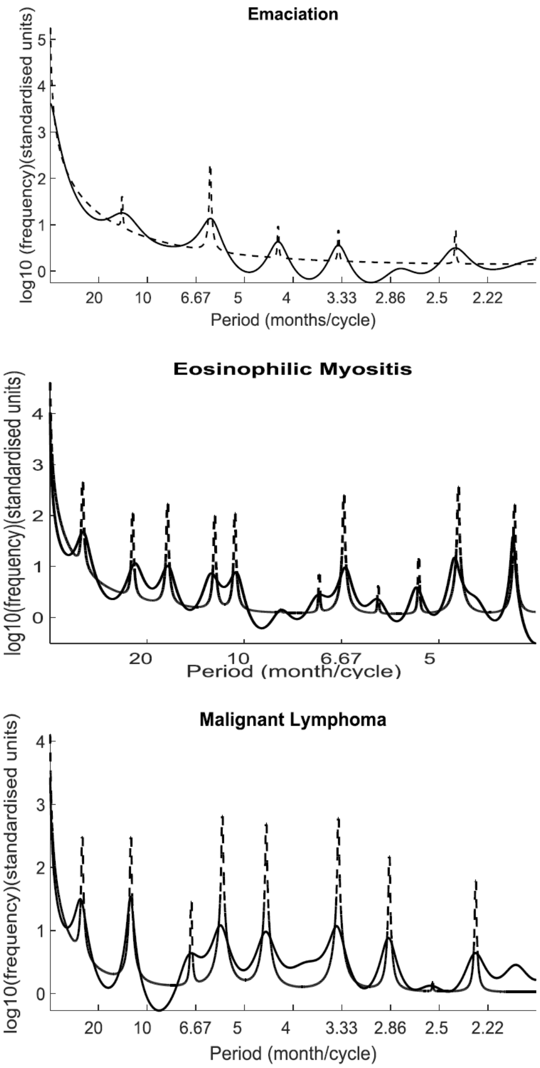

FIGURE 6 | Autoregressive spectrum (23th order) of the reported emaciation (top), eosinophilic myositis (middle) and malignant lymphoma (bottom) condemnation cases in slaughterhouses in California. Solid line indicates the autoregressive spectrum of the carcass condemnation rate. The dashed line indicates the AR spectrum of the DHR model of the carcass condemnation rate using frequency domain NVR optimization.

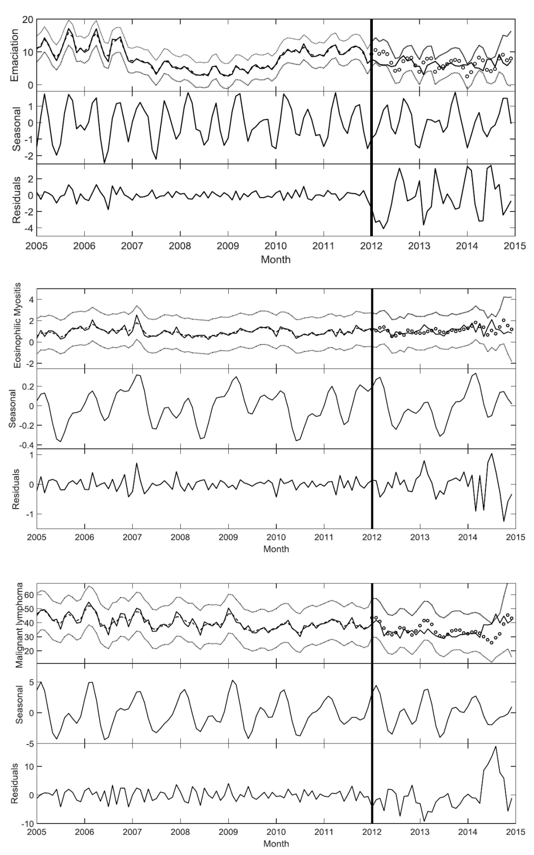

FIGURE 7 | Results of the three Dynamic Harmonic Regression (DHR) models for the carcass condemnation rate [i.e., emaciation (top), eosinophilic myositis (middle) and malignant lymphoma (bottom)] in California from Jan-2005 to Dec-2014. The subplot at the top presents the model simulation results (in dashed line) from Jan-2005 to Dec-2011, the data (solid line) and the two times SE bounds (dotted lines) from Jan-2005 to Dec-2014 and, the 3 month ahead DHR model predictions from Jan-2012 to Dec-2014. The other two subplots present the seasonal component from Jan-2005 to Dec-2014 and the residuals extracted using the discrete-time white noise (Equation 1) from 2005 to Dec-2011 and the prediction error from Jan-2012 to Dec-2014

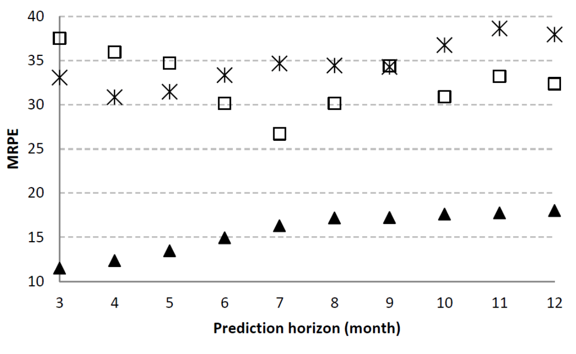

FIGURE 8 | Mean relative prediction error (MRPE) vs prediction horizon for the rate of carcass condemnations of emaciation (star), eosinophilic myositis (square) and malignant lymphoma (triangle) in California. The smaller MRPE indicates a closer fit of the model to the data.

Seasonality was found to be significant only in three condemnation reasons in California: emaciation, eosinophilic myositis and malignant lymphoma (**Figures 6 and 7**), which are representing 3.9, 0.5 and 19.9% of total condemned carcasses in California respectively. The seasonal variation in the number of condemned carcasses could be linked to management and environmental factors that vary seasonally (e.g., market costs, land constraints, or production goals), or to the seasonal occurrence of diseases or syndromes that lead to carcass condemnation. A study in Ontario from 2001 to 2007 (5) found seasonal effects for the number of condemned carcasses showing fewer condemned carcasses in the summer and fall compared to winter. Vial and Reist et al. (3) found seasonal patterns in the number of carcass condemnations in cattle in Switzerland between 2007 and 2012 with a peak in March. A peak in the number of condemned cattle can also occur when more animals are sent to slaughter or when lower quality animals are sent to slaughter in specific seasons. Seasonality could also be associated with forage availability and feed prices among other seasonal changes in management practices and in the environment that have a direct impact animal health (3, 5).

Eosinophilic myositis exact causes are unknown although it has been associated with Sarcocystis cruzi infection (26). A more refined spatial analysis and trace back of where those carcasses are coming from may help to identify high risk areas where particular attention and biosecurity measures should be taken to avoid *S. cruzi* infection in cattle.

Emaciation is associated with wasting conditions caused by chronic diseases. Known causes of emaciation are parasitism, chronic abscess, chronic musculoskeletal pain, advance neoplasias, Johne's disease, and malnutrition (27). Dairy cows contribute most to the number of condemned carcasses in California for these conditions (85%). Our study shows seasonality in the number of condemned carcasses due to emaciation, with a peak in spring and fall and a drop in summer (**Figure 7**). The observed seasonality most likely has to do with the number and type of cows culled in the fall that put adult cows that were not pregnant during the calving season in spring are more likely to be culled in fall after four or five unsuccessful breeding attempts (28).

Malignant lymphoma is the clinical manifestation of an infection caused by the retrovirus bovine leukemia virus (BLV), which infects cattle's lymphoid tissue. Once in the herd, the virus can be transmitted by blood containing infected lymphocytes in shared needles and during dehorning, tattooing or rectal examination (29). When tumors are found at slaughter, carcasses are condemned following federal regulation (30). Malignant lymphoma is the main cause of carcass condemnation in California and other US states (**Figure 1**) and it affects mainly dairy cattle, which contributed to 99% of the all malignant lymphoma condemned carcasses in California from Jan-2005 to Dec-2014 (**Table 1**). In our current study, we found a declining trend in the rate of condemned carcasses due to malignant lymphoma in California. Two previous studies in 1996 and 2007 USDA dairy studies showed a slight decrease in prevalence from 89.0 to 83.9% seropositive dairy herds due to BLV (31). However, the 2007 dairy study also showed higher prevalence in the East (84.4%) than West (78.4%), which could explain the different trends observed between California and the rest of US. Condemned carcasses due to malignant lymphoma peak in winter. Because BLV is a chronic disease, the peak is most likely associated with management decisions. In addition, BLV affects the immune system (making cattle more susceptible to other diseases), and when this is combined with winter environmental factors, cows can be clinically poorer in winter and have decreased milk production, resulting in the cattle being sent to slaughter. Also, since biting insects can transmit BLV (32), changing environmental conditions during the study period can have an impact in insect ecology and disease dynamics. Since distribution of infection is uneven across farms within a region(29), detailed slaughterhouse data with possibility to trace back those cases to the farm of origin could help approximate BLV prevalence in different areas in California where BLV has a big impact on the dairy industry.

In this study we also aimed to evaluate the predictive ability of our models for different time horizons (3–12 month). Therefore, we quantified the model performance predicting the rate of carcass condemnations due to emaciation, malignant lymphoma and eosinophilic myositis from Jan-2012 to Dec-2014. A better understanding of the expected rate of condemnation cases by state, slaughterhouse and farm can definitely help producers and veterinary practitioners better plan and optimize management and disease control strategies to mitigate causes leading to those condemnations. Our results illustrate that the model predicts the rate of carcass condemnations due to emaciation, eosinophilic myositis, and malignant lymphoma quite well for one season (3 months) and up to 12 months ahead with a MRPE of 33, 38 and 11%, respectively (**Figure 8**). Although the MRPE values in our study were not very low, they were around 10% lower than values found by other researchers for similar modeling approaches in similar applications (18). Similarly, the R_T^2 values in our study were considerably high (R_T^2 of 0.98, 0.78 and 0.87 for emaciation, eosinophilic myositis, and malignant lymphoma, respectively) showing a good model fit.

It should be noted that our models did not use any variables as inputs in the data-based model. Taking into account management and climatic factors are expected to further improve the modeling results, although the dynamics of the emaciation, malignant lymphoma and eosinophilic myositis condemned carcasses in slaughterhouses in California were already predicted satisfactorily just based on the cyclic components of the time series. The specific role of environmental and climatic factors in the dynamics of the reasons for carcass condemnation is an ongoing area of research. A dataset covering a longer period might also improve the modeling results. Having access to information on the total animals slaughtered in each slaughterhouse per day, instead of just aggregated by month and by state, will allow us to conduct more detailed spatio-temporal analyses and generating semi-automatic notifications/alerts when the observed number of condemned carcasses exceeds the expected condemned carcasses in a particular slaughterhouse/area. Future studies may also be conducted to understand and predict how long-term environmental and climatic trends (e.g., factors associated with climatic change) and their variability may impact cattle health, animal welfare and livestock production sustainability.

CONCLUSIONS

Slaughter data is a valuable source of information for animal and public health. Our DHR modeling approach provided valuable insights to identify emerging syndromes in the US and quantify the inter-annual changes in the trend and seasonality of the rate of carcass condemnations associated with emaciation, malignant lymphoma and eosinophilic myositis in cattle slaughterhouses in California. Such a data-based modeling approach could be easily extended to other species and can provide the foundations to develop a low-cost syndromic surveillance system using real-time slaughterhouse information to early-detect outbreaks or changes in the number of condemned carcasses in specific locations and time periods. This could be very useful to inform producers and veterinary practitioners to improve farm level management practices and interventions to minimize and prevent the drivers contributing to those condemnations, saving producers and livestock industry millions of US dollars annually.

AUTHOR CONTRIBUTIONS

BM-L and SAH conceived and designed the study. SAH processed the data and conducted the analyses under the supervision of BM-L. BM-L, GV, AE, DM and NSR contributed to the critical interpretation and discussion of the results. All authors read, reviewed, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fvets.2018.00087/full#supplementary-material

FIGURE S1 | Time series of the carcass condemnation rates (number of condemned carcasses/ number of cattle slaughtered; solid line) observed in California from 2005-2014 with the trend line (bold line) and two times standard error bounds (dotted lines) for each of the 35 condemnation reasons.

FIGURE S2 | Time series of the carcass condemnation rates (number of condemned carcasses/ number of cattle slaughtered; solid line) observed in the other US states (without California) from 2005-2014 with the trend line (bold line) and two times standard error bounds (dotted lines) for each of the 35 condemnation reasons.

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Decision Support for Mitigation of Livestock Disease: Rinderpest as a Case Study

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A versatile, interactive model to predict geographically resolved epidemic progression after pathogen introduction into a population is presented. Deterministic simulations incorporating a compartmental disease model run rapidly, facilitating the analysis of mitigations such as vaccination and transmission reduction on epidemic spread and progression. We demonstrate the simulation model using rinderpest infection of cattle, a devastating livestock disease. Rinderpest has been extinguished in the wild, but it is still a threat due to stored virus in some laboratories. Comparison of simulations to historical outbreaks provides some validation of the model. Simulations of potential outbreaks demonstrate potential consequences of rinderpest virus release for a variety of possible disease parameters and mitigations. Our results indicate that a rinderpest outbreak could result in severe social and economic consequences.

Keywords: rinderpest, mechanistic model, spatial model, vaccination, culling, epidemic, mitigation

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1. INTRODUCTION

Rinderpest has been among the most devastating livestock diseases in history (1, 2). Rinderpest virus is a virulent and highly contagious pathogen that infects many cloven-hoofed livestock and wildlife species, resulting in death rates as high as 90%. Rinderpest has ravaged cattle populations in Europe, Asia, and Africa over several centuries. Use of highly effective vaccines in the context of regional and national zoosanitary control and eradication efforts led to a world-wide effort for eradication beginning in 1994. The last wild case was reported in 2001 and rinderpest was declared officially globally eradicated in 2011. It is the first animal pathogen and only the second pathogen (after smallpox virus) that has been eradicated.

While rinderpest does not exist in the wild, several countries maintain rinderpest samples and vaccines as a hedge against reintroduction (3), although this number is decreasing with 3 African countries removing their stores in 2016 (4). Current maintenance of virus and vaccine stocks is a balancing act between risk and reward. The primary purpose of the modeling presented here is to provide quantitative assessment of the consequences of virus release.

Past and potential rinderpest outbreaks are modeled as single epidemic entities amenable to control by a single vaccine, and with a reasonably well-defined presentation and progression of disease. The rinderpest virus is an RNA virus, a member of the *morbillivirus* family; Measles virus is related by divergence in the historical era (5). Several biological features support modeling rinderpest disease as a single epidemic entity. The phylogenetic diversity of the virus is not recognized to contain distinct clades. The empirical observation that vaccination using a single strain is an effective population-level control, coupled with the comparative ease of diagnosis in advanced disease, suggests low phenotypic diversity.

One of the crucial lessons that underpins our approach to modeling rinderpest in this paper is the importance of distinguishing epidemic growth due to spread in new locations vs. continued growth in areas of older infection. This observation may seem self-evident, but consideration of past analyses (e.g., Ebola in West Africa in 2014–2015), demonstrates that models which inappropriately aggregated geography can mask vital dynamics of mitigation and disease control in areas of older infection by relatively uncontrolled epidemic growth in areas of recent infection. A number of technical choices in the formulation of our decision-support oriented epidemiological model are driven by the critical importance of separating geography (see section 2.3) from temporal dependence. Making this separation in a robust way allows for far more reliable differentiation between the fundamental processes of contagion and disease progression, and the human interventions and actions that *modulate* those dynamics. In the modern world, it is these modulations that determine the outcome of epidemics. Aiding the analyses of interventions is the central challenge of epidemiological decision support, and provides the chief drivers for the design of our model in this paper.

The simulation model includes rinderpest disease progression, geographic spread, and the ability to perform historically successful mitigations such as vaccination, culling, and transmission reduction. Both a deterministic and a hybrid stochastic-deterministic version were developed. Challenges in developing compartmental, deterministic models arise from fractions of animals present in compartments–particularly at the start of disease progression in a geographical region. Similarly, fractions of animals can cause deterministic methods to have difficulty modeling extinction events (6). Spread can occur too rapidly and vaccination appears less effective because tiny fractions of individuals can start an outbreak in a new location in deterministic SIR-type models. This drawback of deterministic models was avoided by choosing a threshold for the number of exposed individuals required to start disease propagation in a geographic region. The exact value of this threshold was determined by calibration with the hybrid stochastic-deterministic model.

We first provide an overview of historical rinderpest outbreaks relevant for predicting a novel outbreak in a naïve population outlining important factors determining the scale of consequence as impacted by various mitigations. The epidemic model is then described in detail with a discussion of its accuracy and limitations. Possible outcomes resulting from reintroduction scenarios of the rinderpest virus are presented and discussed.

1.1. Historical Perspective of Rinderpest Outbreaks

We guide our modeling of rinderpest outbreaks in immunologically naïve cattle populations with consideration of historical examples provided in reviews (1, 7, 8). In reviewing this history, we pay particular attention to the rate at which the geographic extent of the epidemic increases, the overall mortality rate, and the circumstances under which the epidemic was ultimately brought under control.

In 1715, Giovanni Lancisi demonstrated effective control of rinderpest outbreaks in areas of Papal Authority through zoosanitary controls (7), such as effective separation of sick and healthy animals including removal of healthy cattle from pastures where sick animals previously resided, fumigating the clothes of shepherds, burial of carcasses in deep pits, and instantly throwing milk from sick cows into a hole in the ground. The killing of animals was preferred over treatment (9). The ability to stop a small outbreak via culling and zoosanitary methods was also demonstrated by control of a 1923 outbreak in Australia through slaughter of 3,000 cattle, sheep, goats, and pigs (10).

A particularly devastating epidemic, The Great Rinderpest Pandemic of ∼1887–1898, was the first in Africa (11) and is described in detail by several authors (1, 11, 12). In eastern Africa, cattle mortality rates were 98% (12) and the Massai (Masai) were devastated by the almost complete loss of their cattle (1, 11). The epidemic spread many hundreds of miles in a single year, whether inland to the Sudan from the coast, from Kenya into Tanzania, or through Zimbabwe into South Africa (1, 11). After being stopped by the Zambesi river for ∼3 years, rinderpest crossed the river and spread rapidly toward South Africa at a rate of 20 mi/day (32 km/day), roughly equal to the distance an ox cart traveled in a day. Control methods of fencing, safety corridors, armed border police, and slaughter by government order (with compensation) were implemented in Transvaal and Cape Colony (South Africa). However, by late 1896 these methods were failing (12). Research into vaccine development in South Africa led to the development of a bile and serum vaccine in 1897 that had some efficacy(12).

