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Rabies Symptoms, Diagnosis, Prophylaxis and Treatment

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Charles Rupprecht, Bernhard Dietzschold
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Rabies Symptoms, Diagnosis, Prophylaxis and Treatment

Special Issue Editors

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About the Special Issue Editors

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Editorial

Special Issue: Rabies Symptoms, Diagnosis, Prophylaxis, and Treatment

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Rabies is an acute, progressive, incurable viral encephalitis found throughout the world. Despite being one of the oldest recognized pathogens, its impact remains substantial in public health, veterinary medicine, and conservation biology. Thus, it is essential to apply existing tools and to seek new methods to improve upon prevention, control, selective variant elimination, and treatment efforts. Advances in diagnosis, vaccinology, pathobiology, and related research techniques continue to afford enhanced insights on rabies. Although rabies is not a candidate for eradication, the results of these innovative communications provide further knowledge to define a more optimal approach to understanding and managing this complex infectious disease of nature on a global basis in a One Health context.

More than 24 papers have been published upon peer review acceptance in this special issue (20 original papers, 1 perspectives piece, and 4 review papers are included). They each contribute to a much better understanding of this disease and to advances concerning the improvements for rabies management. These topics can be summarized as follows:

The clarion call for action was sounded in a perspective by David Durrheim that provides an ideal framework for the ongoing tragedy exemplified by childhood deaths from rabies and application of the necessary steps to end this situation now [1]. Such a thoughtful piece is made all the more enigmatic considering the breakthroughs that have occurred over the millennia, as reviewed by Tarantola [2] and which appear to remain somewhat complicated in practice or ignored in diverse and disparate regions, such as across Asia and in the Caribbean, as reviewed by Buchy et al. [3] at a continental focus, and Seetahal et al. [4] locally for Trinidad (where the unique appreciation of rabies in vampire bats was one of the first examples to be documented and investigated in the New World), respectively.

One underlying theme is paramount: the key to reducing human rabies deaths is the mass vaccination of dogs, which serve as the major global reservoir responsible for the substantial public health burden today. A much better appreciation of the human animal bond, with a focus upon pet prophylaxis as the primary strategy to overcome many public health impacts of rabies, is supported by the work of Palamar et al. [5]. All developed countries have eliminated canine-transmitted rabies. Increasingly, developing countries have also achieved this success, especially in the New World. However, even in North America, reintroduction from abroad or via wildlife is a concern, with a feasible solution for free-ranging dogs in distinct communities such as the Navajo nation, as described by Bender et al. [6]. Additionally, uncontrolled foci at affected borders remain a threat as long as rabies remains in a region, as evidenced in the account from Peru by Castillo-Neyra et al. [7]. Foci remain within Central and South America, as well as the Caribbean. In Haiti, the country most affected by rabies in the New World, Medley et al. [8] present a concept of applied risk assessments combined with a laboratory-based diagnostics focus upon protocols to ensure that exposed individuals receive the needed prophylaxis in a resource-limited environment. In the same vein in the Old World, Lechenne et al. [9] for Chad and Coetzer et al. [10] for Lesotho, discuss the utility of surveillance

and control by mass vaccination of dogs as a critical component for relief of the human rabies burden. Such focal projects there and elsewhere demonstrate the need for a pan-African approach, as championed by Pieracci et al. [11].

As described in the above communications, human rabies may be prevented and dog rabies can be eliminated. However, cross-species transmission complicates what appears to be a somewhat simple system. All warm-blooded vertebrates are susceptible to infection. Beyond dogs, meso-carnivores also act to perpetuate the disease in the Americas, Eurasia, and Africa. Perhaps uniquely among the zoonoses, vaccination against rabies can be applied to such free-ranging populations. In fact, western Europe is largely free of rabies by oral vaccination of red foxes and raccoon dogs. Similar successful programs are operative in North America against gray foxes and coyotes. Subjectively, the meso-carnivore species of greatest concern in Canada and the USA is the raccoon (*Procyon lotor*), as discussed by the following series of related papers. Kirby et al. [12] describe a system for enhanced surveillance of raccoon rabies in the eastern USA. Slate et al. [13] present the data on the use of a tiered system of suspect animals and index of activity centered upon road-killed raccoons. In concert, the use of a decentralized enhanced laboratory-surveillance system using a direct rapid immuno-histochemical test contributes not only to a highly sensitive and specific method concentrated on suspect wildlife in the USA, but also in Canada, as described for a new focus of raccoon rabies in southern Ontario, by Middel et al. [14]. Using the information from public health and wildlife rabies detection, Algeo et al. [15] formulated a model to track raccoon rabies spread over landscape corridors, as an approach to understanding its epizootiology and management from the aerial distribution of vaccine-laden baits. This method is efficient across broad areas but cannot be used easily in urban and suburban ecosystems. Hence, bait stations may prove useful in reaching these distinct raccoon populations, as described in Massachusetts and Florida by Bjorkland et al. [16] and Haley et al. [17].

Besides rabies virus, at least 15 other lyssaviruses cause this disease and more are expected for additional pathogen discovery. Cross-reactivity for all veterinary and human rabies vaccines may be limited against some of these lyssavirus species. To this effect, Kgaladi et al. describe an experimental approach to develop a panlyssavirus vaccine [18]. In addition to prevention or control concerns, relatively little is understood about the pathobiology of these diverse lyssaviruses in their various hosts, such as bats. Suu-Ire et al. describe the results from experimental infection of bats to one major lyssavirus, Lagos bat virus [19]. Similarly, when routine surveillance of wildlife is lacking, enhanced detection may be needed to augment a description of regional lyssavirus reservoirs. Virus neutralizing antibodies are one of the most critical immune effector products in vaccine-mediated immunity in all studied species, regardless of administration route, as well as in abortive infection, but the dilemma in drawing firm conclusions about absolute sero-protection dynamics among wildlife from investigations to date is summarized by Moore et al. [20]. Regarding serology, Tyem et al. utilized sero-surveillance in bat populations to fill in such gaps [21]. However, because many bats are small-bodied, limits to blood volume collection may be a liability for such surveys. Smith and Gilbert [22] describe a micro-neutralization test that can help overcome such issues for focal serological work in laboratory and field applications.