Motivated by this pandemic, and numerous other epidemics throughout Europe, Africa, and Asia, both vaccines suitable for mass vaccination, and molecular diagnostic techniques were developed throughout much of the twentieth century (7, 13). An intensive vaccination program in China from 1950 to 1955 combined with zoosanitary measures successfully eradicated rinderpest from China (7). In other locations, however, vaccination led to tremendous reduction in rinderpest cases, followed by a later resurgence due to incomplete eradication. Joint Project 15 (JP-15) operated in the the field in Africa from 1962 to 1976 involving 22 African countries, 17 of which had active rinderpest (14, 15). This vaccination program eliminated rinderpest from large portions of Africa. However, at its end, small pockets of rinderpest existed in both east and west Africa (1, 16). By the early 1980's these pockets had expanded to cover most of sub-Saharan Africa (1) and expensive international emergency efforts were needed to bring them under control (1, 13).

The 1994 outbreak in the Shangri-La region of Pakistan (17) shows how difficult it can be to extinguish a rinderpest outbreak in the modern era without ongoing vigilance of vaccine quality and communication and education of appropriate hygienic measures to the afflicted population. Fortunately, the inaccessibility of the region, relatively low cattle population densities, and potentially the mild pathogenicity of the viral strain, kept this outbreak confined over the 18 months it took to effectively respond and extinguish this epidemic. Nevertheless, > 80% mortality rates were noted in most affected regions (17).

While some of the more dramatic epidemics were due to strains of high virulence and high reproductive number (R_0), both R_0 and virulence vary across rinderpest strains. Endemic propagation of rinderpest leads to the dominance of milder strains of the virus with both lower virulence and R_0 (18–20). Estimates of R_0 over the different strains range from 1.2 to ∼5 (19).

2. METHODS

To facilitate exploration of the effects of different disease parameters and the utility of a variety of mitigations, the model has been implemented as an interactive application using the Shiny package of R. The code can be found at https://github.com/pfenimore/rinderpest.

2.1. Disease Progression

For clarity, the disease progression model is first described for a single, well-mixed geographical area without spatial spread. **Figure 1** is a schematic of this disease progression with vaccination; culling is omitted. The corresponding differential equations governing the deterministic model, with culling included, are Equations 1–8. β is the transmission parameter with units of inverse time. The subscripts for disease progression rates are the starting and ending states, e.g., k_{IH} is the rate of going from the infectious and mildly ill state, I, to the seriously ill state, H. The rate susceptibles move to the vaccination-given category is k_V. Cattle in states S, E, I, and H can be culled (not shown in **Figure 1**). The rate of culling from state H is k_{Hc}. The total number of live cattle is N.

$$\frac{dS}{dt} = -\beta\frac{(I+H)}{N}S - k_V S - k_{Sc}S \tag{1}$$

$$\frac{dE}{dt} = \beta\frac{(I+H)}{N}(S+V_g) - k_{EI}E - k_{Ec}E \tag{2}$$

$$\frac{dI}{dt} = k_{EI}E - k_{IR}I - k_{IH}I - k_{Ic}I \tag{3}$$

$$\frac{dH}{dt} = k_{IH}I - k_{HD}H - k_{Hc}H - k_{HR}H \tag{4}$$

$$\frac{dD}{dt} = k_{HD}H + k_{Sc}S + k_{Ec}E + k_{Ic}I + k_{Hc}H \tag{5}$$

$$\frac{dR}{dt} = k_{IR}I + k_{HR}H \tag{6}$$

$$\frac{dV_g}{dt} = k_V S - \beta\frac{(I+H)}{N}V_g - k_{V_g V_m}V_g \tag{7}$$

$$\frac{dV_m}{dt} = k_{V_g V_m}V_g \tag{8}$$

2.2. Values of Disease Progression Parameter

Incubation period is defined as the time between inoculation and virus secretion. Rinderpest virus can be shed a day or two before the onset of fever (21), but the level is low and shedding occurs only in a minority of animals (20). Consequently, the incubation period is taken as the time from inoculation to onset of fever. Rossiter and James give a value of 5.6 days (20). States I and H are both infectious, with I denoting the non-specific, non-severe stages of disease and H denoting severe disease. State H is defined as the infectious period after onset of characteristic mouth lesions. The animals also stop eating at this point and are obviously quite sick (14). For cattle with a virulent strain, these lesions become common roughly 3–5 days after fever onset (21).

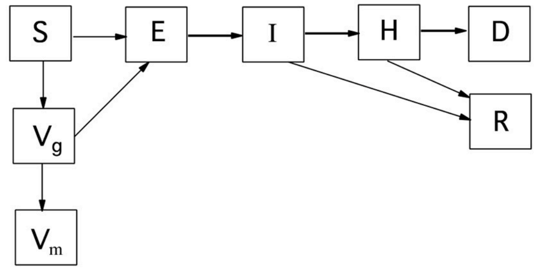

FIGURE 1 | Disease progression for a single well-mixed geographical region. S, susceptible; E, exposed; I, infectious; H, seriously ill; D, dead; R, recovered; V_g, vaccine given; V_m, immune by vaccination. Culling is not shown, but can move cattle from states S, E, I, and H to D.

Disease progression has varied between locales where the virus was present (19). In **Table 1**, the parameters used for our simulation of the 1994 Pakistan epidemic are shown along with parameters for two of the last lineages to be eradicated (19). Lineage-1 was considered to be of moderate virulence and was at one time widespread in Africa. Lineage-2 caused mild disease and was also present in Somalia. The values for time spent in states I and H in **Table 1** are set to be similar to the total time spent in state I in the SEIR model of Mariner et al. (19) for lineages 1 and 2. Mouth lesions last 3 or more days (21), however, for less virulent strains a minority of animals (∼20%) do not develop mouth lesions (20). Consequently, the extra time in the infectious state of the less virulent strains is assigned to state I rather than state H. The fraction of rinderpest infected cattle that die varies greatly with strain, increasing from ∼2 to ∼90% with virulence (20).

2.3. Geographic Spread

Geographic spread is primarily modeled as being due to the movements of cattle and people directly caring for the animals. Spread to market locations can be modeled also. Only for the model of the outbreak in Pakistan is movement along roads modeled. In this section, the methods for modeling spread due to everyday activities as well as to market locations are described.

After initial incidence at a point location, disease spread is implemented on a geographic grid of cattle population with the center block containing the initial point of incidence. Geographic spread of disease is approximated as a force of infection communicating between geographical compartments. The population does not move between compartments (i.e., population grid elements). For illustrative purposes, a simple

TABLE 1 | Rinderpest disease progression parameters.

	Pakistan 1994	Lineage-1	Lineage-2	References
β (1/days)	0.31	1.1–1.4	0.17–0.19	Pakistan 1994; personal communication Paul Rossiter lineages 1 & 2; (19)
Incubation period	5.6	4.5–7	5.5–8	Pakistan 1994; (20)
$1/k_{EI}$ (days)				lineages 1 & 2; (19)
$1/k_{IH}$ (days)	3	1–3	4–8	estimated using (19–21)
$\frac{1}{(k_{HD}+k_{HR})}$ (days)	3	3	3	estimated using (19–21)
Fatality fraction from H	0.9	0.36	0.06	Pakistan 1994; (17). Values for lineages 1 & 2 calculated using rates to R and D in Mariner's SEIR(D) model (19).

geographic grid with 25 blocks is shown on the left in **Figure 2**. (Note: only the grid is shown, population values are not). For the disease to spread out of the initial geographic block, some of the healthy animals in surrounding geographic blocks must be exposed to rinderpest virus. The form of the spatial spread is assumed to be similar to that of foot and mouth disease (FMD) which, as another animal disease affecting livestock, has similar spread mechanisms. The ability of FMD to spread from one location to another is known to decay rapidly with distance when movements of animals between farms and to markets is highly restricted (22, 23). We approximate this decay as exponential with distance and an example of this transmission kernel is shown on the left in **Figure 2**. For each block a one dimensional column in the contact-availability array is computed. In this example, a 25 by 25 matrix, A, is generated.

The contact-availability, $A(i,j)$, of virus from an infectious population of animals in block j, to animals in block i is controlled by the exponentially decaying transmission kernel. Therefore, the spatial extent of the receiving block must be taken into account. This is done approximately as shown below,

$$A(i,j) \propto \int_{r(i,j)-\mathrm{b}/2}^{r(i,j)+\mathrm{b}/2} e^{-x/\mathrm{d}}dx = 2\mathrm{d}e^{-r(i,j)/\mathrm{d}}\sinh(\frac{\mathrm{b}}{2\mathrm{d}}) \tag{9}$$

where b is the size of a square block as defined in **Figure 2 (left)**, $r(i,j)$ is the center-to-center separation of blocks i and j, and d is the characteristic length of the exponentially decaying transmission kernel and has a value of 3 in **Figure 2 (right)**.

For $r(i,i) = 0$, the contact-availability is

$$A(i,i) \propto 2\mathrm{d}(1 - e^{-\mathrm{b}/(2\mathrm{d})}) \tag{10}$$

The total contact availability of an animal should not depend on grid size, b. We also assume that total contact availability is independent of d and normalize it to 1. Therefore, we need,

$$\sum_{i=1}^{n} A(i,j) \leq 1 \tag{11}$$

where n is the number of blocks in the grid. $\sum_{i=1}^{n} A(i,j)$ will be less than one if there is significant availability outside of the geographically modeled region. To facilitate normalization of the contact availability matrix, $A(i,j)$, the simulations should be set-up such that the transmission kernel for the center box decays to approximately 0 at the edges of the modeled region. In practice, if the smallest dimension of the modeled region is 2w, w $\gg$ d. Therefore, the sum of all elements of the contact-availability matrix for the center block, $j = c$, is used to normalize the contact-availability matrix.

$$\sum_{i=1}^{n} A(i,c) = \sum_{i \neq c} 2\mathrm{d}e^{-r(i,c)/\mathrm{d}}\sinh(\frac{\mathrm{b}}{2\mathrm{d}}) + 2\mathrm{d}(1 - e^{-\mathrm{b}/(2\mathrm{d})}) \tag{12}$$

In addition to the non-directional spread described above, directional spread is also implemented; a location of a feed-lot, or market can be specified. The fraction of infectious spread that is directional, f_D, is also specified by the user. If there is only one location that is a gathering point, e.g., a feedlot, then, a long distance contact availability array, $D_{i,j}$ can be defined as,

$$D(i = \text{feedlot}, j) = f_D \quad D(i \neq \text{feedlot}, j) = 0 \tag{13}$$

More generally,

$$\sum_{i=1}^{n} D(i,j) = f_D \tag{14}$$

The total contact availability matrix is then,

$$T(i,j) = (1 - f_D)A(i,j) + D(i,j) \tag{15}$$

and

$$\begin{aligned} \sum_{i=1}^{n} T(i,j) &= (1 - f_D)\sum_{i=1}^{n} A(i,j) + \sum_{i=1}^{n} D(i,j) \\ &= (1 - f_D)\sum_{i=1}^{n} A(i,j) + f_D \leq 1 \end{aligned} \tag{16}$$

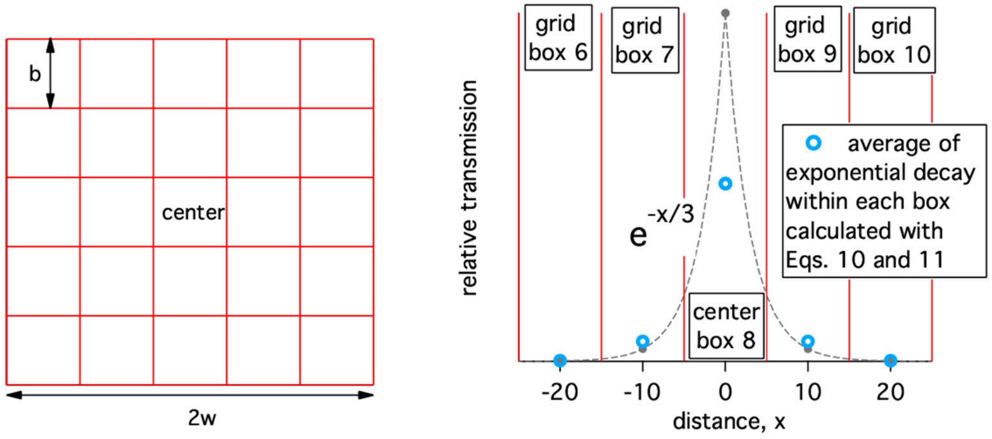

FIGURE 2 | Left: A simple geographic grid with 25 blocks (grid elements). **Right:** Example of the relative availability of an infected animal to infected a healthy animal a distance, x, away. The width of the geographic boxes, b, is 10 and the decay of the transmission kernel is 3.

2.4. Mitigations

Several mitigations are implemented by time-dependent adjustment of rates in Equations 1–8; transmission control, vaccination, and culling. Mitigation implementation times are referenced to the time of disease identification. The disease is identified when the number of cases supersedes a set number specified by the user.

Transmission control reduces the interactions of virus-from infectious cattle- with healthy cattle. It is implemented as a reduction of β. This mitigation can represent, for example, keeping animals in different stalls (i.e., short range movement control) or improved hygiene by the people caring for the animals. The fraction of spread that is due to long range (directional) movement can be reduced. This could represent a ban on the transport of animals to feed-lots or markets.

Vaccination or culling is performed within user defined rings around each block containing symptomatic animals (states I, and H, but not E). The assumption is that once rinderpest has been detected, farmers and veterinarians will be on the lookout for animals with early symptoms. The list of geographical blocks where vaccination or culling is desired is updated once a day.

The number of vaccine doses which can be given in a day, V_d, is a user input. In scenarios for which there is not enough vaccine to vaccinate all of the healthy population within a given radius of symptomatic cattle, an equal fraction of cattle are vaccinated in each of the chosen geographic blocks.

The rate for vaccination in a specific location is $k_V(i)$, where i is the index for a geographic box. In a time step, t_s, with units of days, the number of doses available is $V_d t_s$. The total number of cattle available for vaccination, S_t, is the sum of all susceptible cattle in the region chosen for vaccination. The number of doses administered in a geographic box, i, is then $V_d t_s S(i)/S_t$. We assume that vaccination rates are much faster than the rate at which animals get sick and that culling and vaccination do not occur simultaneously in the same location. The expression for $k_V(i)$ assumes that $S(i)$ decays exponentially with decay constant $k_V(i)$. The change in susceptibles, $\Delta S(i)$, equals the number of doses administered and is given in Equation (17).

$$V_d t_s S(i)/S_t = \Delta S(i) = S(i)(1 - e^{-k_V(i)t_s}) \tag{17}$$

Therefore,

$$k_V(i) = log(1 - V_d t_s/S_t)/t_s. \tag{18}$$

However, if the total number of doses available in a time step is greater than the number of susceptibles needing vaccination, i.e., $V_d t_s/S_t > 1$, this formula fails. In that case, we choose to leave an insignificant fraction of a susceptible unvaccinated. Then

$$S(i)e^{-k_V(i)t_s} = 0.0001 \quad \text{and} \quad k_V(i) = -log(0.0001/S(i))/t_s \tag{19}$$

As with vaccination, culling can be performed in and around geographic blocks that have a symptomatic population including infectious and/or very ill animals. The user has the choice of culling all seriously ill animals (state H), all infectious animals (states I and H), or all animals in states S, E, I, and H. When there are not enough resources to cull all of the chosen animals, the choice of which animals to cull is done analogously to the methods for vaccination.

2.5. Governing Equations

In the geographically-resolved simulation with transmission control, the governing equations for box (grid element) i are given below where c_m is the fractional reduction in β due to mitigation, and r is the fractional reduction in directional spread. We assume that cattle in state H are too sick to move around and hence do not infect cattle in other geographic areas.

$$\frac{dS(i)}{dt} = -k(i)S(i) - k_V(i)S(i) - k_{Sc}(i)S(i) \tag{20}$$

$$\frac{dV_g(i)}{dt} = k_V(i)S(i) - k(i)V_g(i) - k_{V_g V_m} V_g \tag{21}$$

$$\frac{dV_m(i)}{dt} = k_{V_g V_m} V_g(i) \tag{22}$$

$$\frac{dE(i)}{dt} = k(i)(S(i) + V_g(i)) - k_{EI}(i)E(i) - k_{Ec}(i)E(i) \tag{23}$$

$$\frac{dI(i)}{dt} = k_{EI}(i)E(i) - k_{IR}I(i) - k_{IH}I(i) - k_{Ic}(i)I(i) \tag{24}$$

$$\frac{dH(i)}{dt} = k_{IH}I(i) - k_{HD}H(i) - k_{HR}H(i) - k_{Hc}(i)H(i) \tag{25}$$

$$\frac{dD(i)}{dt} = k_{HD}H(i) + k_{Sc}(i)S(i) + k_{Ec}(i)E(i) + k_{Hc}(i)H(i) \tag{26}$$

$$\frac{dR(i)}{dt} = k_{IR}I(i) + k_{HR}H(i) \tag{27}$$

where,

$$k(i) = c_m\beta\frac{1}{N(i)}\sum_{j=1}^{n}[(1 - rf_D)A(i,j) + rD(i,j)]I(j) + H(i) \tag{28}$$

and

$$k_{EI}(i) = \begin{cases} k_{EI}, & \text{if } E(i) > 0.3/(26km^2) \\ 0, & E(i) \leq 0.3/(26km^2) \end{cases} \tag{29}$$

2.6. Deterministic Computational Method

The rate coefficients, $k_{IR}, k_{IH}, k_{HD}, k_{HR}$ do not vary with geographic location. $k_{EI}(i)$, however, depends on location. If the exposed population of cattle is less than a threshold value in a grid element, i, then $k_{EI}(i)$ is set to 0 in that block and there is no disease progression. Accumulation into $E(i)$ however can continue and may lead to disease progression in the grid element at a later time. This restriction on disease progression prevents a miniscule amount of infection from sparking a new location of incidence. **Figure 3** demonstrates that the threshold population needed for disease progression in a grid element can have a large effect on whether mitigations are effective. The value of the threshold population was chosen by matching results of hybrid (section 2.7) and deterministic simulations as discussed in section 2.9.

Linearization of the contagion term in Equation 28 allows us to solve Equations 20–27 as a stepwise eigenvalue problem. We integrated the time-dependent dynamics this way because thresholding the accumulation of initial cases in a geographical cell (Equation 29) makes the net system dynamics integro-differential, obviating proofs of robustness for standard ODE solvers. The eigenvalue solution – in contrast to standard (polynomial) differential equation solvers, has a compact, well-defined inverse Laplace transform, preserving the rate-process structure of most terms in the epidemic model.

Because the $k(i)$ are not constant during each time step, trapezoidal integration is used to improve the approximation. Approximate values of I, H and N at the end of the time step are first determined using k_{start}. These values are then used to determine k_{end}. The values for all states at the end of the time step are then calculated using $k = (k_{end} + k_{start})/2$.

2.7. Hybrid Deterministic-Stochastic Computational Method

Disease progression for geographic boxes in which the number of infected animals is <50 is performed stochastically using Gillespie's τ-leap method (24). For example, to calculate the $S \rightarrow E$ transition, $k(i)$ is calculated using Equation 28, then the number of animals transitioning from $S \rightarrow E$ is calculated by sampling a Poisson distribution with $\lambda = k(i)S(i)t_s$, where t_s is the time step. The other transitions are calculated analogously.

2.8. Initial Conditions

Initial cattle population data are from the Food and Agriculture Organization of the United Nations (25).

A single case of a disease does not always lead to an epidemic. The hybrid method captures this variability when started with a single exposed animal. The goal of this work is to determine what happens when an epidemic is started due to release of rinderpest virus. Therefore, the simulations start with a sufficient number of cases so that rinderpest is very likely to progress for several weeks. The number of starting cases is below the detection threshold that can trigger user specified mitigations. The relative number of cases in each state is based on the disease progression parameters. Specifically lineage-1 is started with 6 animals in state E, 2 in state I, and 1 in state H. Lineage 2 is started with 3 animals in state E, 2 in state I and 2 in state H. When the Pakistan 1994 parameters are used, the simulation is started with 3 animals in E, 2 in I and 1 in H.

2.9. Setting the Timestep and Exposed Population Density Threshold

The timestep for the solution of the differential equations has to be set so that nearly identical answers are obtained if any shorter time step is used. Cumulative cases were simulated using different time steps for the lineage-1 and lineage-2 disease transmission and progression parameters in **Table 5** and for two population densities. The agreement between the hybrid simulations, which use a stochastic computation when the number of diseased animals in a grid element is small, and the wholly deterministic simulations is quite good over this large range of parameters as shown in **Figure 4**. For time steps of 0.02 and 0.05 days the deterministic results are within the errors of the hybrid simulation results. Therefore, the threshold population density needed in the exposed state of 0.3 exposed cattle per 26 km^2 is used for all deterministic simulations. The deterministic results show very little change over a range of 0.01–0.2 day time steps, with the biggest difference in results being 0.36%. Consequently, a time step of 0.2 days is used when deterministic simulations are run. A time step of 0.05 days is used when hybrid simulations are used.

3. RESULTS

3.1. Simulating Historical Outbreaks

3.1.1. 1994 Rinderpest Outbreak in Pakistan

A severe epidemic of rinderpest afflicted northern Pakistan in 1994–1995 (17). The disease was recognized during the first 6 months of the epidemic. At least 40,000 animals died and possibly

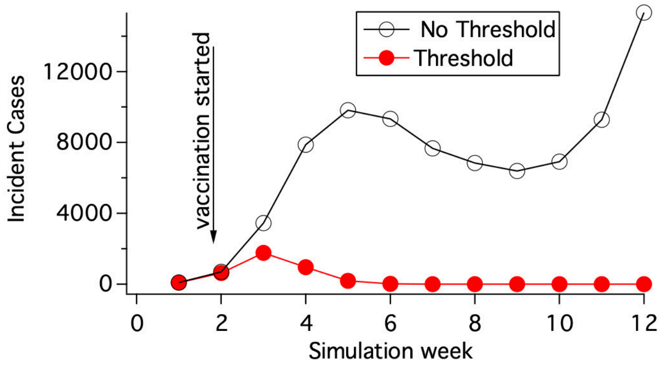

FIGURE 3 | Vaccination at a rate of 7,500 vaccinations per day was begun 7 days after 5 cows had obvious symptoms (state *H*). The vaccination radius was 6 km. The population density was 60 cattle/km^2. The threshold population in state E needed for disease progression was 0.3 cattle per grid element when calculating the red curve.

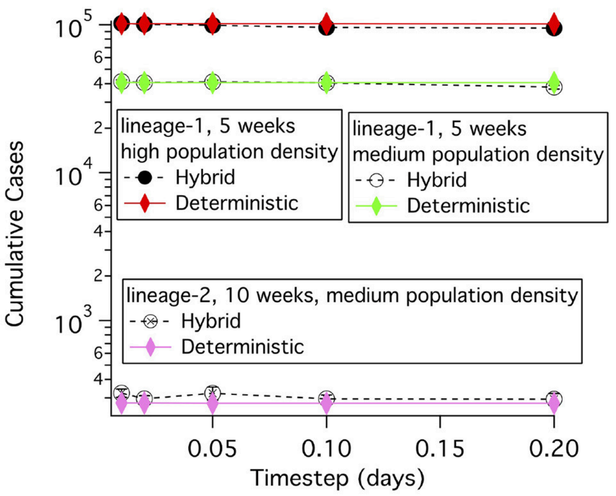

FIGURE 4 | Cumulative case counts at simulation ends vs. the time step used in the simulation for both deterministic and hybrid simulations. For the hybrid model, the mean and standard deviation of 5 simulations each averaging 30 runs of the model are shown.

TABLE 2 | Pakistan Rinderpest outbreak 1994–1995: Parameters.

	Inital value	Simulation time when changed	New value
β (/day)	0.31	110 days ~July 20*	0.25
d, characteristic length of transmission kernel (km)	1.3	–	1.3
long distance spread (%)	0.5	110 days ~Jul. 20*	0.08
vaccinations (/day)	0	140 days ~mid August	200
1/e time for immunity due to vaccination (days)	5	–	5
Fraction of infected animals that die	0.9	–	0.9

**Assuming the epidemic started in the beginning of March.*

as many as 50,000, approximately 7,000 of which died in the first 5 months. The first reported case was in Parri, a village south of Gilgit, in March 1994. Morbidity rates were near 100% in villages where animals were not vaccinated. The Pakistani government confirmed that the disease was rinderpest in August. Vaccination also began in August, but some of the vaccine used was later found to be subpotent and many vaccinated animals were afflicted by rinderpest. By October, rinderpest was prevalent in the upper Indus watershed including the Hunza and Gilgit valleys[1] (17). The FAO and European Union then provided nearly 4 million vaccine doses. However, due to the onset of winter and poor road conditions, only limited vaccination was performed. A final vaccination effort was started in April 1995. The outbreak ended in a village to the west, Khaplu, in November 1995 (17).

In the 1994 Pakistan outbreak, the infection moved along the roads as cattle were taken to market or to relatives. Therefore long distance movement was modeled by having the epidemic moving along the roads. The road data were taken from the OpenStreetMap project[2]. The cattle trade in this region is directional, with cattle rarely traveling south from the junction of the Gilgit with the Indus river (personal communication, Paul Rossiter). Consequently, southern movement of the epidemic along roads south of this junction was forbidden in the simulation.

For the first ~160 days of the simulation, March–August of 1994, no vaccination is performed in accordance with known facts about the epidemic. Subsequently, vaccination is performed at a rate of 200 effective cattle vaccinations per day with a

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[1] *The World Without Rinderpest*. Available online at: http://www.fao.org/docrep/003/w3246e/W3246E06.htm (Accessed February 2016).

[2] Available online at: http://download.geofabrik.de (Accessed January 16, 2016).

vaccination ring of 30 km to simulate the use of subpotent vaccine. When more effective vaccine did arrive, winter had set in and only limited vaccination was performed. Consequently, we continue at a rate of 200 effective vaccinations per day until the simulation ends in mid-winter.

Short range, isotropic movement was modeled with an exponentially decaying function with a decay constant of 1.5 km. Movement along the roads was always in a distance range of 28–42 km. The percentage of disease spread occurring via the road network is initially 0.5%. However starting in September 1994, the amount of long distance spread is reduced by a factor of 5 under the hypothesis that if people couldn't move around very well to vaccinate cattle, the virus was not spread long distances either. Parameters are summarized in **Table 2**. Gridded cattle population data from the Food and Agriculture Organization of the United Nations (25) were reduced by a factor of 1.3 to account for the increase in population between 1994 and 2005 (26).

With so many cattle in the area dying it is expected that hygiene would be improved even before confirmation that the disease was rinderpest and the commencement of vaccination. However, reports from the time indicate that hygiene was generally poor (17). Consequently, β is reduced by 20% only 30 days before vaccination is begun.

The results of the simulation are consistent with known features of the epidemic. After week 20 of the simulation, i.e., in August 1994, ∼7,300 cattle are dead consistent with the ∼7,000 reported by Rossiter (17). Furthermore, **Figure 5** shows that rinderpest was prevalent in the Hunza and Gilgit valleys as reported. At the end of the 41st simulation week, there are ∼42,500 dead animals consistent with an end number of ∼50,000 or less in Nov. of 1995 following a summer of effective vaccination. Therefore, geographical and temporal spread are satisfied simultaneously with reasonable parameterization.

3.1.2. Fremantle, Australia: Control by Culling and Quarantine

The 1923 outbreak in Fremantle, Australia near Perth was controlled by culling and quarantine (10). To roughly model this

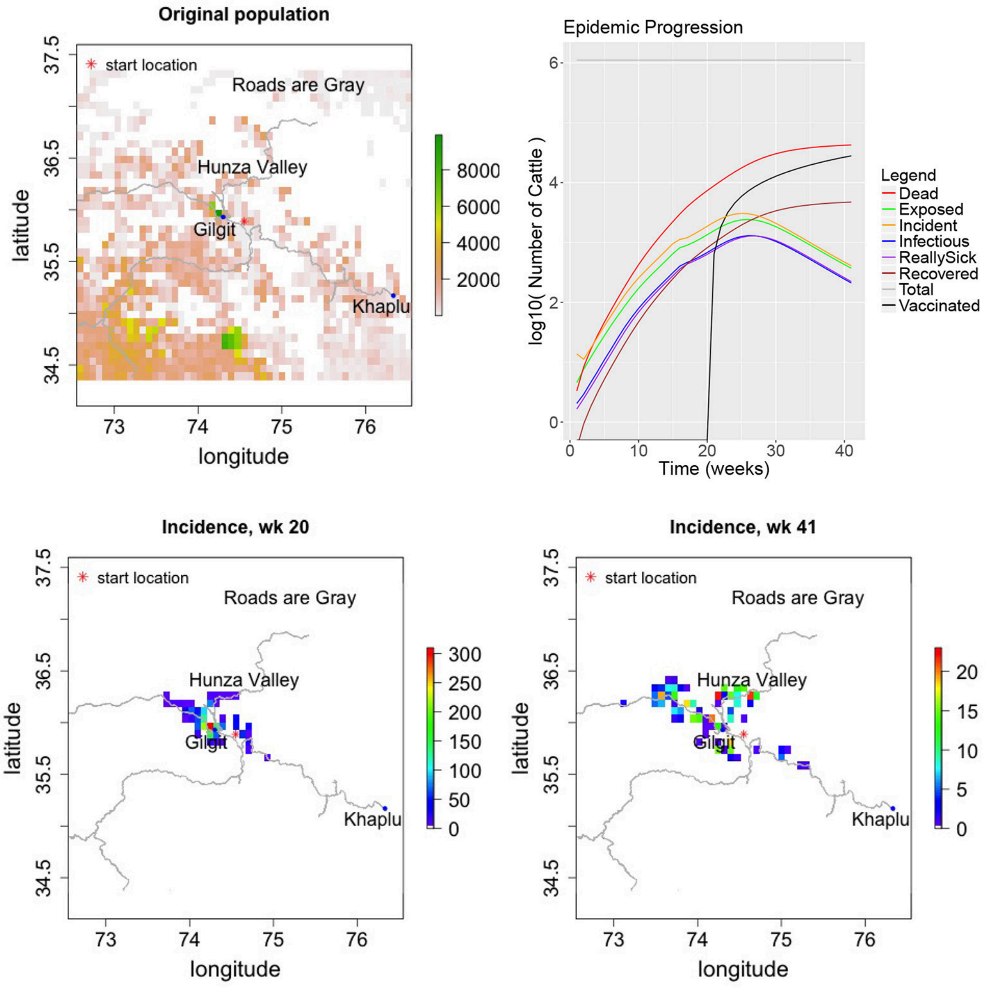

FIGURE 5 | Results of simulating the 1994–1995 rinderpest outbreak in Pakistan.

TABLE 3 | Fremantle parameters.

β (1/days)	1.0
Incubation period ($1/k_{EI}$) (days)	5.6
$1/k_{IH}$ (days)	3
$1/(k_{HD} + k_{HR})$ (days)	3
Fatality fraction from H	0.8
Starting cases (exposed)	5
No. of animals that have had obvious symptoms when rinderpest is detected	5

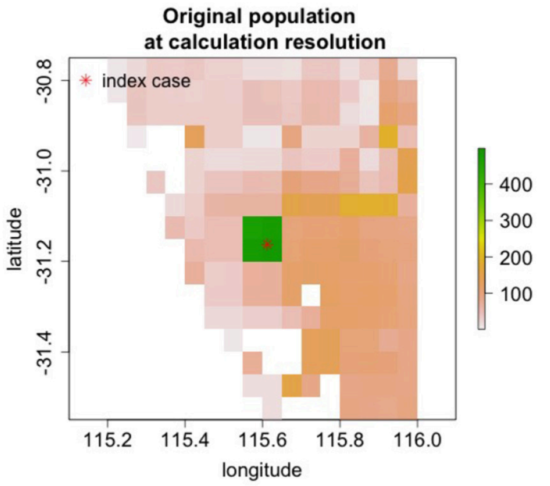

FIGURE 6 | Starting population in Fremantle, Australia.

TABLE 4 | Simulation results for Fremantle, Australia.

	Cumulative cases	Dead at 10
No mitigations	27,500 at 8 weeks*	14,800 at 8 weeks*
Cull 1,500 /day	418	14,297
Cull 1,500 /day with 50% transmission reduction	144	10,820

**Rounded to the nearest hundred. And still increasing.*

outbreak, a rinderpest epidemic starting just north of Fremantle was simulated. Since no specific information is available on the value of β, we assumed $\beta = 1$ as a stringent test of whether the epidemic could be extinguished with culling and transmission control. Mitigations began 2 days after disease detection. Culling was performed at a total rate of 1,500 animals per day with the culling radius set at 7 km and a 1 day delay between animals becoming infectious and when they are culled. Quarantine was modeled as a factor of 2 reduction in transmission. The parameters are in **Table 3**. The starting cattle population in **Figure 6** shows that the population density is low with most of the cattle in a very localized region.

Culling alone was able to extinguish the epidemic as shown in **Table 4**. With the addition of quarantine (50% transmission reduction), the epidemic was extinguished with fewer dead cattle. This level of transmission reduction alone does not end the epidemic. Without culling, the outbreak would continue and many more cattle would die after the 10 week simulation period. In the 1923 outbreak, ∼3,000 animals were culled. This smaller number of culls is likely due to the lower animal population in 1923.

3.2. Simulation of Rinderpest Virus Release in a Naïve Population

Nine regions around the world with varying population densities were modeled to understand potential epidemics resulting from the introduction of rinderpest into a naiv̈e population. The regions were chosen to have relatively uniform population densities and a few examples are shown in **Figure 7**.

Rinderpest varies in virulence (20), consequently simulations were performed with several parameter sets. Parameters of the strain in the 1994 Pakistan outbreak and lineage 1 and 2 from Mariner et al. (19) are shown in **Table 5**. For all simulations the characteristic distance for spreading was 1.3 km and the characteristic time for the vaccine to become effective was 5 days.

3.2.1. Uncontrolled Release

Cumulative case counts at the end of simulations are shown as a function of population density in **Figure 8**. Simulations with lineage-1 parameters were run for 5 weeks, while simulations with lineage-2 parameters were recorded for 5 and 30 weeks. When case counts are low as for the 5 week simulations using lineage-2, the results are nearly independent of initial populations because S/N is approximately 1 (See Equations 20, 28, 23). However, when case counts rise and S/N falls in the lower population regions, a strong dependence on population density is seen; the lineage-1 results at 5 weeks and the lineage-2 results at 30 weeks in **Figure 8**.

Before rinderpest is detected it could spread to locations distant from the original outbreak. This type of spread occurred in the 2001 FMD outbreak (27). Once rinderpest is detected, we assume that long distance movement of cattle will be banned (and stopped). Nonetheless, the initial spread leads to a more rapidly progressing epidemic. The open circle in **Figure 8** demonstrates this increase at 5 weeks for lineage-1 parameters when 1% of the spread is to long distance locations until long distance movements are stopped 2 days after rinderpest is detected.

A final input to investigate is the characteristic distance of spread, d. Increasing d from 1.3 to 2 km increases the number of cases (after the start phase of the simulations) as shown in **Figure 9**. This increase is greater for areas with lower populations. This population dependence can be explained as the increased spread providing a larger susceptible population. The variation in the general trends are due to the fact that these real world populations are not homogeneous.

3.2.2. Effects of Mitigations

Three mitigations are modeled: reduction in transmission, vaccination, and culling. As discussed in the historical perspective section, transmission can be reduced by better hygiene and by movement control. These measures can slow an epidemic, but do not extinguish it. Vaccination and culling both have the potential to extinguish an epidemic, but the the details of implementation

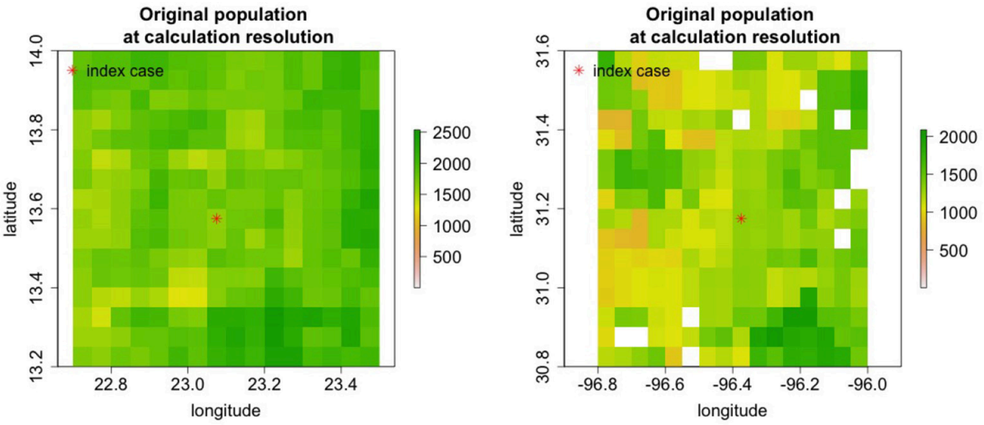

FIGURE 7 | Cattle populations in two of nine regions where rinderpest introduction was simulated are shown in areas 0.8^{O} longitude by 0.8^{O} lattitide. For some simulations the area modeled was increased.

TABLE 5 | Simulation starting parameters.