The blood–brain barrier is a formidable concern when trying to deliver certain biologics to the CNS, especially in the treatment of encephalitis. To this end, data on further technological improvement for the use of a highly attenuated rabies virus recombinant vaccine in disease prevention and potential treatment is offered by Lebrun et al. [23]. Needs for improved passive immunity via alternative methods to polyclonal immune globulins, such as monoclonal antibodies (MAb), were first described at the end of the 1970s. Since then, numerous studies have shown the utility of these products as a potential replacement for rabies immune globulin. To this effect, another example of some of the epidemiological complexities posed by bat rabies virus variants, for a broadly reactive MAb candidate is discussed by Franka et al. [24].

Finally, Warrell et al. [25] can be understood in a similar context as the issue discussion began—rabies can be prevented by rapid and appropriate postexposure prophylaxis, but retains

the title as the entity with the highest case fatality. As such, once clinical signs manifest, frustration and futility ensue for all involved. Rather than be treated as a pariah, at a very minimum, modern medicine offers palliation to the victim afflicted with this heinous affliction, as attempts for treatment continue. Towards this latter point, a challenge is presented to veterinarians at large to use their considerable time, talent and treasure to vaccinate all companion animals at risk and begin to develop safe and effective measures to treat clinical rabies as it presents in the domestic animals under their charge, given their oath and the biomedical tools at hand now, first by palliation at the very least and predictably by intervention. All veterinarians and their staff should be vaccinated, relieving a major concern from the unvaccinated physicians and nurses that care for rabies patients now. In this manner, a better collective approach will evolve for all species at hand, including *Homo sapiens*. Please recall that Pasteur, a biochemist, embarked on this controversial path from animal models to Joseph Meister and, at one time, canine vaccination was viewed as an unrealistic fantasy—it is far time the veterinary profession accepted the same responsibility towards the ‘incurable wound’ as true One Health demands.

In retrospect, the comparative number and the diversity of papers, the depth of the topics and the geographical reach of the authors from the Americas, Africa, Eurasia, and Australia in this special issue on rabies confirm the continued collective major interest in this area. This eclectic open access collection contributes to a much better understanding on the detection, prevention, control, selective elimination, and eventual therapy of this ancient zoonosis. We hope that you may enjoy digesting their content as much as we were pleased to share them with an international audience and look forward to future opportunities to broaden such success to the field at large. Optimistically, if even a single individual is inspired by a new thought in one of these manuscripts, then our objective is accomplished.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Four Thousand Years of Concepts Relating to Rabies in Animals and Humans, Its Prevention and Its Cure

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Abstract: The epitome of the One Health paradigm—and of its shortcomings—rabies has been known to humankind for at least 4000 years. We review the evolution through history of concepts leading to our current understanding of rabies in dogs and humans and its prevention, as transmitted by accessible and surviving written texts. The tools and concepts currently available to control rabies were developed at the end of the 19th Century, including the first live, attenuated vaccine ever developed for humans and the first post-exposure prophylaxis (PEP) regimen. No progress, however, has been made in etiological treatment, leaving clinicians who provide care to animals or patients with symptomatic rabies as powerless today as their colleagues in Mesopotamia, 40 centuries ago. Rabies remains to date the most lethal infectious disease known to humans. Widespread access to timely, effective, and affordable PEP in rural areas of developing countries is urgently needed.

Keywords: rabies; vaccine; history; One Health; post-exposure prophylaxis; Galtier; Roux; Pasteur; Semple; dog

Preamble

Rabies is an ancient and much-feared disease. Over the centuries, many different authors—clinicians, veterinarians, surgeons, pharmacists but also writers, philosophers, and poets—have mentioned rabies in their writings. The sequence of research and surviving writings on rabies described below is far from exhaustive. Rather, it aims to identify the work of those who made remarkable contributions to the current stage of knowledge on dog-mediated rabies, its cause and its prevention, control or management. Sources that conducted research on rabies but proposed alternate views of causation now considered misleading (such as spontaneous generation) have purposefully been left aside. Furthermore, no further potential sources from Ancient China, India, pre-Columbian America, or Africa could be identified or accessed.

1. Rabies in Sumerian and Akkadian Civilizations

Humans have lived alongside domesticated dogs for 14,000 years at least, with estimates reaching back to 32,000 years [1,2]. They have also long been familiar with their diseases, which became more prevalent as populations and their animals congregated in the cities that arose in Mesopotamia [3–6]. Two cuneiform tablets (Figure 1) discovered at Tell Abū Harmal, Baghdad Governorate, Iraq in 1945 and 1947, recount the Laws of Eshnunna, a Sumerian and later Akkadian city-state located in present Tell Asmar, Iraq [7]. This city was most prominent during the Isin-Larsa period, ca. 1950–1850 BCE and the tablet is dated ca. 1770 BCE [8]. Distinct copies of another source date back to ca. 1930 BCE. These describe Sumerian rules and regulations attesting to the fact that a causal link between the bite of a rabid animal and a human death from rabies was well recognized almost 4000 years ago [9]:



“If a dog becomes rabid and the ward authority makes that known to its owner, but he does not watch over his dog so that it bites a man and causes his death, the owner of the dog shall pay forty shekels of silver; if it bites a slave and causes his death, he shall pay fifteen shekels of silver.”

Figure 1. Excerpts from the Sumerian Laws of Eshnunna, Northern Babylonia ca. 1930 BCE. (a) Tablets of the Laws of Eshnunna; (b) One possible translation of Paragraphs 56–57 of the Laws of Eshnunna (A iv 20–24) [10]. Another possible translation speaks of a dog becoming “furious” or “vicious” [8,9]. Even 15 shekels was a considerable sum: The Hammurabi code mentions the cost of a boat of sixty “gur” at two shekels. (Source: <http://legacy.fordham.edu/halsall/ancient/hamcode.asp>). Acknowledgement: Dr. Mark Weeden, Lecturer in Ancient Near Eastern Studies, School of Oriental and African Studies, London, UK.