	Lineage-1	Lineage-2
β (1/days)	1.2	0.18
Incubation period ($1/k_{EI}$) (days)	5.6	6.8
$1/k_{IH}$ (days)	3	7
$1/(k_{HD}+k_{HR})$ (days)	3	3
Fatality fraction from H	0.36	0.06
No. of animals that have had obvious symptoms when rinderpest is detected	5	5

are critical to both the success of the effort and the final impact of the epidemic. In the following we look at how the efficacy of mitigation is affected both by controllable parameters such as the number of vaccinations per day as well as by uncontrollable parameters such as population density and characteristic spread distance. Results are presented only for lineage-1 because the results are a more stringent test of a mitigations efficacy.

3.2.2.1. Vaccination

In the simulations, 10,000 doses were assumed to be available each day and the vaccination radius is set to 6 km. Vaccination started 7 days after the disease was identified which occurred after only 5 cattle showed clear signs of rinderpest. The length of time needed to extinguish the epidemic, the cumulative cases and the number of cattle immunized are given in **Table 6** for several different regions with varying average cattle population densities. Ending an epidemic solely by vaccination is more difficult in higher population density regions. For the highest population densities, these vaccination conditions were not sufficient to stop the epidemic. If highly effective transmission reduction, begun only 1 day after disease identification, is combined with these vaccination conditions, then the epidemic can be extinguished as shown on the right of **Table 6**.

The effect on epidemic progression of the start time for vaccination was studied. **Figure 10A** shows that for a region with a cattle density of roughly 68 cattle/km^2, the number of cattle immunized goes up rapidly with the delay in starting vaccination until the available 10,000 doses/day is not enough to control the epidemic. For a population density of 126 cattle/km^2 which was shown as not controlled in **Table 6**, the epidemic can be controlled if the start delay is reduced from 7 to 5 days. The length of the epidemic is reduced further as the delay is decreased. For a population density of 150 cattle/km^2 the epidemic can only be brought under control by vaccination alone if vaccination begins after a nearly impossible delay of only 2 days after detection. Clearly, the delay in starting vaccination is a critical parameter.

All the results thus far concerning vaccination have been obtained using a characteristic spread distance of 1.3 km. While this parameter can not necessarily be controlled as part of a mitigation, it is important to know how stable our results are to the variety of geographical spread conditions which may occur in different parts of the world. **Table 7** contains results for a spread distance of 2 km for the geographic area having an average population density of 68 cattle/km^2. With this increase in geographic spread, the epidemic can no longer be controlled by the same vaccination parameters used to obtain the results in **Table 6**. Rather, both the number of available doses and the radius around active cases where vaccination is performed must be increased.

3.2.2.2. Vaccination with transmission reduction

With extensive movement controls transmission of highly infectious diseases can be reduced by roughly a factor of 2 as was shown for FMD in an analysis of the 2001 outbreak (23). The right three columns of **Table 6** show that it is possible to extinguish a rinderpest epidemic with vaccination under nearly ideal circumstances; (i) the disease was identified when only 5 cattle had become seriously ill and before any infected animals were transported and the disease spread to distant locations, (ii) effective vaccination was begun 7 days after disease identification and veterinarians were able to vaccinate when and where needed, (iii) hygiene and movement control reduced transmission by a

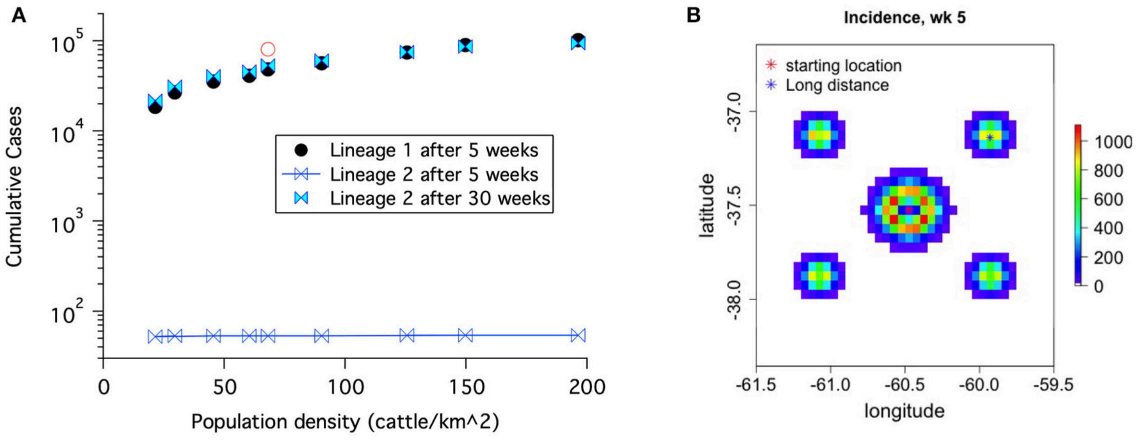

FIGURE 8 | **(A)** Cumulative case counts at the end of the simulation increase with population density. **(B)** Geographical spread of disease including both local and long distance spread for lineage-1 at 5 weeks. The red open circle in **(A)** corresponds to results with the 1% spread to the four long distance locations shown in **(B)**.

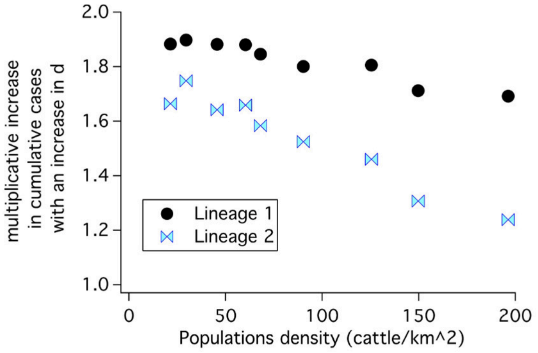

FIGURE 9 | The characteristic distance of spread, d, was increased from 1.3 to 2 km. The cumulative cases at 30 weeks increased for both sets of disease parameters for uncontrolled epidemic propagation.

factor of 2 one day after disease identification. However, if some of the circumstances are not ideal, such as the disease spreading before identification and a delay in starting vaccination then the outbreak may not be extinguished. Even in a location of medium population density (68 cattle/km^2) more resources are needed for greater vaccination delay and with a long enough delay the epidemic can not be controlled as shown in **Figure 10B**.

3.2.2.3. Culling

The potential to stop the lineage-1 strain of rinderpest by culling was examined for several areas with different population densities using the parameters from **Table 5**. The maximum rate of killing cattle was assumed to be 10,000 per day and cattle were culled regardless of disease state. The area of the geographic bins was reduced by a factor of 4 for this work, so that the radius of culls around an infectious grid element could be examined at higher resolution. In the initial examination of culling for control, the culling radius was set at 3 km, culling was started 7 days after the epidemic was detected, and the time between an animal becoming infectious and the start of culling in that grid element and the surrounding elements defined by the cull radius was set at 2 days. The left side of **Table 8** shows that culling with these fairly optimal parameters does not stop the epidemic in areas with high cattle population. When very effective transmission reduction is achieved starting only 1 day after disease detection, then culling can stop the epidemic albeit with large numbers of dead cattle that were never sick.

Figure 11 demonstrates that the delay between when the first animal in a grid element becomes infectious and when animals in that grid element and the specified surrounding area are culled is a critical parameter determining how many animals must be culled before epidemic extinction. An increase in this local delay increases the number of animals that must be culled in order to extinguished the epidemic increases. Furthermore, in the presence of initial long distance spread, **Figure 11B**, the epidemic can not be controlled if the delay between when the animals become infectious and when animals in that grid element are culled is 2 days or longer.

The radius of culling around locations with identified cases is a very important practical parameter. Owners of livestock generally do not want to have their apparently healthy animals slaughtered. Culling only locations where animals are sick can extinguish low transmissibility strains such as lineage-2 (results not shown), but not high transmissibility strains such as lineage-1. However, even for the high transmissibility strains, the culling radius around identified locations with infected animals does not need to be large for the relatively small characteristic distances of spread likely to hold for livestock (especially under movement restrictions). A characteristic spread distance of 2 km is used in the investigations of culling radius and an area of 1° × 1.5° was modeled. The effects of increasing the cull radius depend critically on the delay between disease identification and culling as well as on slaughter and disposal resources. **Table 9** shows results for both a 7 day initial delay combined with a 3 day local delay, and a 3 day initial delay combined with a 4 day local delay. For these two scenarios different cull radii and limitations on

TABLE 6 | Extinguishing lineage-1 epidemics by vaccination.

	No transmission reduction			50% Transmission reduction		
Population density (cattle/km^2)	Vaccine doses given*	Cumulative cases**	Weeks to extinguish	Vaccine doses given*	Cumulative cases**	Weeks to extinguish
22	21,000	1,300	8	13,000	300	7
30	36,000	1,700	9	21,000	400	7
46	79,000	2,300	10	28,000	400	8
60	96,000	2,800	11	36,000	500	8
68	137,000	3,800	12	53,000	500	8
90	168,000	5,000	11	60,000	600	8
126	NC	NC	NC	141,000	800	10
150	NC	NC	NC	168,000	1,000	10
196	NC	NC	NC	262,000	1,600	11

**Rounded to nearest thousand.*

***Rounded to nearest hundred.*

^{NC}*The epidemic is not controlled within 15 weeks.*

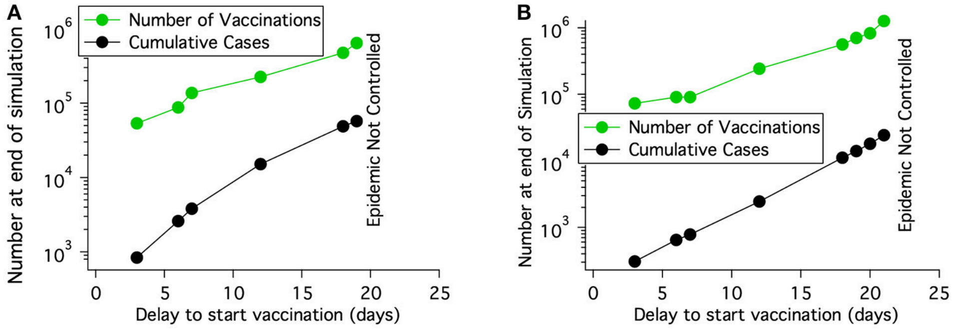

FIGURE 10 | For a fairly uniform population density of 68 cattle/km^2 and a rinderpest virus having the parameters of lineage-1, the number of vaccinations needed to control an epidemic increases rapidly with the delay in starting vaccination after rinderpest is identified. If the delay is long enough, the epidemic can not be extinguished. **(A)** No long distance spread and no transmission reduction. **(B)** Long distance spread is allowed to the locations shown in **Figure 8B** until 1 day after disease detection. Transmission is reduced by a factor of 2 also 1 day after disease detection.

available culling resources are considered. If an attempt is made to expand the culling area, but resources are inadequate to cull the entire area, then the number of dead animals can actually increase with "mitigation" if areas with infectious animals are not culled. If sufficient resources are available to cull the entire chosen area each day, then otherwise uncontrollable epidemics can be extinguished or reductions in the number of dead animals can be achieved. A consequence of this result is that a large cull radius is more important at the start of an epidemic when fewer resources are needed.

TABLE 7 | Effects of vaccination radius and available doses for a characteristic spread distance of 2 km and average population density of 68 cattle/km^2.

Vaccination radius	Doses/day	
	10,000	13,000
6 km	NC*	NC*
9 km	NC*	Controlled

**Not controlled at 15 weeks.*

4. DISCUSSION

4.1. Decision Support

Most epidemics begin with exponential growth until either there is a depletion of the susceptible population or control measures are put in place. A general scenario for incidence, cumulative cases and dead is shown in **Figure 12**. The first mitigation to be implemented for any disease is usually transmission reduction, often through better hygiene or separation of animals. Subsequently, either culling or vaccination is needed.

The 2001 European FMD epidemic provides insight into the practicalities of culling that are likely to arise if rinderpest were to escape from the lab. Nearly 600,000 cattle were slaughtered as a disease control measure in England (28). Based on the epidemic progression (28) and the dates when culling was performed on farms contiguous to infected farms, we estimate that the majority of culling took place over a 6 week period from about March 11th to April 29th and that on average 10,000 cattle were

TABLE 8 | Extinguishing epidemics by culling.

	No transmission reduction			50% transmission reduction		
Population density (cattle/km^2)	**Dead***	**Cumulative cases****	**Weeks to extinguish**	**Dead***	**Cumulative cases****	**Weeks to extinguish**
22	17,000	700	4	10,000	200	4
30	22,000	900	5	14,000	300	4
46	42,000	1,100	5	20,000	300	4
60	54,000	1,200	5	30,000	300	5
68	71,000	1,500	6	34,000	300	5
90	104,000	1,900	7	46,000	300	5
126	233,000	5,900	8	82,000	400	7
150	NC	NC	NC	110,000	500	7
196	NC	NC	NC	146,000	700	7

*Rounded to nearest thousand.

**Rounded to nearest hundred.

^{NC}Not Controlled.

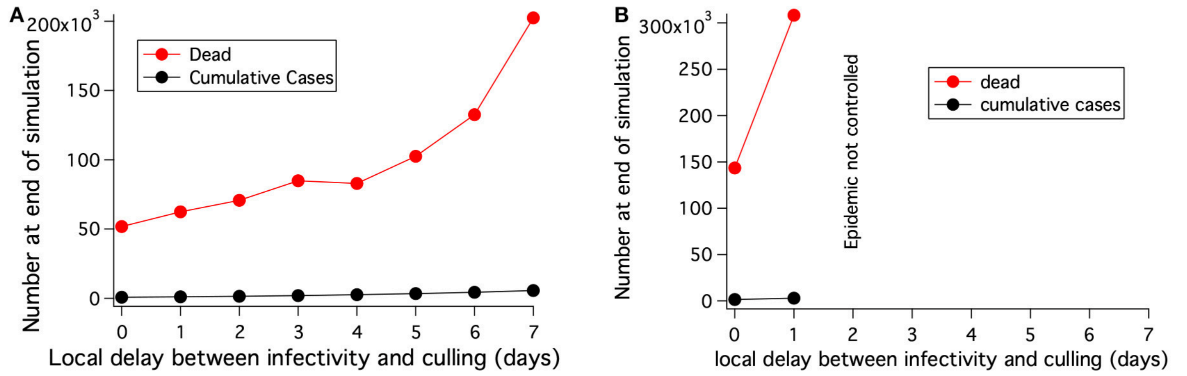

FIGURE 11 | For a fairly uniform population density of 68 cattle/km^2, the number of cattle that must be killed to extinguish an epidemic with culling depends on the delay between when the cattle at a location become infected and when they are killed. **(A)** No long distance spread and no transmission reduction. Area modeled is 0.8 × 0.8 degrees. **(B)** Long distance spread is allowed to the locations shown in **Figure 8B** until 1 day after disease detection. The epidemic can not be extinguished if the delay between when the cattle at a location become infected and when they are killed is 2 days or more. Area modeled is 1.3 × 2 degrees.

TABLE 9 | Number of dead cattle for different culling scenarios* using a fairly uniform population density of 68 cattle/km^2.

Initial delay to start culling (days)	**Local delay (days)**	**Cull radius (km)**	**Culls/day**			
			8,000	**10,000**	**11,000**	**12,000**
7	3	3	NC	NC	311,000	245,000
7	3	5	NC	NC	278,000	239,000
7	3	7	NC	NC	517,000	295,000
3	4	3	NC	NC	NC	163,000
3	4	5	266,000	135,000	118,000	104,000
3	4	7	NC	174,000	104,000	133,000

*The characteristic distance of spread was 2 km instead of 1.3 km as was used for most of the simulations.

culled per day. Both the Netherlands and the United Kingdom experienced difficulties in safely disposing of the carcasses (28, 29). Furthermore, there was significant resistance to culling from farmers (30).

In this work concerning rinderpest, the high culling rates of the FMD epidemic were used (10,000 /day). Even with an extremely rapid start of 5 days after disease detection, culling is unable to extinguish highly contagious epidemics at the highest

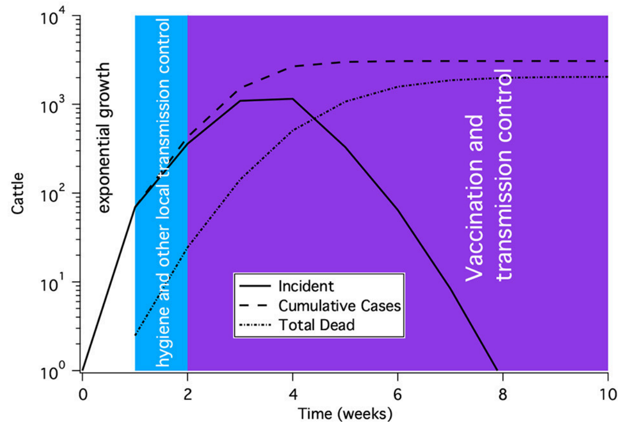

FIGURE 12 | A representative time course for a rinderpest epidemic subject to modern epidemic response and control. The shape of the curve shows initial exponential rise, a slow down in growth due to transmission reduction and finally control of the epidemic after several weeks of vaccination. This time course is not meant to represent a particular outbreak, but rather to illustrate the general features of an outbreak.

population densities. When culling does extinguish a weeks-to-months old epidemic, the result can be hundreds of thousands of dead cattle that were never sick.

Vaccination has the advantages of being more farmer-friendly and has no carcass disposal problems, but requires a large ready supply of effective vaccine and a means to administer it. Simulations were performed assuming 10,000 vaccinations could be performed per day. This rate is not sufficient to stop epidemics of highly virulent rinderpest in the highest cattle population areas on earth. Only in combination with highly effective hygiene control that cuts transmission in half can vaccination end epidemics in these types of scenarios. The assumption of a 50% reduction in β is based on analysis of the 2001 FMD epidemic (23). However, this large reduction may not always be achievable (17).

The presented simulations of rinderpest epidemics in naïve populations demonstrate the importance of several skills and mitigation resources. (1) The rapid detection of a rinderpest outbreak. (2) The ability to stop the movement of cattle and prevent any (further) spread to distant locations. (3) The implementation of better zoosanitary hygiene and animal isolation. If vaccination is to be used to stop an epidemic, then the ability to start an effective vaccination program of up to 10,000 animals per day on only a few days notice is needed. If culling is to end the epidemic then other resources are needed; the ability to slaughter and dispose of up to 10,000 animals per day on short (1–2 days) notice including the ability to slaughter animals on proximate sites where no animals have been diagnosed.

History demonstrates that an outbreak of a serious and highly transmissible disease such as rinderpest is likely to have severe economic and sociological consequences. As noted in section 1.1, the rinderpest outbreak in the late nineteenth century caused famine amongst the Masai people of Africa (11). Livestock have considerable social value in some parts of sub-Saharan Africa as described by Catley et al. (31). Other parts of the world are not immune from the socio-economic consequences of livestock disease. The 2001 outbreak of FMD, another serious and highly transmissable livestock disease, caused economic hardship both for farmers with herds affected by infection and/or culling as well as for otherwise unaffected farms due to movement and trade restrictions (32). The epidemic was very costly with the UK Department of Environment Food and Rural Affairs (DEFRA) spending >£3 billion (33).

4.2. Comparison of Our Techniques to Other Modeling Methods

There is a significant body of work modeling the geographical spread of infectious disease. Much of the early work focused on spread between isolated cities and these models are sometimes referred to as metapopulation and/or patch models (34–36). However, in many areas of the world, human and animal populations are fairly contiguous and a model of well separated cites or farms is not appropriate. There is a large body of research on which transport model is best under which conditions (37, 38) and a conceptual analysis of process-driven (i.e., mechanistic) modeling frameworks has been published (39). Our rectangular tiling of population and spatial spread kernel are both simple to implement and understand. As noted in Mancy et al. (39), when working at the research–policy interface non-complex models have advantages if they can be implemented without sacrificing accuracy. Additionally, there is evidence that the exponentially decaying spread kernel is appropriate from the 2001 foot and mouth disease outbreak (22, 23).

This combination of gridded population data and a spatial transmission kernel has been previously used in modeling the spatio-temporal spread of epidemics. For example, models incorporating airline transport over the entire world have been developed (40, 41). The effects of pixel size in the grid have been investigated using a stochastic SIR model (42). A model of Rift Valley fever (43) and a model of rinderpest in the US (44) used deterministic progression and spread within "counties" combined with stochastic spread between "counties."

One of the challenges for deterministic models is constructing realistic spatially explicit models (45). The model presented here addresses that challenge without the considerable computational cost and large data requirements of agent-based models, nor the infinite spectrum of approximate eigenvalues in a non-linear partial differential equation. Our deterministic simulation can be run in seconds for an area of 88 × 88 km on a laptop computer. Therefore, effects of a variety of mitigations can be analyzed rapidly. A screen shot of the graphical interface is shown at the end of this paper (**Appendix**).

5. CONCLUSIONS

The described simulation methods can model the time course of historical data using the known mitigation methods and disease progression parameters. Additionally, the time-dependence of the geographic extent of the simulated epidemic compared with the known extent of the historical outbreaks provides significant further constraint of the model. This geographically-resolved epi-model, developed using historical data, provides information for present-day decision-support.

Mitigation of a rinderpest outbreak without serious or even devastating effects on human society depends on disease recognition when case numbers are still small, and requires a response in a few days or 1–2 weeks depending on the transmissibility of the virus and the cattle population density. Even large-scale, effective vaccination within a week of noticing 5 cattle seriously ill with rinderpest in the worlds densest cattle population areas will not eliminate the epidemic on a timescale of weeks to months for the most virulent strains. The outbreak of a virulent strain in an area of dense but reasonably common cattle population would lead to hundreds of thousands of dead cattle assuming a nearly optimal response. Mitigation of epidemics involving less virulent strains should be possible with culling or vaccination, particularly when combined with transmission reduction. Responding to an epidemic requires resources and constant preparation including; an established and highly functional veterinary infrastructure, resources for carcass disposal and a large stockpile of effective vaccines. The perceived benefit from continued storage of rinderpest virus must be considered in the context of consequences due to a potential release of an already eradicated disease.

AUTHOR CONTRIBUTIONS

BM and PF had the original idea for the basic structure of the epidemiological model. They also wrote the first version of the code. JM has taken the original code and made substantial revisions to both the underlying mathematical structure and to the interface based on discussions with PF, CM, and BM. CM has made critical inputs regarding the inner workings of the algorithm.

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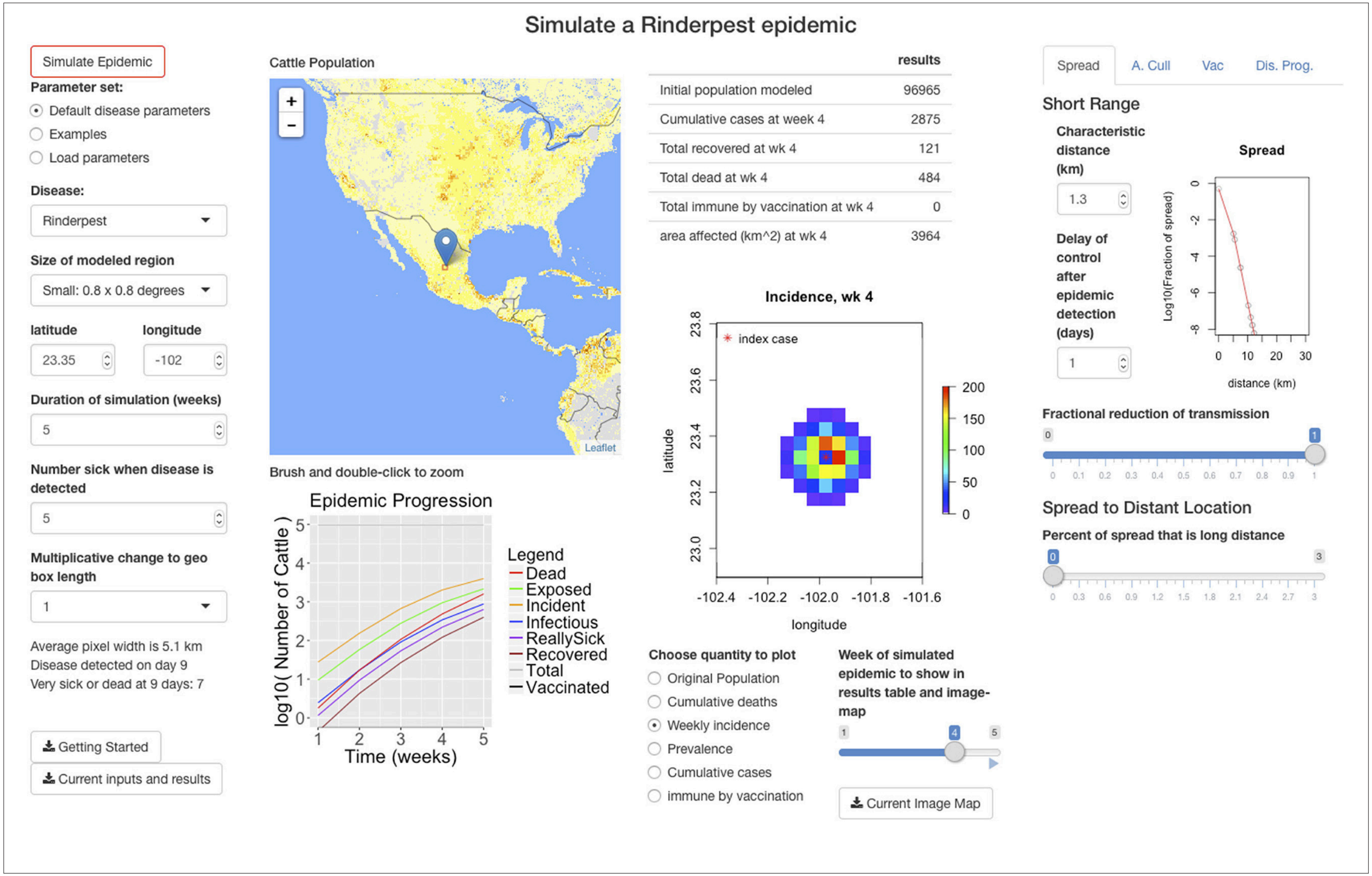
APPENDIX



Simulate a Rinderpest epidemic

Simulate Epidemic

Parameter set:
- ● Default disease parameters
- ○ Examples
- ○ Load parameters

Disease: Rinderpest

Size of modeled region: Small: 0.8 x 0.8 degrees

latitude: 23.35

longitude: -102

Duration of simulation (weeks): 5

Number sick when disease is detected: 5

Multiplicative change to geo box length: 1

Average pixel width is 5.1 km
Disease detected on day 9
Very sick or dead at 9 days: 7

Getting Started

Current inputs and results

Cattle Population

Brush and double-click to zoom

	results
Initial population modeled	96965
Cumulative cases at week 4	2875
Total recovered at wk 4	121
Total dead at wk 4	484
Total immune by vaccination at wk 4	0
area affected (km^2) at wk 4	3964

Choose quantity to plot
- ○ Original Population
- ○ Cumulative deaths
- ● Weekly incidence
- ○ Prevalence
- ○ Cumulative cases
- ○ immune by vaccination

Week of simulated epidemic to show in results table and image-map: 4

Current Image Map

Spread | A. Cull | Vac | Dis. Prog.

Short Range

Characteristic distance (km): 1.3

Delay of control after epidemic detection (days): 1

Fractional reduction of transmission: 1

Spread to Distant Location

Percent of spread that is long distance: 0

frontiers
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REVIEW
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Pathogenesis of *Mycobacterium bovis* Infection: the Badger Model As a Paradigm for Understanding Tuberculosis in Animals

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Tuberculosis in animals is caused principally by infection with *Mycobacterium bovis* and the potential for transmission of infection to humans is often the fundamental driver for surveillance of disease in livestock and wild animals. However, with such a vast array of species susceptible to infection, it is often extremely difficult to gain a detailed understanding of the pathogenesis of infection—a key component of the epidemiology in all affected species. This is important because the development of disease control strategies in animals is determined chiefly by an understanding of the epidemiology of the disease. The most revealing data from which to formulate theories on pathogenesis are that observed in susceptible hosts infected by natural transmission. These data are gathered from detailed studies of the distribution of gross and histological lesions, and the presence and distribution of infection as determined by highly sensitive bacteriology procedures. The information can also be used to establish the baseline for evaluating experimental model systems. The European badger (*Meles meles*) is one of a very small number of wild animal hosts where detailed knowledge of the pathogenesis of *M. bovis* infection has been generated from observations in natural-infected animals. By drawing parallels from other animal species, an experimental badger infection model has also been established where infection of the lower respiratory tract mimics infection and the disease observed in natural-infected badgers. This has facilitated the development of diagnostic tests and testing of vaccines that have the potential to control the disease in badgers. In this review, we highlight the fundamental principles of how detailed knowledge of pathogenesis can be used to evaluate specific intervention strategies, and how the badger model may be a paradigm for understanding pathogenesis of tuberculosis in any affected wild animal species.

Keywords: tuberculosis, *Mycobacterium bovis*, badgers, pathogenesis, infection, vaccination

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INTRODUCTION

The presence of tuberculosis in wild animals has attracted scientific attention primarily because they are implicated in transmission of infection to livestock and other economically important species, and the risk of zoonotic transmission to humans. In Ireland and the UK, the European badger (*Meles meles*) is the principal wild animal species involved (1, 2). Elsewhere, wild boar (*Sus scrofa*) (3), goats (*Capra hircus*) (4) and species of deer, notably red deer (*Cervus elaphus*), fallow (*Dama dama*), and roe deer (*Capreolus capreolus*) are affected in continental Europe (5). In New Zealand, the brushtail possum (*Trichosurus vulpecula*) is the key species affected (6). In North America, white-tailed deer (*Odocoileus virginianus)* (7) elk (*Cervus canadensis*) (8) and bison (*Bison bison*) (9) are among the known reservoirs of infection. In Africa, many species of outstanding conservation merit are infected, posing a threat to the survival of local populations (10). With limited resources available to conduct surveillance programs, the gathering of basic information to develop an understanding of pathogenesis is rarely undertaken in natural-infected hosts. A key reason is often the physical size of the animal and the volume of tissues and specimens required to ensure maximum sensitivity and specificity of diagnostic procedures. Added problems include the availability of suitable samples and the potential for bias arising from misinterpretation of data because sampling is only from advanced disease cases or from cases identified by imperfect diagnostic tests.

There has been much written about the value of laboratory animals as surrogates in studying tuberculosis (11). This is mainly in the context of human tuberculosis where there is a drive to understand the host–pathogen interactions in great detail with a view to developing new therapies and vaccines (12). Those involved in trying to study the disease in a particular species are often reliant on information generated from laboratory animals, which may or may not be particularly relevant. Other than providing insights into pathogenesis at the animal level, laboratory animal models cannot contribute substantially toward understanding the epidemiology of human or animal tuberculosis at a population level. With the exception of the most commonly used laboratory animals, progress has also been hindered by the almost universal lack of reagents for specific animal species: this has constrained the development of diagnostic tests and limited the ability to understand how animals might respond to vaccines.

Nevertheless, animal models have been used extensively in tuberculosis research and proved invaluable in improving the understanding of pathogenesis and defining the subtle interactions between the pathogen and the host immune system (13, 14). The mouse model has been particularly useful and has revealed detailed functional information on many aspects of the host immunological responses to infection (15). The relative costs involved, the wide availability of immunological reagents, and the development of genetically modified lines have made the mouse model the pragmatic choice for many laboratories. However, the mouse is not considered to be a natural host for tuberculosis and study results often differ depending on the mouse strain used (16). This can be a cause for concern when extrapolating to different species. Other animal models including rabbits, guinea pigs, zebrafish, non-human primates, and cattle (a natural host of *M. bovis*) are all subject to the same constraints when applying the interpretation of the results across species (11). Notwithstanding the availability of reagents and the logistics and welfare of housing animals, there may be differences in the host response influenced by, for example, route of infection and pathogenesis. This all poses particular challenges for the study of tuberculosis in more exotic natural susceptible hosts and particular care needs to be taken to translate the results of studies from one model animal to another species. Key to this is deciding which pieces of information are relevant to the target species and how this can be used to develop a complete picture of the pathogenesis of infection. In Ireland, we have compared natural and experimental *M. bovis* infection models of badgers with a uniform level of postmortem examination, histology, and bacteriology. This has provided a unique opportunity to evaluate and gain insights into pathogenesis in both model systems.

TUBERCULOSIS IN BADGERS

The involvement of badgers in the epidemiology of tuberculosis in cattle is well established in Ireland and the UK (1, 17). Results from the four area badger removal trial in Ireland and the Randomized Badger Culling Trial (RBCT) in England provided evidence of a positive effect of badger culling on incidence rates of tuberculosis in associated cattle herds (18, 19). Arising from these studies, current policies to eradicate the disease are largely focused on surveillance testing of cattle supplemented with badger population control measures in areas of Ireland and England considered as high risk for cross-species transmission (1, 20). Analysis of *M. bovis* prevalence rates in approximately 5,000 badgers culled in Ireland in response to tuberculosis breakdowns in cattle herds has revealed a decrease in the overall prevalence from 26 to 11% between 2007 and 2011 (21). Nevertheless, large-scale culling is considered to be unsustainable in the long term, although it is recognized in Ireland, the UK, and other countries that eradication of tuberculosis in cattle is unlikely if the infection reservoir of *M. bovis* infection in badgers, and maybe other maintenance species, is not adequately addressed (22–24). The development of a vaccination strategy targeted at badgers is judged as a potentially feasible option; a key objective of vaccination is to reduce the transmission rate of infection within the badger population by reducing the level of susceptibility to infection or to alter the pathology of the infection in vaccinated badgers where protection is less than 100% to the extent that it decreases the rate of excretion of *M. bovis* and transmission to cattle (25). Until relatively recently there was limited detailed information relating to susceptibility of badgers to tuberculosis, and whether they were capable of resisting *M. bovis* infection through the generation of protective immune responses. A considerable body of research work has been carried out in Ireland and the UK to gain a greater understanding of the disease in badgers with the objective to develop and implement a vaccination strategy for badger populations (26). A critical step in this development stage is establishing a model system that closely mimics the natural infection state in free-living populations.

PATHOGENESIS OF TUBERCULOSIS IN BADGERS

Insights into the pathogenesis and a baseline for the evaluation of experimental infection studies have been gained in badgers to a degree not previously undertaken in any other natural-infected species (27). Badgers are considered to be highly susceptible to *M. bovis* infection (28). As in many species, tuberculosis is principally a respiratory disease in badgers but in a natural-infected population there appears to be a second route of transmission by contamination of bite wounds, which is less prevalent but still significant. Pulmonary infection is established following inhalation of infectious aerosols and this leads to protracted progression toward a clinical state of disease (29). Aerosol infection results in a chronic disease and infected animals can express a variety of disease states ranging from latent subclinical infection (i.e., no visibly detectable lesions or clinical manifestations of disease) to moderate disease (with size-limited pulmonary and extrapulmonary lesions) and to severe overt disease with generalized pathology. In the majority of aerosol-infected badgers, however, infection remains latent and the proportion of badgers that develop generalized disease is small (29, 30). By contrast, bite wound infection results in a more rapid and progressive infection with typically generalized infection and lesions (29, 31). Despite the absence of lesions in the majority of infected animals, badgers with any state of infection may pose a risk of transmission to susceptible hosts where there is close and frequent contact.

More refined insights into the pathology of disease have been revealed from detailed postmortem studies of culled badgers (31, 32). The sequence of events in the pathogenesis, including the early dissemination of infection from the lungs, is best demonstrated when the distribution of infection in the badgers is examined in a broad repertoire of anatomical sites (**Figure 1**). Gross visible lesions are commonly found in the thoracic cavity (lungs, tracheobronchial and mediastinal lymph nodes), with the head and body lymph nodes the most frequently affected extrathoracic sites. Visible lesions are scarce in the abdominal cavity; however, a broad range of tissues and organs may be infected. In badgers presenting with only a single site of infection, the distribution pattern is much the same as that in badgers with multiple infection sites. This could signify that host–pathogen interactions during the initial stages postinfection, rather than tissue predisposition, dissemination, or progression of disease, govern the infection distribution. Tuberculosis can also develop when bite wounds become infected with saliva containing infective bacilli. The spectrum of infected bite wounds can range from circumscribed subcutaneous granulomas, lacerated wounds draining abscesses, to large open ulcerated areas devoid of skin. The pathogenesis of infection following bite wound contamination differs from that after aerosol infection in that there is rapid progression of infection, a greater number of lesions, and wider distribution and severity of infection (27).

The presence of discrete tuberculous granulomas is the characteristic of infection in badgers as also occurs in other reservoir hosts including cattle (33), possums (34), and ferrets (35). These are composed largely of epithelioid cells, macrophages, and sporadic lymphocytes. Lesions are typically cellular and proliferative to a large extent, with limited necrosis, mineralization, or fibrosis (29, 36–38). Histologically, there is a wide variety in the size and structure of lesions present in an animal, and even in individual tissues (**Figure 2**). As the severity and size of lesions increase and the granulomas expands, the central mass of epithelioid cells increase and are enclosed by a peripheral rim of lymphocytes with the outer layer composed of macrophages and neutrophils, bounded by a narrow uneven fibroblast layer. As the infection progresses, lesions coalesce and may form large areas of necrosis

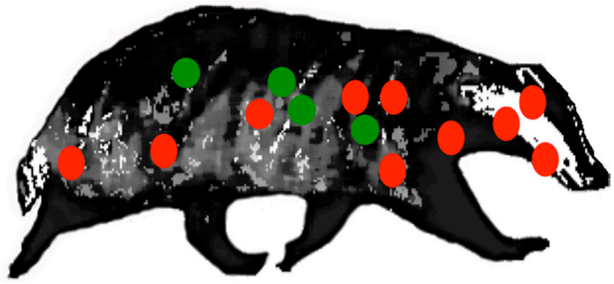

FIGURE 1 | Location of lymph nodes (red) and visceral organs (green) examined for gross lesions, and samples for histological/bacteriological examination during systematic detailed postmortem of natural-infected wild badgers. Reproductive tract tissues (not shown) were also examined (31).