At least five old Mesopotamian “dog incantations” (ca. 1900–1600 BCE) such as the one below (Figure 2) clearly reflect the notion of rabies being caused by something present in the saliva of the afflicted animal, akin to the poison transmitted by a snakebite or scorpion sting [9–12]. An herb seems to have been used after a dog bite and the biting dog’s movement was restricted [12]. Dogs were thought more likely to become rabid when a lunar eclipse occurred at year’s end [9].



*“Its [seed] coagulates on its [dogs’] teeth.
Where it has bitten it has left its [consequence].”*

Figure 2. “Dog incantation”, ca. 1900–1600 BCE (a) Tablet; (b) Translation, adapted from [11].

Finally, clay tablets (Figure 3) unearthed by H.V. Hilprecht in 1889 at the Nippur site (3rd dynasty of Ur III, 21st- 20th-century BCE) of what is now Nuffar in Iraq display Akkadian incantations, to which healers resorted when medicine failed [9,13,14]. This dialogue between Marduk, the God of Healing, and his father Enki was recited by priests over (thus blessed) water which was then administered orally.

These incantations are striking, marked as they are by the caveat of likely—however divine—failure, and certain death should rabies develop.

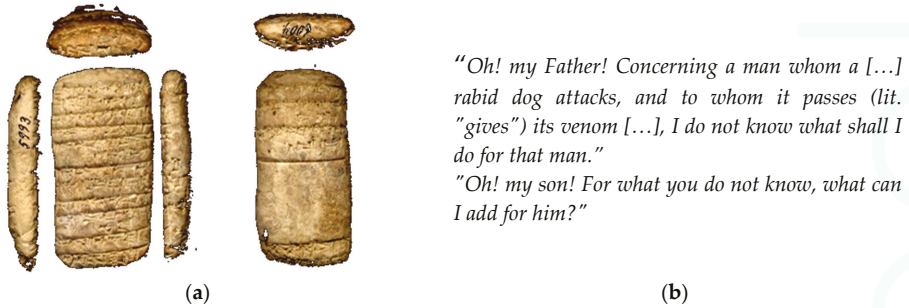


Figure 3. Ur incantations. (a) Tablets of the Ur III incantations (<http://cdli.ucla.edu/P142047>); (b) Translation. Acknowledgement: Prof. N. Veldhuis, Professor of Assyriology, University of California, Berkeley, CA, USA.

Just like Yama, a Hindu god of death, the Babylonian Goddess Gula, patroness of doctors and a healing deity, was represented in the 14th–7thC BCE with a dog at her feet [15,16] (Figure 4). In one ancient tale, a Nippur man bitten by a dog, self-referred for treatment to a temple in Isin, the city of Gula [9]. As ancient deities of the Near East were shown mounting or otherwise dominating animals to demonstrate their power, it can be hypothesized that this association represented dogs both positively (the dog as a protector) and negatively (the dog as a source of danger, including rabies) (Prof. T. Ornan, personal communication, 11 December 2015).



Figure 4. Goddess Gula represented on her throne, a dog at her feet on a *kudurru* of Nebuchadnezzar I (12th Century, BCE) [16]. Acknowledgement: Prof. Tallay Ornan, Hebrew University of Jerusalem, Department of Archaeology & the Ancient Near East Department.

Although this remains disputed [17], the origin of “rabias”, the Latin word for rabies, may originate from “rabhas” or “rabhasa” (रभस) in Sanskrit (<http://www.webcitation.org/6os2XRrN8>), perhaps crossing Indo-European cultures and centuries [18]. Indeed, rabies is mentioned in many ancient texts, from the Vedic period (in ancient India ca. 1750–500 BCE) [19–21], to ancient China [22–25], Egypt [26] and the Middle East [27] as well as Greece and Rome [19,28]. Attempts at prevention or treatment of clinical rabies, however, remained faith-based, magical or otherwise exotic [19,29–31].

2. Rabies in Classical Antiquity

Aristotle, Hippocrates, Pliny, Ovid, Cicero... a great many texts by physicians and other authors of classical antiquity attest to a progressively improved comprehension of rabies. They—and especially Caelius Aurelianus, who also wrote an early description of palliative care in rabies patients [32]—provide accurate and detailed descriptions of symptoms, whether in dogs or in humans [19,28,31,33,34]. Galen noted the absence of symptoms in bite victims before the onset of rabies [34]. Both Dioscorides (ca. 4–90 CE) and Philomenos (1stC CE) discuss a latency period of varying duration after an infective bite, generally lasting six weeks but sometimes lasting up to several years [32]. In his “Emergency Formulas to Keep up One’s Sleeves” (Zhǒu Hòu Jiù Zú Fān, 肘後備急方), Ge Hong (葛洪) of the Jin Dynasty (around 300 CE) also described prolonged incubation periods in humans (but unfortunately recommended the application of the biting dog’s brain tissue to the bite wound to prevent rabies) [35].

Primary prevention of rabies through the prevention of bites by suspected rabid dogs was recommended in the Persian Avesta, composed in 200–400 CE, perhaps from much more ancient texts [36]. Around 60 CE, Columella’s *De Re Rustica* described shepherds’ habit of cutting puppies’ tails when they are 40 days old, as a preventive measure against rabies in dogs should they be bitten, perhaps one of the earliest known example of One Health, or at least One Medicine, which sees disease prevention in humans as intimately linked with the health of the animals to which they are exposed [32,37–39]. Many different treatments were on offer to prevent rabies in dogs after they had been bitten [40].