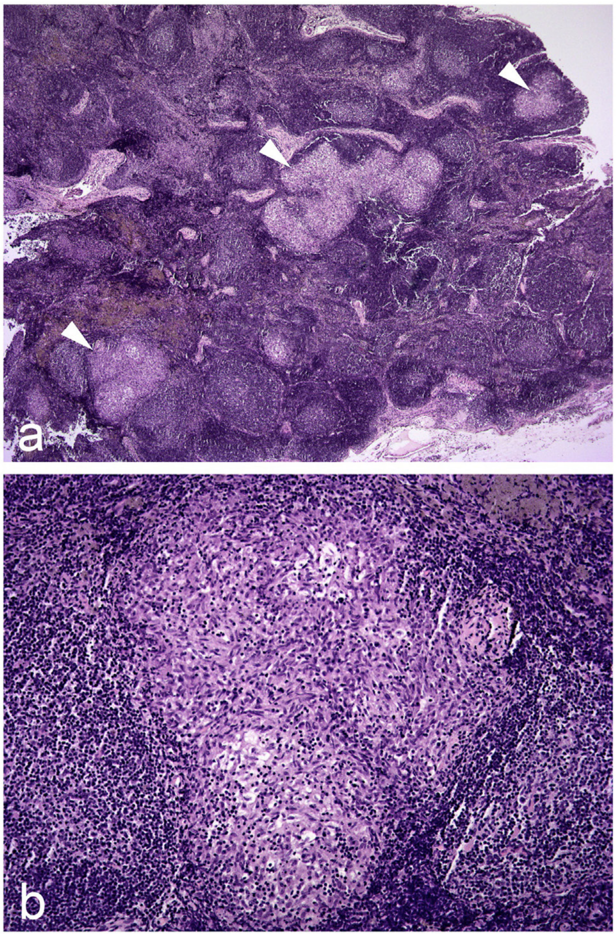

FIGURE 2 | Photomicrographs illustrating **(A)** three granulomas of varying size indicated by white arrowheads in a hyperplastic/reactive lymph node of a badger; **(B)** higher magnification image of one of these granulomas. Hematoxylin and eosin stains, magnification ×20 **(A)** and ×100 **(B)**.

and caseastion, sometimes associated with mineralization. The presence of acid-fast bacilli (AFB) becomes more common as the area of necrosis increases and then may be extracellular. In lung lesions, there may be erosion of bronchi and bronchioles walls, and AFB may be present in cellular debris in the lumina.

The specific immunological responses to infection with *M. bovis* can also offer important insights into key aspects of pathogenesis. However, measurement of these responses in badgers or other wild animals can be problematic not only because of a lack of reagents but also the requirement to repeatedly capture and collect samples from animals. In an experimental setting, this is feasible and it is possible to monitor changes in responses as the infection progresses (39, 40) As in all species studied, the predominant early specific immune response following *M. bovis* infection of badgers is T cell-mediated (CMI), leading to proliferation of T lymphocytes, secretion of interleukin-2 (IL-2) and release of pro-inflammatory cytokines including interferon-γ (IFN-γ) (41–43). The responses can be measured *ex vivo* following antigenic stimulation of blood or purified peripheral blood monocytes with tuberculin or the specific mycobacterial antigens ESAT-6/CFP10 cocktail. Where longitudinal studies have been conducted in wild badgers, the strength of the initial IFN-γ responses correlates with the progression of infection (44). Serological responses are associated with a later stage of infection when visible lesions are likely to be present and severity of disease is high (45). The immune-dominant serological antigen is restricted to the mycobacterial antigen MPB83, although the sensitivity of detection of antibodies recognizing this antigen is relatively low across the full spectrum of infection severity (46, 47). The innate immune response to infection with tuberculosis is regulated through the activity of macrophages; the key cells that are permissive for the growth of intracellular mycobacteria. Activated macrophages produce reactive nitrogen intermediates that are directly inhibitory for the growth of a wide range of intracellular organisms including *M. tuberculosis* (48) and *M. bovis* BCG (49). Nitric oxide (NO) is produced through oxidation of L-arginine (50) in a reaction catalyzed by an inducible nitric oxide synthase (iNOS) (51). However, studies in badgers have revealed that blood monocyte-derived macrophages do not produce NO or upregulate iNOS expression following *in vitro* activation of macrophages (52). This intriguing finding might imply that badgers should lack the ability to control infection *via* the innate response, though there is no strong pathological evidence to support this.

EXPERIMENTAL INFECTION MODELS IN BADGERS

While the use of natural-infected animals has proved invaluable for investigating pathology across the broad spectrum of disease ranging from early infection to clinical disease, experimental models have the advantage of allowing study of the kinetics of disease progression and immunological responses starting from a fixed dose and a fixed point of time (28, 39, 40). This can facilitate reproduction of disease in a format necessary for the development of diagnostic tests and for evaluating vaccines. In order to understand fundamental aspects of pathogenesis, the experimental model needs to be framed around relevant infection routes, and plausible challenge doses that are reflective of natural transmission. The profile of infection that is generated should also be characteristic and consistent within the recognized spectrum of the naturally occurring disease. Principally, this requires evenness in the profile of lesion development and distribution of infection as found in natural *M. bovis* infection. In developing the badger infection model for tuberculosis in Ireland, the key factors that were considered to achieve this end were the choice of *M. bovis* strain, route of infection, the infective dose, and the kinetics of infection. The strain of *M. bovis* used for experimental infection was first isolated from a lesion in a clinically diseased badger (28). Spoligotyping revealed that the strain type was common in both infected badgers and cattle. In an initial study, the infective doses used were <10 colony forming units (CFU) (low dose), ~100 CFU (medium dose), and ~3,000 CFU (high dose) with delivery by the endobronchial route of infection, to mimic the dominant respiratory route of natural infection (28).

The results of this study showed that badgers were very susceptible to infection: all of the badgers had established infection across each of the doses used when animals were euthanized at 17 weeks postinfection (28). The results also demonstrated that the dose of *M. bovis* had a little effect on the distribution of infection but as the dose increased so did the rate of disease progression. There was a consistent profile of infection among the groups exposed to each dose according to the measures employed: distribution and number of lesions, severity score of gross lesions, distribution and number of infected tissues, levels of extrathoracic infection, and distribution and number of histological lesions. The inoculation resulted in a variety of infection states, ranging from latency (absence of gross lesions), to gross lesions in the lungs, draining and extrathoracic lymph nodes, and pleura. The experimental infections appeared to mimic the more severe end of the spectrum of lung disease found in natural-infected badgers in that pulmonary lesions ranged from 1- to 2-mm diameter discrete tubercles, to extensive miliary lesions, with consolidation and pervading caseation of lobes (**Figure 3**). Cavitation and liquefaction were not seen (37).

The CMI responses, as determined by antigen stimulation of peripheral blood mononuclear cells (PBMCs) with bovine purified protein derivative tuberculin (PPD-B), increased to levels associated with the infective dose and pathology recorded postmortem (39). The badgers infected with the highest dose of *M. bovis* developed the earliest immune responses at 3 weeks postinfection. In addition, the highest infective dose correlated with the most consistent CMI response. In those animals with latent infection the CMI responses were weak and indistinguishable from non-infected control animals over most of the study period. In contrast to the CMI response, the humoral antibody responses of the badgers were intermittent over the time period of the study and not strongly influenced by the dose of *M. bovis*.

The aim of the follow-up study was to describe pathological, bacteriological, and immunological changes over a 24-week infection period using only the high-dose rate (46, 53). Inoculation by

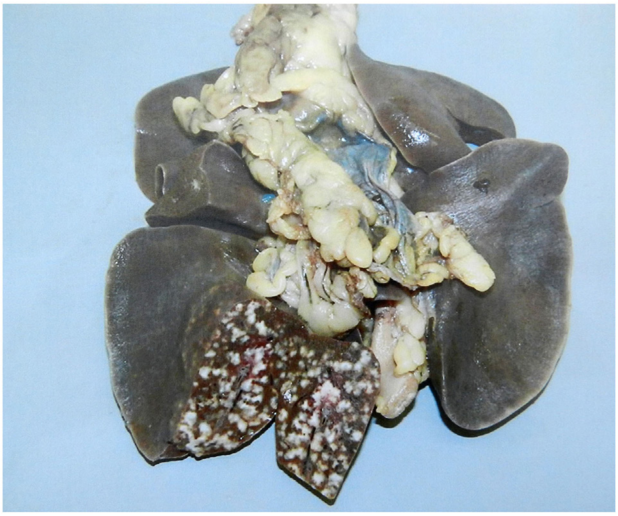

FIGURE 3 | Experimental infection. Lesions of experimental pulmonary tuberculosis with miliary lesions of a uniform size in the inoculated lobe. In natural-infected badgers typically fewer pulmonary lesions are observed and they vary in size.

the endobronchial route resulted in a non-progressive infection in most of the badgers, with limited dissemination of infection from the thoracic cavity. Where this occurred it was principally to the mesenteric and hepatic lymph nodes. However, there was a lack of uniformity in the progression of disease across time. There was contraction in the distribution of infection from that seen at 6 weeks to that at 12 weeks, but then progression of infection between 12 and 18 weeks but similar results for 18 and 24 weeks. The analysis suggested that changes in the distribution of infection between time points arose from non-uniform development of lesions within badgers and that local resolution of lesions may have also occurred. The CMI responses to PPD-B were first detectable 3 weeks after infection, and over the study period the responses of the PBMC to antigen stimulation with PPD-B and the specific antigen CFP-10 were positively associated with the presence of gross lesions in the infected badgers. There was no evidence of a direct correlation between the strength of the response and the severity or distribution of observed lesions. The serological response was largely restricted to MPB83 across all states of infection. An interesting finding from this study was the generation of immunoglobulin-G (IgG) recognition of MPB83 coincident with the CMI response at 3 weeks postchallenge.

A key feature of the badger experimental model has been the dose-dependent recreation of different states of infection, ranging from latency to clinical disease, all characteristic of natural infection. This is unique among natural hosts of infection in that each state in the badger can be defined by a combination of pathological and immunological parameters. In human tuberculosis, latency (skin test and IFN-γ immunological responses but no clinical or radiographic signs) is significant in the epidemiology of disease (54, 55). Latency is also an important feature in natural-infected badgers where, in a high proportion of infected badgers, infection is not associated with any clinical signs or gross pathology (31). The apparent lack of immunological responses in the natural or experimentally reproduced latency may reflect how the different immune systems control very low levels of infection, or strains of low virulence, e.g., bacillus of Calmette–Guérin (BCG), or may point to differences in pathogenesis of infection in the different hosts (56).

BCG VACCINATION AND PATHOGENESIS OF DISEASE IN BADGERS

Having established an experimental infection model that mimics the characteristics of moderate-to-severe natural disease, the studies progressed to evaluating BCG vaccine protection using the endobronchial experimental infection model (57, 58). A second objective was to determine if vaccination altered the pathogenesis of disease. The first study examined the effect of vaccination on the distribution of experimental infection. The BCG vaccine was administered in two different ways: subcutaneous injection (~5×10^5 CFU) and application to mucosal membranes (nasal and conjunctival mucosa with total final vaccine dose of 4×10^5 CFU). This latter route of immunization facilitated the delivery of BCG directly to an available mucosal surface, as a proxy route for delivery of an oral vaccine. With this protocol, the BCG was likely to be presented to the immune system *via* lymphoid tissues of the nasal cavity and/or conjunctiva. Inhaled aerosol particles may also have been exposed to the lymphoid tissues of the lower respiratory tract.

Following vaccination and endobronchial challenge (~10^4 CFU *M. bovis*) infection was established in all vaccinates and in all of the control group. Compared with the controls, vaccinates had fewer sites of infection, sites of extrathoracic infection, and sites with gross lesions. In the group vaccinated by the subcutaneous routes, all these measures were lower than for those vaccinated by the mucosal route. Vaccination also altered the distribution of gross lesions following challenge with no extrathoracic gross lesions in the vaccinates. These effects were more pronounced for the subcutaneous group. It was 2 weeks after *M. bovis* challenge that the subcutaneous vaccinated group responded to PPD-B, whereas the remaining groups responded from 4 weeks postchallenge. Over the period of infection, the highest immune responses were recorded in the non-vaccinated/infected control group. The immune profiles observed also associated with lesion severity scores measured at postmortem in all groups. Vaccination was also expressed by a significant delay in seroconversion to MPB83, which correlated with the levels of severity of the disease in all groups. From an immunological perspective, an intriguing finding was the lack of CMI activity in response to vaccination, as measured by IFN-γ production. This has been found in other captive badger studies and there is evidence that it correlates positively with the dose of vaccine delivered (59). It is tempting to speculate that this lack of CMI activity following vaccination is mechanistically related to the absence of similar responses in latent-infected badgers. In both cases, it might suggest that innate T cells play a prominent role for limiting multiplication of bacilli and maintaining them at low numbers. In recent years, there is growing evidence of non-specific protective effects generated by vaccination with BCG and other live vaccines (60). It is thought to be mediated by cross-reactivity of T-cell responses to related and unrelated pathogens, and/or by "trained immunity" whereby the innate immune system is modified by epigenetic programming following initial exposure, in order to increase resistance to reinfection (61, 62). The repeated exposure of badgers to environmental mycobacteria may provide the conditions necessary for training of the innate immune system to maintain subsequent exposure to *M. bovis* as a latent infection in most cases, and to limit the requirement of a CMI response in response to BCG vaccination.

Further studies examined the effect of oral BCG vaccination (endo-esophageal instillation of ~10^8 CFU BCG encapsulated in a lipid formulation) on protective effect and the distribution of infection after experimental challenge (63, 64). The predominant effect of BCG vaccination was seen as a reduction in the severity and number of gross lesions, decreased mycobacterial load in the lungs, and reduced number of sites of infection. However, vaccination did not alter the thoracic–extrathoracic pattern of infection. The CMI responses recorded in each of the vaccine groups were consistent with protection when expressed by pathology severity scores.

The most effective way of validating an experimental vaccination model is to compare it to vaccination of free-living animals in their natural environment. This allows vaccine protection to be assessed in a scenario of natural transmission. It also addresses an inherent limitation of captive animal vaccine studies where the infective dose and time of infection are controlled, leading to a relatively homogenous infection level in all animals from using relatively high-challenge doses. Two vaccine field trials have been carried out in badgers, in the UK and Ireland (65, 66). An injectable BCG vaccine for badgers was used in the UK trial and the results reported a 74% reduction in seropositivity among vaccinated badgers as compared with non-vaccinated badgers (65). Further analysis using a combination of diagnostic tests revealed a decreased risk of cubs testing positive as the proportion of adults vaccinated increased (67). In the Irish study, there was also a clear reduction in the rate of seroconversion among vaccinated badgers as compared with the non-vaccinated badgers, and in those vaccinated animals that did seroconvert, there was a significant time delay to when seroconversion occurred relative to the non-vaccinated badgers (66). The delayed time to seroconversion was consistent with that recorded in the captive badger vaccine studies where vaccine protection was also measured by time to seroconversion following endobronchial *M. bovis* challenge (33). A follow-up analysis using a different serology test confirmed that the proportion of seropositive animals was reduced in the vaccinated population compared with the non-vaccinated animals over the course of the trial (68).

BCG VACCINATION AND DISSEMINATION OF INFECTION IN BADGERS

In all of the experimental vaccine studies, the protection levels generated by vaccination with BCG did not prevent establishment of infection; this may have reflected the severity of challenge resulting from endobronchial delivery of virulent *M. bovis*. However, vaccination did not appear to substantially change the pathogenesis of disease. One notable finding of the experimental vaccine protection studies was the level of dissemination of infection from the lung in all infected badgers including vaccinates. The principal measure of vaccine-induced protection was a reduction in the severity of thoracic and extrathoracic lesions rather than a reduction in the wider distribution of infection. The BCG vaccine is known to limit disseminated disease when delivered to children but is less effective in protecting adults against pulmonary disease (69–71). During the early innate stages of the immune response dissemination from the initial site of infection may occur before migration of the mycobacteria to a lymph node stimulates the development of a CMI response (72). Lesion development characteristic of tuberculosis will not commence until a sufficiently potent CMI response is generated, and this may take several weeks, dependent on the potency of the immune response and the severity of the infective dose. In the badger experimental model the average time to detect a measurable CMI response is 2–4 weeks (58, 63). During the period preceding this response, infected macrophages can use the lymphatic system to pass through the draining lymph nodes and circulate throughout the host. When a sufficiently dominant CMI response is induced, infected macrophages can become immobilized in lymph node tissue and further migration is restricted. Lesions can then develop and progress at sites where infected macrophages are resident, including those areas of the lung where infection was initiated. Following vaccination, antigen specific T-cells still take time to accumulate, allowing the infective bacilli to multiply and spread. In the badger model, the earliest time point for measuring postinfection responses is 2 weeks. The data indicate that responses in vaccinates appear around this time point and earlier than in non-vaccinates. Nevertheless, it still allows time for dissemination of infection to occur in the days following establishment of infection, by translocation of infected macrophages *via* the lymphatic system or the blood stream. Local dissemination may occur by movement of infected macrophages within tissues or following accumulation of infected debris in the lymphatics, or blood vessels, airways, renal or gastrointestinal system (29). This may partly explain why vaccination is particularly successful in reducing the severity of disease but has limited impact on distribution of infection.

EXPERIMENTAL INFECTION MODELS FOR DOMESTIC SPECIES AND WILD ANIMAL TUBERCULOSIS

Studies in natural susceptible hosts and experimental studies in captive natural susceptible hosts are indispensable in advancing the understanding of the pathogenesis of tuberculosis. This in turn can generate confidence in the interpretation of results from experimental vaccine—challenge studies. In many domestic species, e.g., cattle, goats, and deer, the presentation of tuberculosis is similar to that observed in the greater majority of infected humans in that it is a chronic, slowly progressive disease with pathology predominantly confined to the lower respiratory tract, with associated cellular immune responses (73, 74). However, the occurrence and prevalence of latency in these species has not been clearly established and is difficult to identify in either natural-infected or experimentally infected animals.

Many of the pathogenesis studies and infection models developed in livestock and wild animals have been motivated by the desire to develop vaccines to control inter-species spread of tuberculosis (75). With this in mind, development of a reliable experimental infection model is important for a number of reasons including (a) the need to establish infection with an appropriate dose, (b) keeping the dose biologically plausible, (c) obtaining a balance ensuring infection is successful in all animals including vaccinates, (d) ensuring that the infection route used and the pathology generated is at least within the spectrum of the disease in its natural state, and (e) there are appropriate and measurable parameters for quantifying protective immunity.

Unlike laboratory animal model systems, livestock and wild animals are usually natural hosts for infection and are relatively outbred. Therefore, replicating the pathogenesis of natural infection might be confounded by the differing environmental conditions experienced by free-living and captive animals, and also different levels of natural susceptibility to infection. Experimental infection of domestic cattle with low doses of *M. bovis* (10^2 to 10^3 CFU) by intratracheal/endobronchial inoculation or by aerosol-generating systems has resulted in lesions similar to those detected in the lungs and associated lymph nodes of natural-infected animals (76, 77). A natural challenge system of housing tuberculosis-free cattle with natural-infected animals has been presented as a biologically plausible challenge system but with a highly variable degree of success (78, 79). Experimental infections using endobronchial (*M. caprae*) or aerosol delivery (*M. bovis*) have been carried out in goats (80, 81). In both cases, the distribution of lesions mimicked those seen in natural infection. Further studies in goats experimentally infected with selected members of the *M. tuberculosis* complex (*M. bovis*, *M. caprae*, *M. tuberculosis*) revealed different clinical outcomes with lesion scores ranking highest with *M. bovis*, then *M. caprae* and *M. tuberculosis* (51). These results highlight a good example of host tropism associated with closely related mycobacterial species. Sheep have also been experimentally infected with *M. caprae* by the endotracheal route resulting in granulomatous caseous and necrotizing lesions in the lung and associated lymph nodes, typically found in natural cases of sheep tuberculosis (82). In addition, there were similar measured immunological, pathological, and bacteriological parameters as found in experimental *M. caprae* infection of goats. In deer naturally infected with *M. bovis*, the distribution of lesions differs from other domestic animals in that lesions are found predominantly in the retropharyngeal lymph nodes followed by lung and thoracic lymph nodes. Experimental infection models in deer routinely target inoculation of the tonsillar crypts to mimic natural infection and lesion distribution (83, 84). Additional studies in white-tailed deer have demonstrated that dissemination from the tonsil is an infrequent event involving low numbers of bacilli (85).

Ferrets (*Mustela Furo*) are susceptible to infection with *M. bovis* and are considered as part of the epidemiology of *M. bovis* transmission in New Zealand (86). As mustelids related to badgers, they offer some advantages as a model animal species in that they are available from licensed suppliers, and are relatively easy to house and maintain in captivity. Experimental infection models have been established where *M. bovis* was delivered to captive ferrets by the oral or aerosol route (35, 87). In both models, infection was found in the thoracic cavity and also in the mesenteric lymph nodes. The mechanism of dissemination from the primary site of infection in both models is not clearly understood. To date, experimental challenge by the endobronchial route has not been reported; this would allow for direct comparison of different aspects of pathogenesis with badgers, which might reveal subtle differences in specific host–pathogen interactions influencing dissemination.

The wild boar (*Sus scrofa*) is considered as a key maintenance host for tuberculosis in Spain with prevalence rates >50% in areas with high-density populations (88). Vaccination is being explored as a potential strategy to control the level of disease in these wild animals. In the first challenge study, a field isolate of *M. bovis* was delivered to boar by the oropharyngeal route using a range of infective doses between 10^2 and 10^6 CFU (89). All four challenged wild-boar-developed lesions and severe generalized lesions were observed in two animals exposed to the highest dose: this was not considered typical of the lesion distribution encountered in natural infections.

Routes of infection not observed in natural-infected wild animals have been used in experimental infections to achieve particular outcomes. Such alternative inoculation routes have been used to experimentally infect New Zealand brushtail possums to counter the high susceptibility of these animals to experimental infection and the rapid progression of disease. Delivery of *M. bovis via* conjunctival instillation resulted in established infection in a dose-dependent manner (90). The infection progressed slowly in the possums, generating palpable lesions in superficial lymph node lesions, and widespread distribution of macroscopic and microscopic lesions, all characteristics of the disease in wild, natural-infected possums. Percutaneous inoculation of *M. bovis* suspension into the paws resulted in gross lesions in superficial lymph nodes as is observed in natural-infected possums (91). As with the conjunctival challenge route, percutaneous infection prolonged the postchallenge survival period. However, although these two alternative routes of infection do result in gross lesion distribution as seen in natural-infected wild possums, they significantly underestimate the distribution of infection as seen when more detailed postmortem examination procedures are employed (6). Natural transmission between infected and susceptible captive possums was also investigated as a possible method of evaluating vaccination in captive possums but proved unsuitable (92). The rate of *M. bovis* transmission was lowest when animals were mixed at random; however, transmission increased significantly with mixing of more sociable possums. This indicated that transmission was influenced by the proximity of susceptible and infected possums leading to increased frequency and duration of social interactions.

Though the presence of visible lesions, whether in experimentally infected or in natural-infected animals, can point toward the likely route of natural infection, caution must be taken in interpreting such data when only a limited number of infection parameters are measured. This highlights one of the difficulties when working with relatively large animal species to understand pathogenesis. The sensitivity of detection of infection can be severely compromised in large animals, such as cattle and deer, simply because of the difficulty of detecting small lesions or infection in large organs and lymph nodes (93). This can lead to biases when analysis is based on visible lesion detection. In all animals, there is the possibility of microscopic lesions or small bacteriological loads existing in the carcase but too small to detect, this being an increasing probability as the mass of the animal increases. Sampling from natural-infected populations can also compound this when only animals considered likely to have large lesions, or strong reactors to the skin test are recruited for analysis. The problem here is that it represents a relatively late stage of disease progression and the distribution of lesions may not reflect the early stage of pathogenesis and the likely route of infection.

Even when using natural-infected animals across a broad spectrum of disease, the interpretation can be subjected to bias if insensitive protocols for bacteriology and histology are employed. In studying the disease in natural-infected badgers, we tried to minimize these biases by randomly sampling from the infected population and by using sensitive bacteriological and histological procedures on a predetermined set of 36 tissues covering a wide range of anatomically diverse tissue samples in each animal. In addition, the tissues for bacteriology were collected aseptically so that the bacteriological detection of infection was maximized and tissues were individually cultured for the same reason (31). This intensity of pathological investigation has not been repeated in any other natural-infected animal species but for the badger, at least, it provides a reference point to compare natural-infected animals with those from experimental infection studies.

CONCLUDING REMARKS

The pathological and bacteriological examination of natural-infected badgers with tuberculosis when using sensitive post-mortem examination procedures has provided an important and essential baseline for understanding the pathogenesis and epidemiology of tuberculosis in this species. It has also provided the framework for developing experimental infection studies, which has allowed the evaluation of diagnostic assays and the measurement of vaccine efficacy. There is a growing awareness that *M. bovis* infection is naturally endemic in a diverse range of domestic and wild animals, as well as in humans. Experimentally, at least, it seems that most animal species are susceptible to infection. With the continuous threat of infection spreading from wild animals to livestock and the risk of onward transmission to humans, the continued improvement in knowledge of pathogenesis and epidemiology will serve to lessen the transmission risks into the future.

AUTHOR CONTRIBUTIONS

EG and LC contributed equally to this review including critical analysis of published data and preparation of the manuscript.

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Preliminary Results Indicate That Inactivated Vaccine against Paratuberculosis Could Modify the Course of Experimental *Mycobacterium bovis* Infection in Calves

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Although paratuberculosis (PTB) vaccination has been recognized as an effective tool to control the disease, its use has been limited in countries undergoing bovine tuberculosis (bTB) eradication programs because of its interference with the diagnostic techniques. Due to this restraint, little is known about the effect of vaccinating against PTB on the progression of bTB infection. To assess this topic, an experimental infection was carried out including the following three groups of five calves each: non-vaccinated infected with *Mycobacterium bovis* (NVI), vaccinated against PTB infected with *M. bovis* (VI), and vaccinated against PTB non-infected (VNI). The level of infection attending to pathological and bacteriological parameters was evaluated at necropsy in collected tissue samples. Infection was confirmed in all challenged animals being the lung and thoracic regions most affected for all studied parameters. The VI group presented 15.62% less gross lesions in the thoracic region than the NVI, although no significant differences were found. Only one vaccinated animal presented gross lesions in the lung, compared to three non-vaccinated calves. NVI animals showed an average of 1.8 lung lobes with gross lesions whereas in the vaccinated group the average number of affected lobes was 0.2, representing an 89% reduction. Significant differences were not found, although a tendency was observed ($p = 0.126$). Pathological and culture scores showed the same tendency. Vaccination induced a 71.42 and 60% reduction in lesion and culture scores in the lung as well as a 23.75 and 26.66% decline, respectively, in the thoracic region. The VI group showed lower positivity in the rest of the areas for all measured criteria except for the head. In order to reinforce our results, further research on a larger sample size is needed, but the results from this study suggest that PTB vaccination could confer certain degree of protection against bTB infection, supporting the view that PTB vaccination could increase resistance to the main mycobacterioses that affect animals.

Keywords: paratuberculosis, bovine tuberculosis, vaccine, interference, crossed-protection

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INTRODUCTION

Bovine tuberculosis (bTB) and paratuberculosis (PTB) are mycobacterial diseases that have a huge economic impact on cattle, especially on dairy herds (1, 2). Both present a widespread distribution, affecting domestic hosts (3, 4) and wildlife species (5, 6), promoting the successful dissemination of their etiological agents.

Paratuberculosis or Johne's disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Progression of the disease causes a chronic gastrointestinal granulomatous inflammation. Different factors, such as the long incubation period of the disease, fecal-oral route transmission, intermittent excretion periods added to high resistance of mycobacteria in the environment, and limited performance of diagnostic methods, make control of the infection difficult to achieve. PTB vaccination has been proven to restrain the disease in cattle (7), sheep (8), and goats (9). Excretion of bacterial burden is considerably reduced, containing the spread of the infection and therefore diminishing the number of clinical cases (10, 11). Nevertheless, the interference induced by PTB vaccination with the current interpretation criteria (12) of the bTB official diagnostic tests (13) results in the restricted use of PTB vaccination. MAP-based vaccination in cattle is not allowed by the Animal Health Authorities of most countries. Despite the diagnostic interference, a certain degree of containment of the lesion dissemination from the target organs after a bTB infection has been previously reported in PTB-vaccinated goats (14). This suggests that if interference issues are avoided, PTB vaccination can be used for PTB control and might also be beneficial against bTB conferring some degree of protection.

The degree of interference of PTB vaccination with official bTB diagnostic tests has been evaluated previously in cattle (13, 15) and goats (16, 17). Cross-reactivity in the skin test has proven to be limited if the comparative intradermal test is used in bTB-free bovine herds (13). These findings have been based on an exhaustive analysis of results from a vaccine clinical trial under field conditions. To evaluate the effect of PTB vaccination on bTB infection and the interference due with bTB diagnosis, an experimental infection with *Mycobacterium bovis* in bovines previously vaccinated against PTB was performed. Results on interference using alternative diagnostic criteria and specific antigens have been reported in a separate paper (12) while pathological and bacteriological changes associated with vaccination in a bTB experimental infection are the goal of this report.

MATERIALS AND METHODS

Ethics Statement

Animals used in this study had their origin in commercial farms. With the purpose of obtaining data for this trial, calves were submitted to clinical practices standardized and regulated by the European, Spanish and Regional Law and Ethics Committee. The experimental design underwent ethical review and approval by NEIKER's Animal Care and Use Committee and by the Agriculture Department (PARAPATO-1264-BFA).

Animal Selection

Thirty Friesian calves from 13 different farms in northern Spain were selected in a feedlot. For final selection of animals, absence of previous contact with mycobacteria was considered. IFN-γ release assay (IGRA) with standard tuberculins (A-PPD, B-PPD) as well as with more specific antigens (ESAT-6/CFP10 and Rv3615c) (18) already tested for the diagnosis of bTB was the assay used for this purpose. The first blood sampling was carried out at week 0, at the age of 2 months. After the blood samples were collected, 20 randomly selected animals were vaccinated subcutaneously in the dewlap with 1 ml of a heat-inactivated vaccine (Silirum® CZV, Pontevedra, Spain) and 10 remained unvaccinated. In order to reassure the absence of previous contact with *M. bovis* or other mycobacteria, blood samplings at the feedlot were repeated twice, at weeks 4 and 12. Finally 10 vaccinated and five non-vaccinated animals with negative results for IGRA and without evident pathologies were selected and transported to the biosafety level 3 (BSL-3) facilities in NEIKER where three groups of five animals each were formed.

M. bovis Challenge

A 2-week adaptation period was established for the calves after their arrival at the BSL-3 facilities. At week 18 post-vaccination, five vaccinated and five non-vaccinated animals were challenged by the endotracheal route with 10^6 colony-forming units (CFUs) of a *M. bovis* field isolate suspended in 2 ml of phosphate-buffered saline after intramuscular sedation with Xylazine (10 mg/50 kg). The *M. bovis* challenging isolate was spoligotype SB0339 according to the *M. bovis* Spoligotype Database website (http://www.mbovis.org). The final experimental groups were as follows: PTB vaccinated and *M. bovis* infected (VI), PTB vaccinated and *M. bovis* non-infected (VNI), and PTB non-vaccinated and *M. bovis* infected (NVI).

Postmortem Studies

The animals were slaughtered at week 14 post-infection in three consecutive days, five calves per day. Upon sedation with XILAGESIC® 2% (10 mg/50 kg lw) (Laboratorios Calier, S.A., Barcelona, Spain), animals were euthanized by an intravenous injection of T61 (4–6 ml/50 kg) (Intervet International GMBH, Unterschleissheim, Germany). Complete necropsy was performed although special focus was set on the respiratory system. All tissue specimens were individually collected and processed for pathological and microbiological analysis. Collected tissues per region included were: head [tonsils, nasal turbinate, and parotid and retropharyngeal and mandibular lymph nodes (LNs)], thorax (tracheal, tracheobronchial, mediastinal, pulmonary, and prescapular LNs), lung (right and left cranial and caudal lobes and medium and accessory lobes), abdomen (liver, spleen, and hepatic LNs), and others (prefemoral LNs).

Gross Pathology

All tissues were visually inspected for lesions compatible with TB infection. Scoring of lesions according to Palmer et al. (19) was performed independently by two researchers, and the mean value of both scores was used. Briefly, the scoring system for lung was as follows: 0: no visible gross lesion, 1: no visible external gross lesion but internal detected after splicing, 2: less than five lesions smaller than 10 mm, 3: over five lesions smaller than 10 mm, 4: more than one lesion bigger than 10 mm, and 5: gross confluent lesions. In the case of LNs, scoring was as follows: 0: no visible gross lesions, 1: focal lesions of 1–2 mm, 2: a lot of small foci, and 3: extended lesions. Once scores were assigned to each lesion, total and regional scores per animal were calculated by adding them.

Bacterial Culture

Bacterial tissue culture was performed as described previously (20). Briefly, 2 g of tissue samples were homogenized in 10 ml of sterile distilled water. Five milliliters of the suspension was decontaminated and processed for liquid culture in BBL MGIT tubes supplemented with BBL MGIT PANTA and BACTEC MGIT growth supplement (Becton Dickinson, Franklin Lakes, NJ, USA) following manufacturer's instructions. BBL MGIT tubes were incubated for 42 days in a BACTEC MGIT 960 System. The remaining 5 ml were decontaminated in hexadecyl-pyridinium chloride 0.75% (w/v) for 12–18 h for solid culture. After a 5 min centrifugation step at 2,500 × *g*, pellets were cultured in Coletsos tubes (bioMérieux SAF-69280 Marcy l'Etoile France) at 37°C during 4 months.

Once the MGIT incubation period finished, DNA extraction was performed on culture from all positive tubes and some negative tubes. PCR was carried out subsequently to confirm that growth was due to *M. bovis* (21).

After the incubation period for solid culture was completed, colonies were visualized under a stereoscope and scraped for DNA extraction and *M. tuberculosis* complex PCR confirmation (21). All isolates were confirmed as *M. bovis* SB0339 by spoligotyping (22). A culture score was defined in order to categorize the infection level of each tissue. In this case, scores were as follows: 0: no growth, 1: less than 10 colonies, 2: between 10 and 50 colonies, and 3: over 50 colonies (20).

A tissue was considered positive for culture when it gave a positive result by solid culture, liquid culture, or both.

Statistical Analysis

Reduction by vaccination was calculated by the formula (1 − VI/NVI) × 100 for each of the following parameters: solid culture score, number of positive tissues by culture, gross lesion score, and number of affected areas by gross pathology.

For the analysis, the number of tissues with positive cultures, with bTB compatible gross lesion and the solid culture and lesion scores, was calculated per area and animal. Differences in the degree of pathology and bacterial burden (lesion scores and culture scores) were compared using the Mann–Whitney *U*-test. Differences in the number of affected tissues by gross pathology and culture were assessed using Student's paired two-sample *t*-test, whereas Fisher's exact test was used for proportions of animals with lesions. Spearman's correlation test was applied to assess the association between culture and gross pathology results. In all cases, significance of the differences among groups for all variables was accepted at $p < 0.05$.

RESULTS

Clinical Signs

All animals included in the study went through the whole experiment, and no adverse reactions were reported after vaccination or challenge. No clinical signs of bTB such as wasting and coughing were observed in any of the animals after challenge. Infection of all challenged animals was confirmed by bacteriological and pathological analysis. The VNI group did not present gross lesions compatible with bTB or culture-positive tissues.

Postmortem Analysis

Detailed pathology results from the infected groups of the study are shown in **Table 1**. The thoracic region and lung were the areas presenting a higher number of affected tissues and the NVI presented slightly higher scores as expected. Culture results from the infected groups of the study are shown in **Table 2**. In this case, again thorax and lung were the most affected areas, although the VI group only presented one animal with one positive tissue. The analysis has been focused separately on head, thorax, and lung as well as on the total where all areas of the animal have been considered. The analysis of number of tissues presenting pathology and culture positive results is shown in **Table 3**.