Rabies prevention after a bite in humans made few advances. Similar incantations to that found at Nippur/Nuffar were spoken in Greek-speaking Egypt around the 3rdC CE [41]. Along with Aulus Cornelius Celsus in his *De Medicina* (published between 18 and 39 CE in Rome) [42,43], the only author who may have had some impact on the replication of viral inocula in wounds made by rabid animals, was Pédianus Dioscorides (ca. 40–90 A.D.), of Anazarba in Cilicia, founded by the Assyrians but a then Roman city, now in Adana Province of southern Turkey. A physician and a pharmacologist, he is said to have described rabies accurately and like Celsus, proposed cauterization of the bitten part as prevention [28]. But all attempts at treatment of clinically-declared rabies cases remained based on hopeful conjecture [44] or were denounced as unnecessarily brutal, as by Asclepiades of Bithynia in the mid-2ndC CE [45].

3. The Middle Ages

The list continues with great mediaeval practitioners of medicine and botany, in Europe and elsewhere [19,28,31,33]. Despite religious antagonism against dogs, considered unclean, and recommendations for their containment [40] the mediaeval Middle East was rife with stray dogs [26,27,46]. Works by Mohammad-e Zakariya-e Rāzi (Rhazes) [47,48], Ibn-Sīnā (Avicenna) [47,49], Moshe ben Maimon a.k.a. Mūsā ibn Maymūn (Maimonides) [27,29] and many others [32], all discuss or refer to dog-mediated rabies (Figure 5). Authors continued to accurately describe the disease in animals or humans, including the notion of paralytic rabies [40], the absence of hydrophobia in rabid dogs [40], or of a—in some cases considerable—delay [29] before symptoms onset of rabies in humans and its lamentable prognosis. No further remedy to clinically-declared rabies was identified.

At the end of the 13thC, Arnaldus de Villanova insisted on the importance of careful and thorough wound cleansing as prevention of rabies after a dog bite [50]. Bartholomew Glanville (mid-13thC) is said to have referred to a poison, “growing” and “multiplying” in bite wounds although this author found no primary source [40]. Prevention and treatment otherwise made no significant progress. Medical or surgical management delineated in Ancient Greece or Rome became increasingly tinted with religion. In Europe a miracle cure was deemed to be found at several specialized religious sites [51], such as the church of the village of Andage, renamed Saint-Hubert, where Louis I the Pious, one of Charlemagne’s sons and his successor, authorized the transfer of the eponymous saint’s thighbones in 826 CE. This abbey located near Liège, Belgium became a specialized center for rabies prevention. At the time, prevention before a bite took the form of applying a white-hot Key of

Saint Hubert to dogs so they would not contract the disease [52,53]. An example of this amulet can be seen at <http://www.webcitation.org/6os1x82Ty>. Contrary to what was practiced in other reputed sites such as San Bellino [17], near present-day Rovigo in Italy, or in Saint-Tügen’s chapel in Primelin, France, this method must have been considered too cruel or too unreliable in humans bitten by suspected rabid animals. In humans, the preferred method of rabies prevention after a bite was based on incision of the forehead and implantation of threads from the Saint’s supposedly miraculous stole, accompanied by prayers and fasting [19,25,52–54]. In spite of Ambroise Paré—who after the siege of Turin in 1536 discontinued the practice of cauterization to heal wounds [55,56]—Dioscorides’ and Celsus’ cauterization approach remained widespread in the management of rabies risks well into the 19thC [31,57]. This may be because cauterization was performed to inactivate a “poison” and perhaps also because their work was never lost to practitioners in Europe in spite of the fall of the Roman Empire [58,59]. Patients, however, found little recourse should prevention fail: at Saint-Tügen chapel, patients with declared rabies were stifled between mattresses until the beginning of the 19thC.



Figure 5. Outdoor scene with a mad dog biting a man. Folio from the ‘Kitab al-Hashaish’, an Arabic translation of the *Materia medica* by Dioscorides (ca. 40–90 C.E.) copied by Abdallah ibn al-Fadl, Baghdad, A.H.621/1224 A.D. Freer Gallery of Art and Arthur M. Sackler Gallery, Smithsonian Institution, Washington, D.C.: Purchase—Charles Lang Freer Endowment, F 1953.91.

4. After 1492: Emergence and Control

Rabies continued to concern populations and medical writers of the Renaissance. Julien Le Paulmier (1520–1588) wrote seven medical textbooks in all, one specifically on rabies [60,61].

The preventive practices at Saint-Hubert were condemned by the Sorbonne as superstitious in June 1671 [28] but remained in use in the Ardennes well into the 19thC [19,28,31,54]. The protective effect of thorough wound washing, and described anew in a publication dated 1796 cited by C. Ménécier, was by now well established among clinicians [62,63]. The converse was also true: the potentially deadly role of saliva was put to use by Polish-Lithuanian artillery general Kazimierz Siemienowicz (c. 1600–c. 1651), who in an early attempt at biological warfare, is said to have fired hollow shells containing saliva of rabid dogs in 1650 [64,65].

“Madstones”—bezoars or gallstones—thought to absorb or otherwise neutralize the agent of rabies were used extensively as amulets in mediaeval Europe and well into the 19thC by early European

settlers in North America [31,66,67]. Dog-mediated rabies circulated in Europe, in Africa and in Asia [26,31,68]. Human deaths associated with bat bites were already identified in the mid-1500s in Latin America [69]. Although there were Nahuatl (Aztec) words for rabies and rabid dogs in what is now central Mexico, canine rabies was noticeably rare if not entirely absent from Central and South America [25,31,66,69]. Dog-mediated rabies, however, seems to have circulated more intensely and widely in both the Old and the New World after the landings of seafaring European conquerors and their dogs [19,25,68,70,71]. The 18thC was marked by intense rabies epizootics in the Americas and by the emergence of rabies on many islands of the Caribbean and the Indian Ocean [25,28,68,72]. Rabies became rampant among mongooses introduced in the Caribbean to eliminate rats pillaging sugar fields [73–76]. Colonial powers increasingly documented animal and human rabies cases in southern Africa in the 18th–19th Centuries [77].

Although circulation of rabies had reportedly increased, especially in Europe, great progress was being made in the prevention of dog bites in European cities [28,40]. Regulations for keeping dogs or for the containment of domestic dogs and elimination of stray dogs were passed in a city (Utrecht, Netherlands) in 1446 [78], in a Dutch province (Friesland) in 1714 [61], and in a country (Prussia) in 1787 [72,79,80]. A similar approach led to the successful elimination of dog-mediated rabies from Denmark, Norway and Sweden by 1826 [81]. Other long-known approaches including muzzling were implemented in other cities or territories [28,40,72]. In a 1793 communication, Samuel Bardsley proposed to quarantine local and imported dogs to “eradicate rabies from the British Isles” [40,82]. The decision to implement an international plan to control canine rabies was made at the 2nd International Veterinary Conference in Vienna on 21–27 August 1865. Cities and states legislated, integrating and applying early forms of what are now termed One Health principles [20,53].

The understanding of the physiopathology of rabies also evolved: in 1546, Girolamo Fracastoro hypothesized that rabies was transmitted by *semina* (“seeds”) present in the saliva [64,72,83,84]. Edward Topsell, translating Conrad Gessner’s work dated ca. 1555, mentions that rabies transmission is instant after the bite of a rabid dog [17]. Martin Lister added in 1698 that the risk of transmission varies according to the anatomical site of the bite [32], a notion comprehensively described by John Hunter in 1793 [85]. Joseph-Ignace Guillotin proposed in 1766 that biting dogs remain in 15-day observation to ascertain the risk of rabies transmission to a bite victim [40]. Van Swieten in 1775 declared saliva to be the source of rabies transmission and provides a clinical description in humans that remains relevant to date [86]. Hunter also spoke of many animals being, like humans, susceptible to rabies without being capable of transmitting the virus, and of that susceptibility being variable among species [85]. In 1776 and 1793, respectively, both Guillotin and Hunter proposed (dog bite) inoculation experiments to better understand the physiopathology of rabies, including in prisoners awaiting capital punishment [24,85,87]. Like Pasteur’s similar considerations plainly laid out in a letter to the Emperor of Brazil dated September 22, 1884, ten months before the post-exposure vaccination of Joseph Meister, these fortunately were never put into effect [87,88].

The understanding of post-bite rabies prevention in animals or in humans, however, still made no progress. Published on 17 June 1684, the first edition of *Medicina Curiosa*, the first English-language journal wholly dedicated to medicine, describes post-exposure prevention failure in a suspected human case of rabies acquired from a cat [89]. “Treatment” after a bite remained faith-based [90] or otherwise fanciful, based for example on applying hair of the biting dog (“hair of the dog”) to the wound [28,66] or omelets flavored with “dog-rose root” (*Rosa canina* or cynorrhodon, as already suggested by Pliny the Elder in the 1stC CE) [91–95]. The same was true outside Europe [96]. Suggested therapies—some even based on homeopathic approaches—were rightly criticized as ineffective [97]. The fact that rabies is not transmitted in all cases even after the bite of an evidently rabid dog or wolf contributed to the illusion that each of the many preventive “treatments” had been effective.

These are all too easily disparaged as ludicrous recommendations made by self-assured and pompous clinicians, steeped from old-wives’ remedies. They are, however, sure signs of desperate and all-out efforts by health providers of the time to save their patients from what to this day remains

an intractable disease. Vigorous approaches continued to be used well into the mid-19thC: In 1830s London, children bitten by potentially rabid dogs still underwent surgery or cauterization of the wound [57] (still discussed by Babes in 1912 [72]). Patients with clinically declared rabies were plunged into cold water or hot oil as recommended by Celsus [31,86], or were later euthanized by being stifled between mattresses or made to bleed to death [17,90,98,99].

5. Pasteur and His Time

Around the turn of the 19thC, the scientific approach improved the understanding of the physiopathology and clinical epidemiology of rabies, which was remarkably summarized by Samuel Cooper in 1823 [100].

Much experimental work was done on the transmission of rabies [26,101–103]—and its prevention through the amputation (Helmann, cited in [72]) or immunization of animals [28,72,102,104]. In 1804 in Jena (in present-day Germany), Georg Zinke transmitted rabies experimentally (without a bite) by applying the saliva of rabid dogs to animals' tissues [28,31,101–103,105]. The same was demonstrated in 1813 by Hugo Altgraf zu Salm-Reifferscheidt [106] and prior to 1814 by François Magendie and Gilbert Breschet, this time using saliva from a human rabies patient [107–109]. In 1805 in Turin, Francesco Rossi reported having experimentally transmitted rabies to dogs by inserting sciatic nerve segments of rabid cats into a fresh wound [110]. Clinicians progressively identified the seat of rabies infection in the midbrain [28,102] and nerve ending density was positively correlated with risks of infection and migration [72,111].

In the struggle pitting the microbial theory against spontaneous generation, subsequent experiments provided solid scientific evidence to support the long-suspected transmission of rabies by “filterable” infectious agents present in the saliva [101,102,104]: Magendie in 1842 suspected that the agent was not a poison but a “virus” capable of multiplying and developing in the host [112,113]. Magendie, then Casimir Davaine in 1872, experimented on virulence, increased by serial passage (but these were with septicemia and anthrax bacteria, not with viruses) [114–116]. In 1880, Edmond Nocard succeeded in separating saliva into two components, one non-infective and the other infective [117]. These agents were now considered to progressively ascend from the infected wound to the brain not through the blood but through the nerves—as initially hypothesized in 1879 but not established by Paul-Henri Duboué [118]—before diffusing centrifugally [31,72,102,112].

Resorting to nerve section as a means of prevention had been contemplated by George Hicks in 1807 [119]. Duboué—who communicated his findings to Louis Pasteur on 12 January 1881 [117]—also postulated that the rabies “virus” could be destroyed *in situ* or prevented from reaching the medulla oblongata [111]. This paved the way for the advent of post-exposure prophylaxis, based on the notion of taking advantage of the latency period and rapidly building the patient's immunity through timely and adequate vaccination [102].