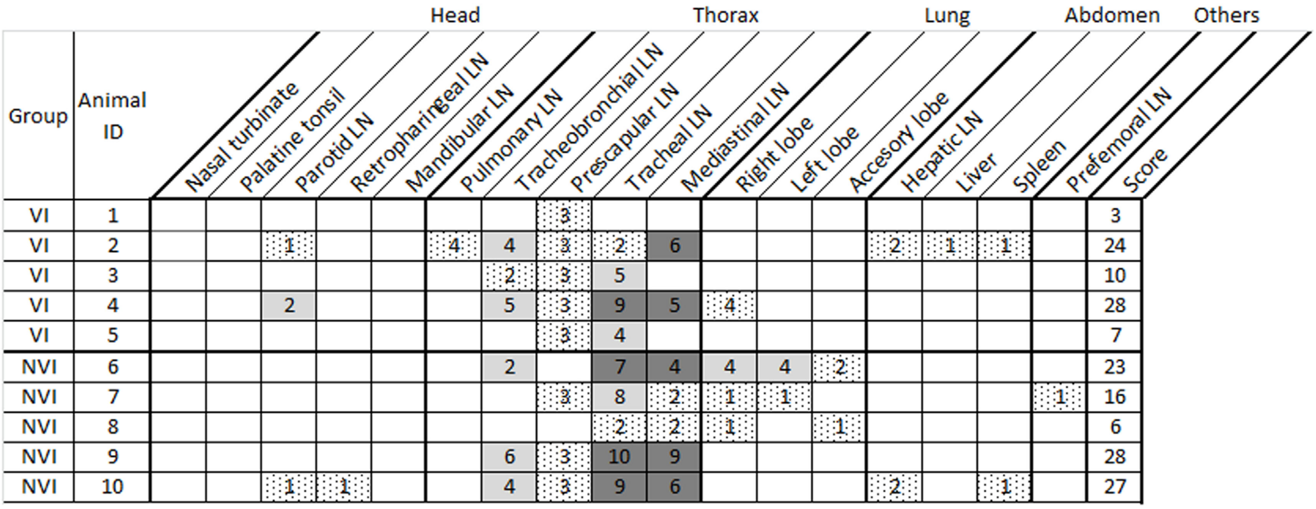
In the thoracic region, the NVI group presented a mean of 6.4 LNs with gross lesions with a minimum of two and a maximum of 10 affected LNs in each animal compared to the VI group with an average of 5.4 LNs affected with a minimum of two and a maximum of nine affected LNs. Significant differences among groups were not observed ($p = 0.643$). However, the reduction due to vaccination was of 15.62%.

Of the six lung lobes evaluated, the NVI group presented an average of 1.8 lobes with gross lesions with a minimum of zero and a maximum of five affected lobes per animal, much higher than the mean of 0.2 affected lobes found in the VI group, which had a minimum of zero and a maximum of one affected lobes. Although significant differences were not observed between the number of affected lobes, the tendency should be considered (*t*-test, $p = 0.126$). This represents an 89% reduction due to vaccination. Only one animal (1/5, 20%) from the VI group presented lesions in the lung compared to three animals (3/5, 60%) from the NVI group (Fisher's test; $p = 0.189$).

The number of affected tissues was always lower in the VI group than in the NVI groups except for the area that compromised the head. In this case, two animals presented gross lesions, one in one tissue and another in two in the VI group, whereas the NVI group presented one animal with gross lesions in two tissues. Tissue culture positivity was also slightly higher in the head in the VI group where an average of 1.4 ± 0.4 tissues with detectable bacteria was observed compared to the mean of 1 ± 0.55 tissues in the NVI group. In the VI group, four animals presented positive tissue culture (4/5, 80%) compared to three in the NVI group (3/5, 60%).

Pathology and culture scores were always lower in the VI group than in the NVI group (**Table 4** and **Figure 1**) except for the head where lesion scores were slightly higher in the VI group (0.6 ± 0.4 vs. 0.4 ± 0.4). In any case, significant differences were

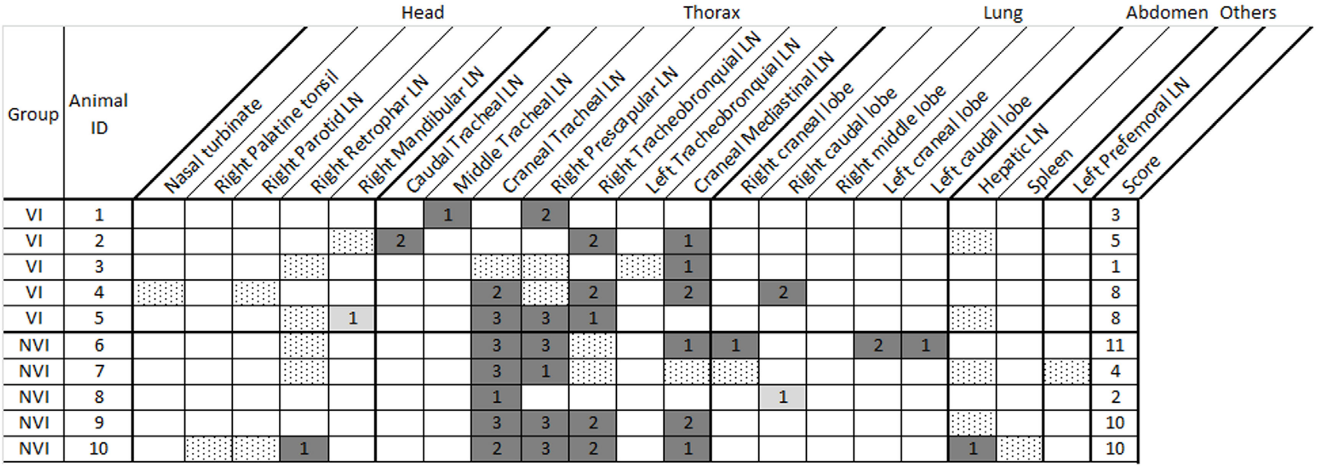
TABLE 1 | Tissues with tuberculosis compatible lesions in the infected groups of the study.



Group	Animal ID	Head					Thorax					Lung			Abdomen			Others	
		Nasal turbinate	Palatine tonsil	Parotid LN	Retropharingeal LN	Mandibular LN	Pulmonary LN	Tracheobronchial LN	Prescapular LN	Tracheal LN	Mediastinal LN	Right lobe	Left lobe	Accesory lobe	Hepatic LN	Liver	Spleen	Prefemoral LN	Score
VI	1								░ 3										3
VI	2			░ 1			░ 4	▒ 4	░ 3	░ 2	▓ 6				░ 2	░ 1	░ 1		24
VI	3							░ 2	░ 3	▒ 5									10
VI	4			▒ 2				▒ 5	░ 3	▓ 9	▓ 5	░ 4							28
VI	5								░ 3	▒ 4									7
NVI	6							▒ 2		▓ 7	▓ 4	▒ 4	▒ 4	░ 2					23
NVI	7								░ 3	▒ 8	░ 2	░ 1	░ 1					░ 1	16
NVI	8									░ 2	░ 2	░ 1		░ 1					6
NVI	9							▒ 6	░ 3	▓ 10	▓ 9								28
NVI	10			░ 1	░ 1			▒ 4	░ 3	▓ 9	▓ 6				░ 2		░ 1		27

VI, vaccinated infected; NVI, non-vaccinated infected; LN, lymph node.
One LN affected (right or left): dotted; two LNs affected (both right and left or two of three, cranial, caudal, or medial): light gray; three LNs affected (cranial, caudal, and medial): dark gray.
Lesion score is the sum of the scores of all tissues.

TABLE 2 | Tissues with positive culture results in the infected groups of the study.



Group	Animal ID	Head					Thorax							Lung					Abdomen		Others	
		Nasal turbinate	Right Palatine tonsil	Right Parotid LN	Right Retrophar LN	Right Mandibular LN	Caudal Tracheal LN	Middle Tracheal LN	Craneal Tracheal LN	Right Prescapular LN	Right Tracheobronquial LN	Left Tracheobronquial LN	Craneal Mediastinal LN	Right craneal lobe	Right caudal lobe	Right middle lobe	Left craneal lobe	Left caudal lobe	Hepatic LN	Spleen	Left Prefemoral LN	Score
VI	1							▓ 1		▓ 2												3
VI	2					░	▓ 2				▓ 2		▓ 1						░			5
VI	3				░				░	░		░	▓ 1									1
VI	4	░		░					▓ 2	░	▓ 2		▓ 2		▓ 2							8
VI	5				░	▒ 1			▓ 3	▓ 3	▓ 1								░			8
NVI	6				░				▓ 3	▓ 3	░		▓ 1	▓ 1			▓ 2	▓ 1				11
NVI	7				░				▓ 3	▓ 1	░		░	░					░		░	4
NVI	8								▓ 1						▒ 1							2
NVI	9								▓ 3	▓ 3	▓ 2		▓ 2						░			10
NVI	10		░	░	▓ 1				▓ 2	▓ 3	▓ 2		▓ 1						▓ 1	░		10

VI, vaccinated infected; NVI, non-vaccinated infected; LN, lymph node.
MGIT culture positive: dotted; solid culture positive: light gray; both MGIT and solid culture positive: dark gray.
Culture score is the sum of the scores of all tissues.

not observed among analyzed areas. However, reductions in lung of 71.42 and 60% in lesion and culture scores, respectively, and of 23.75 and 26.66% in the vaccinated group for the same parameters in thorax should be noted.

Correlation between both diagnostic methods considering number of affected tissues was best fit in lung ($r = 0.988$, $p < 0.0001$), followed by head ($r = 0.746$, $p = 0.013$), total ($r = 0.667$, $p = 0.035$), and thorax ($r = 0.655$, $p = 0.04$).

DISCUSSION

Although the efficacy of PTB vaccination has been repeatedly demonstrated (7, 11, 18, 23), its use has been restricted due to the cross-reactivity with current bTB diagnostic methods. Interference issues have probably led to the lack of knowledge on the effect of PTB vaccination in the development of bTB infection. This is the first study examining this topic in bovines under experimental conditions.

In this particular experimental setting, all animals became infected regardless of their vaccination status showing positive culture results and macroscopic lesions compatible with bTB. In both groups, thorax and lung were the most affected areas. The majority of tissue sites affected by infection in terms of lesion development or bacterial colonization, as well as the highest lesion and culture scores appeared in these two areas. However, PTB vaccination seemed to induce a moderate protective

TABLE 3 | Gross pathology and bacteriology analysis considering number of affected tissues per area.

	Pathology				Bacteriology			
	Mean (SEM)		*t*-Test		Mean (SEM)		*t*-Test	
	NVI	VI	*p*	% R	NVI	VI	*p*	% R
Head	0.4 (0.40)	0.6 (0.4)	0.733	0.00	1.0 (0.55)	1.4 (0.4)	0.572	0.00
Thorax	6.4 (1.43)	5.4 (1.50)	0.643	15.62	3.4 (0.60)	3.4 (0.40)	1.000	0.00
Lung	1.8 (0.92)	0.2 (0.20)	0.126	89.00	1.0 (0.55)	0.2 (0.20)	0.207	80.00
Total	9.2 (1.74)	6.8 (2.35)	0.436	26.10	6.6 (1.40)	5.4 (0.93)	0.495	18.18

NVI, non-vaccinated infected; VI, vaccinated infected; R, reduction due to vaccination.

TABLE 4 | Gross pathology and bacteriology analysis considering lesion and culture score per area.

	Pathology				Bacteriology			
	Mean (SEM)		*U*-test		Mean (SEM)		*U*-test	
	NVI	VI	*p*	% R	NVI	VI	*P*	% R
Head	0.4 (0.40)	0.6 (0.40)	0.606	0.00	0.2 (0.20)	0.2 (0.20)	1.000	0.00
Thorax	16.0 (4.13)	12.2 (3.60)	0.401	23.75	6.0 (1.58)	4.4 (1.07)	0.344	26.66
Lung	2.8 (1.85)	0.8 (0.80)	0.288	71.42	1.0 (0.77)	0.4 (0.40)	0.521	60.00
Total	20.0 (4.10)	14.4 (4.90)	0.530	28.00	7.4 (1.83)	5.0 (1.37)	0.248	32.43

NVI, non-vaccinated infected; VI, vaccinated infected; R, reduction due to vaccination.

response against *M. bovis* challenge, which led to a reduction of the pathological and bacteriological results in both areas of the VI group. The effect of vaccination was most evident in the lung as seen by the fact that only one animal (1/5, 20%) of the VI group presented lesions and detectable bacteria in this area compared to the NVI group where three animals were affected (3/5, 60%). Same tendency was observed in the remaining studied areas except for the head, where the protective effect was not evident. Although differences among groups were not statistically significant and only tendencies have been observed, the degree of protection observed can be considered biologically relevant. To reinforce the appealing results obtained from this first trial, it would be necessary to carry out further studies with a larger sample size.

These results suggest that a certain degree of heterologous protection against *M. bovis* infection takes place after PTB vaccination, and although the protection conferred is probably not enough to impede the establishment of the disease or prevent horizontal transmission within a herd, it may contain the infection to some extent. Results from this experiment indicate that this containment would clearly benefit the lung (60–89% reduction in lesions and bacterial burden) since reduction due to vaccination in the thoracic region was less notorious and absent in the head area. This may be important considering that the main excretion route of *M. bovis* is through the respiratory system, and therefore, the reduction of the bacterial load in the lung may lead to a reduction of this figure in the environment.

These findings partially agree with the results obtained by Pérez de Val et al. (14) in goats after PTB vaccination and subsequent challenge with *M. bovis* where, lesions in vaccinated goats appeared only in the lung and corresponding LNs whereas non-vaccinated animals showed an increased dissemination frequency of the lesions. In that case (14), goats were challenged through the endobronchial route and, therefore, infection progressed mainly affecting the lower respiratory tract. In another study in goats in which the transthoracic route was used, lesions were mainly located in lung and mediastinic and mesenteric LNs (24). In our study, thorax was the primary focus and extrathoracic and extrapulmonary dissemination of bacteria to the upper respiratory tract or head area (retropharyngeal, mandibular, parotid LNs, and nasal turbinate) occurred in four animals of the VI group and three animals of the NVI group. This could be due to a pulmonary dissemination to the head by mycobacterial shedding in the tracheobronchial secretions and subsequent ingestion as hypothesized in previous reports (25). Bacteria and gross lesions were detected in spleen of one animal of the NVI group, whereas only gross lesions were observed in spleen and liver of one animal of the VI group indicating that systemic circulation of mycobacteria had occurred.

Added to the route, the challenging dose can be crucial for the pathological outcome of the infection. In previous studies in goats (14, 24, 25), animals were challenged with lower doses of *Mycobacterium caprae* (10^2–1.5×10^3 CFUs), but as stated before in those studies, the inoculum was directly deposited into the lung. The selected dose (10^6 CFU) and infection route (endotracheal) may be responsible for the wider spreading of the lesions in our study. This high dose was applied to guarantee infection for vaccine evaluation. In any case, considering the fact that in experimental conditions the bacterial load administered for challenge is most probably many logs higher than the amount of *M. bovis* that an animal will be in contact with in field conditions

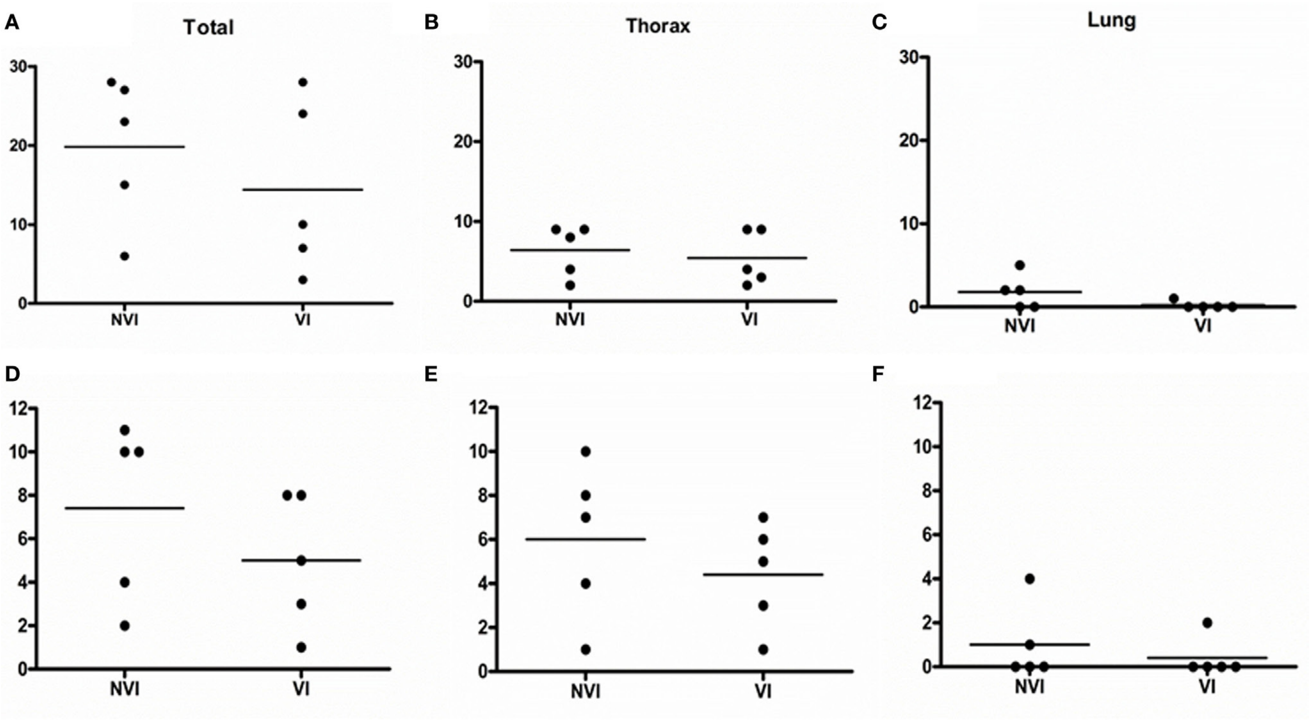


FIGURE 1 | Bovine tuberculosis lesion and culture scores for total **(A,D)**, thorax **(B,E)**, and lung **(C,F)**. The solid lines present median values.

it could be expected that higher protection levels would be observed in these cases.

Positive correlations were found between pathological and bacteriological techniques as expected. These were best fit in the lung, area that poses the most noticeable partial protective effect by the vaccine.

The results presented here suggest that vaccination against PTB modifies the course of experimental bTB infection by decreasing the severity of the lesions and the bacterial burden. Although our results are not conclusive, they support the view that mycobacterial vaccines could potentially be useful tools for disease control in specific settings where vaccination does not interfere with eradication programs.

ETHICS STATEMENT

With the purpose of obtaining data for this trial, calves were submitted to clinical practices standardized and regulated by the European, Spanish and Regional Law and Ethics Committee. The experimental design underwent ethical review and approval by NEIKER's Animal Care and Use Committee and by the Agriculture Department (PARAPATO-1264-BFA).

AUTHOR CONTRIBUTIONS

RJ, JG, and IS conceived the study. MS, EM, MG, IS, and NE carried out the laboratory work. RJ, NE, and MS compiled and analyzed the data. MS, NE, and IS collated the results. MS, NE, IS, and JG drafted the preliminary manuscript. All authors participated in the review and the editing of the final draft and also read and approved its final version.

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