Variolation—the use of dried-out scabs containing attenuated smallpox virus to directly immunize against and prevent more severe smallpox—had been performed by intranasal insufflation in China since the 10thC, and inoculation was later extensively used in the Ottoman Empire [120,121]. This hazardous procedure was described by Emanuele Timoni in 1714 and subsequently experimented by Hans Sloane in English prisoners in 1722, after being championed by Mary Wortley Montagu [120–126]. Vaccination—the inoculation of virus causing much milder cowpox—to provide cross-immunization against smallpox had been pioneered by Benjamin Jesty in 1774, Peter Plett in 1790–1792 and Edward Jenner in 1796, perhaps based on John Fewster's earlier work [121,127,128]. Putting John Hunter's recommendations into practice, Eusebio Valli, an Italian physician, claimed to have carried out experimental infections and successfully immunized dogs by injecting the saliva of other dogs after submitting it to gastric juices of frogs in 1799. He claimed to have inoculated this mixture to at least two people in Pisa bitten by a suspected rabid dog and who did not contract rabies [24,129,130]. If confirmed, this would make Valli the initiator of the first attenuated vaccine and rabies vaccine, although the small numbers discussed and the absence of laboratory confirmation would not prove

preventive effectiveness. Valli in 1816 made a fatal attempt at self-inoculation, not with rabies virus but with yellow fever, a few days after landing in Cuba to assist in an epidemic [26,131–133]. Although this author was unable to access original sources, Apollinaire Bouchardat, a pharmacist of the Veterinary Faculty in Lyons, is cited as having postulated in the 1850s that dogs could be immunized against rabies as a public health measure [134]. Available sources from 1882–1884 show Bouchardat discussing vaccination against infectious diseases, citing Pasteur's work. In 1879, at the Veterinary school also in Lyons, rabies pioneer Pierre-Victor Galtier inoculated rabies to a rabbit through cutaneous injection, administered rabid dog saliva intravenously to a sheep which did not contract rabies but became immunized, theorized post-exposure prophylaxis and began experimenting on vaccination of dogs [102,135–144]. Henry Toussaint—another veterinarian—conducted research in Lyons on heat- and subsequently carbolic acid-attenuated anthrax vaccine in 1880 [144,145]. Paul Gibier from the Faculty of Medicine and the Muséum d'Histoire Naturelle de Paris, showed in 1883–1884 that the rabies virus lost virulence after dessication and that this approach could be used in humans [146,147].

It is in this already extremely rich and advanced research context that Louis Pasteur and his colleagues at the Ecole Normale Supérieure in Paris began to apply their systematic, rigorous and data-driven scientific methods to the study of rabies in December of 1880 [92,118,146]. Pasteur and his team had already developed an effective attenuated fowl cholera vaccine [148], were working on an attenuated anthrax vaccine and strove to apply their techniques to rabies—a much-feared and highly symbolic disease, albeit known to be controllable by veterinary measures alone [117,149]. An experimental model of rabies was developed by Paul Emilio (Emile) Roux in dogs inoculated after trepanation, and later in the noticeably more manageable rabbit [92,102]. A “fixed”, adapted, rabies virus strain of “exalted virulence” with shorter incubation times and unailing transmission could then be selected through successive passage in the rabbit, thereby paving the way for an experimental and methodical approach. After discussing it in 1881 [150], Pasteur and his team endeavored in 1882 to develop a canine “vaccine” (thus named in honor of Jenner), using after 1884 the desiccation technique also developed by Emile Roux to attenuate this live, highly virulent virus [151–154]. Rabies virus attenuation was first validated by experiments which Pasteur and his team reported in 1884, documenting survival of dogs vaccinated by live, attenuated vaccine before viral challenge. The prototypal vaccine against rabies was first used as salvage therapy in humans presenting signs of declared clinical rabies, with rapid documented failure in at least one instance: that of the child Antoinette Poughon in late June 1885 [92,155]. The vaccine, however, was to meet resounding success in patients exposed to rabies virus but with yet no signs of declared infection.

History remembers a 9-year-old schoolboy, Joseph Meister (Figure 6), attacked and bitten 14 times by Mr. Théodore Vonné's dog while on an errand in Maisongoutte (Meissengott), in then German-occupied Alsace, on 4 July 1885 [152]. Joseph Meister suffered deep bites to the right hand and to the thighs and leg. The owner of the dog, Mr. Théodore Vonné (or Vone) also received one bite to the arm by the same dog before it was shot by the police; the bite being delivered through cloth (untorn) and leaving no wound, Mr. Vonné received no prophylaxis and survived [72,154,156,157]. Dr. Eugène Weber, a local medical doctor with a practice in nearby Villé, made a call to the Meister home that evening and cleansed the wound thoroughly with carbolic acid, 12 h after the attack [158]. As he waited in a café for the coach to return home, Vonné spoke of the event with clients and was told that Pasteur had developed prevention against rabies [158]. He went back to the Meister home to inform the family and Meister, accompanied by his mother and Vonné left the next morning by train and arrived in Paris on 6 July. Although a medical doctor, Emile Roux did not inject the vaccine into Meister. This may be because he was not regularly working in the laboratory at the time or had not practiced medicine for too long, but published sources agree it clearly was because Roux had unequivocally stated his concern that the rabies vaccination procedure developed in dogs was insufficiently tested and too risky to be used in humans [92,131,159,160]. Jacques-Joseph Grancher therefore administered subcutaneously the first doses of live attenuated rabies vaccine on 6 July 1885, at 8:00 PM in the presence of Louis Pasteur—who, as a chemist, was not authorized to perform injections—and Alfred

Vulpian. The first injection was derived from the chord of an inoculated rabbit which died of rabies on 21 June (15 days earlier) [92]. Over the 10 following days, Joseph Meister received 12 additional doses of attenuated and progressively more virulent virus to quickly generate an immune response, in an attempt to beat the virus in a deadly race against time [19,33,72]. Meister survived.



Figure 6. Joseph Meister in 1885, the first human to have received Pasteur's live, attenuated rabies vaccine on July 6, 1885 (© Institut Pasteur-Musée Pasteur).

This successful attempt was repeated in late October 1885 in a second case, that of a 15-year-old shepherd, Jean-Baptiste Jupille from Villers-Farlay, Jura, who sustained on October 14 a deep bite to the left and right hands after an attack by a furious dog [92,152]. Jupille was referred to Pasteur by the town mayor and received rabies post-exposure prophylaxis (PEP) in Paris from 20 to 30 October, 1885. Following Grancher's accidental exposure to the attenuated vaccine during Jupille's PEP, Adrien Loir and Eugène Viala became the two first humans to receive *pre*-exposure rabies vaccination [134]. Having become a laboratory assistant in the Pasteur team, Meister was also the first to receive rabies vaccine boosters when he underwent a—reportedly less well-tolerated—second PEP in 1890 after being bitten by a guinea pig with experimental rabies (M-T. Meister, personal communication, 16 May 2016).

For the very first time since its first recorded description 3800 years earlier, and despite some failures due mostly to delayed referral [92,154], clinicians now had a proven and effective means of rabies prevention in humans. This led to Louis Pasteur's laboratory at École Normale Supérieure, Paris routinely offering PEP services. Around one year after the first PEP, L. Pasteur in August 1886 reported 3 (0.2%) deaths (whether the case of Louise Pelletier is included among these deaths is unclear) among 1235 PEP recipients [92], while another source speaks of 21 (1.0%) deaths among 1986 recipients (including one from Bombay, India) by 22 August of that same year [161]. In 1887, Vulpian documented 12 (0.7%) deaths among 1726 PEP recipients, for an expected number of approximately 264 (15.3%) rabies deaths if PEP had not been administered [162].

The rabbit cord used in the Pasteur vaccination protocol was known to preserve its virulence despite preservation in carbolic acid [152]. It was, however, not stabilized and therefore not usable outside Paris unless "transported" by/in inoculated rabbits. Patients therefore had to travel to access PEP, in some cases across continents or oceans [163,164]. After PEP spared the lives of 16 of 19 Russian patients who came to Paris from Smolensk after being attacked by a rabid wolf [165], Elie Metchnikoff was named director of the first center established specifically to produce rabies vaccine (which benefited from Louis Pasteur's support) and implemented the "Pasteur treatment" in

Odessa in June 1886, [166–168]. The not-for-profit, non-governmental Institut Pasteur Foundation was incorporated in France by a decree on 4 June 1887. The Institut Pasteur itself was built and inaugurated on 14 November 1888, after an unprecedented national and international movement and fundraising campaign to further disseminate the technique and to pursue research [19,118,163].

6. Modern Developments

Over the decades that followed the development of PEP by Pasteur and his team, many rabies prevention centers or “Pasteur institutes”—some affiliated with the Institut Pasteur in Paris, most not [167]—appeared across the Old and the New World. In 1909 there were 75 such centers worldwide, including in then Indochina [72,169]. These centers cultured *in vivo* then attenuated highly virulent rabies virus (RABV) locally. In Saigon, animal bite victims received PEP as early as 1891, becoming the first to receive rabies PEP in Asia, Africa or Latin America (Figure 7) [170–174]. This was facilitated by RABV preservation techniques in glycerin, also developed by Emile Roux and Albert Calmette [30,118], which no longer made uninterrupted sequences of RABV inoculation to successive unfortunate rabbits every ten days a requirement to preserve live virus.



Figure 7. Albert Calmette and the first two patients to receive rabies PEP in Asia (excluding the Russian Empire), Africa or Latin America, 18 April 1891. The handwritten legend indicates that these were Malay children referred from Singapore (© Institut Pasteur-Musée Pasteur).

Post-exposure prophylaxis biologicals and procedures were improved in the ensuing decades. The rabies vaccine was further refined by Emile Roux [170,175], Victor Babes [72], Follen Cabot [176], Claudio Fermi, Endre Högyes [177] and especially David Semple [177–179]. Babes and M. Lepp in 1889 first described immunity as a correlate of vaccine response and protection, discussed inactivated rabies vaccines and experimentally demonstrated protection of animals by antiserum in 1891 [72,177,180]. Solutions of attenuated virus mixed with serum immunoglobulin were experimented at the Pasteur Institute as early as 1902 [169]. Rabies antiserum was administered in humans to interrupt replication of the virus in bite wounds by Fermi in 1911 and the use of rabies-specific immunoglobulin was generalized in the 1950s [31,181–186]. Monoclonal antibodies (produced either in animals or by yeasts or plants) are now being developed to replace unaffordable equine—let alone human—rabies immunoglobulin (RIG), so far with mixed but promising results [187–194].

Semple’s killed-virus vaccine, developed in 1911 at the Pasteur Institute in Kasauli, India, using sheep brain tissue, remained the most used worldwide into the 2000s. Although the vaccine had limited immunogenicity and required a tedious protocol (and was painful, as experienced first-hand by the author in West Africa as a child in the 1970s) it was affordable and for decades saved countless

human lives, especially in the developing world. The League of Nations' health organization's bulletin reported 115,859 PEP worldwide recipients in 1932-May 1934 among whom 439 (0.4%) were considered to have died of rabies [195]. After initial attempts at the Institut Pasteur in 1913 [196], the rabies virus was successfully cultured *in vitro* through several passages in 1936 [197]. In the 1960s, harvests of RABV grown in tissue cultures became increasingly pure [198] and normative methods were developed to standardize the potency of the various vaccines [31,199,200]. Vaccines were developed on suckling mouse brains [201] or on duck or chicken embryos [202], until the advent of new, highly antigenic, better-tolerated cell-culture vaccines [33,203–206]. This allowed for the tedious Pasteur then Semple protocols to be progressively replaced by the shorter Essen and Zagreb protocols [207]. An oral vaccine was developed for wildlife in 1971 [208]. Through canine population regulations and control, rabies was eliminated from cities in the industrialized world and elsewhere, including Shanghai in 1949 and Malaya in the early 1950s [25,209].

Research on the rabies virus itself made rapid advances. In 1903, Adelchi Negri described the first RABV-neuron interaction and Lina Luzzani-Negri described its diagnostic value in infection with "street" rabies virus [210–212]. The rabies virus itself was first observed by electron microscope in the early 1960s [213–215]. The molecules produced by RABV (transcriptional mapping) were described in 1978 [216] and the viral genes which code for them were sequenced in their entirety in 1988 [217]. Direct and indirect diagnostic methods were developed to reliably confirm infection and antibody protection [200,218,219].

These advances led to the validation of rabies vaccine effectiveness, of shorter and dose-sparing regimens and of the equivalence of the intradermal vaccination route [220–222]. It also enabled the identification of nonfatal cases of RABV infection in animals [223–226] and in humans [227–229]. Human survivors of clinical rabies were first documented, mostly in the New World following bat exposure [230–236].

Whether or not these survived thanks to attempted treatment remains hotly debated [33,237–239]. Despite our dramatic advancements in the knowledge and prevention of rabies, and with a handful of exceptions to date [229], all documented patients with clinically-declared rabies have died within a few hours or days. Efforts to test some traditional medicines, in Ethiopia for example, have failed [240]. Antivirals are currently being explored as a therapeutic resource, so far with little success [241].

7. Conclusions

Our understanding of the mechanisms and primary and secondary prevention of rabies in animals and in humans has profoundly changed since the Laws of Eshnunna were introduced by one of the earliest known civilizations. Yet despite this, and great progress in symptomatic management of encephalitic patients, clinicians caring for animals or patients with symptomatic rabies remain as powerless today as they were 4000 years ago. Rabies remains today the most lethal disease known to man and this author is not aware of any other disease for which—once the disease is declared—modern medicine has offered no tangible improvement. We wait in hope for researchers to identify antiviral agents capable of controlling progression of clinically-declared rabies.

Rabies became a neglected disease when it was eliminated from Europe and North America. It is emerging in some island territories and remains uncontrolled in most of the developing world, where surveillance of dog bites, rabies exposures (syndromic or laboratory-confirmed) or rabies deaths, is poor [242,243]. The prevention of human rabies deaths in the 21stC still rests on tools and strategies developed in the 19thC: Effective primary prevention of animal bites and responsible dog ownership as delineated by Fleming (in 1872) [28]; canine vaccination as proposed by H. Bouley (in 1884) [72] and timely and effective rabies post-exposure prophylaxis (developed by Pasteur and his team and first administered in 1885).

An estimated total of 59,000 humans die of rabies each year, more than twice the estimated 28,600 deaths caused by the tragic 2014–2016 Ebola outbreak in West Africa [244,245]. The World Health Organization, the World Organization for Animal Health and the Food and Agriculture

Organization of the United Nations are currently spearheading an effort to eliminate dog-transmitted rabies worldwide by 2030 [246]. While we strive for all dogs to be vaccinated, a major effort is urgently needed to make the time-proven and well-tolerated vaccine (and immunoglobulin) geographically and financially accessible in a timely way to those people who remain the most vulnerable to rabies: the rural populations of developing countries [247,248].

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Perspective

Childhood Rabies Deaths and the Rule of Rescue

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Abstract: Every childhood rabies death is potentially preventable. The vaccine that prevents rabies disease has a formidable safety and efficacy track record. Rabies vaccination of dogs and timely pre- and post-exposure vaccine administration are life-saving and cost-effective, and yet nearly 60,000 people, mainly children, die unnecessarily each year. Poor performance by many veterinary and public health systems, and neglect by complicit authorities is in stark contravention of the Convention on the Rights of the Child. The ethical principle of beneficence and the rule of rescue demand re-energised commitment to eradicating childhood rabies deaths.

Keywords: rabies; child; death; vaccination; immunization; ethics; rights; beneficence; rescue

1. Introduction

The burden of human rabies falls heavily on young children in developing countries with most of the approximately 59,000 annual deaths due to rabies occurring in children younger than 15 years of age in Africa and Asia with virus transmission largely from rabid dogs [1,2]. Almost every one of these deaths reflects a failure of the public health and veterinary systems as the vaccine that is available is highly effective in preventing disease in dogs and humans [3]. However, in addition to the primary failure of not reaching all dogs with vaccine by weak veterinary programs, and the secondary failure in not providing ready access to post-exposure treatment with rabies vaccine and immunoglobulin by deficient health systems, either through inability to secure stock or to follow guidelines, there is a potential third failure, that of not administering pre-emptive protective vaccination to children in high-risk rabies-endemic areas where the perpetual weakness of the public health and veterinary systems in delivering education and vaccination is well recognised [4]. A recent systematic review of the safety and immunogenicity of pre-exposure rabies prophylaxis found that it is safe, effective and should be considered in areas “where access to post-exposure prophylaxis is limited or delayed, where the risk of exposure is high and may go unrecognised, and where controlling rabies in the animal reservoir is difficult” [5]. However, only two countries, Peru and the Philippines, have thus far implemented this strategy.

In addition, accelerated regimens (all vaccine doses administered within a week) and administration by intradermal rather than intramuscular route were found to be highly immunoprotective. Thus we have at our disposal effective tools to eliminate childhood rabies deaths but to date the will to fully embrace this challenge in many endemic countries and the international community has been lacking.

2. Convention of the Rights of the Child

It is timely to remind ourselves of our shared obligations under the Convention of the Rights of the Child (<http://www.unicef.org/crc/>). This international legally binding instrument enjoys remarkable acceptance with all United Nations states, excepting Somalia and the United States of

America, having ratified the Convention [6]. Article 6 (Survival and Development) is particularly pertinent to the childhood rabies death sentence: “Children have the right to live. Governments should ensure that children survive and develop healthily”. Thus individual governments in rabies endemic areas should not tolerate poor performance of their veterinary or public health sectors in achieving high dog rabies vaccination or ensuring the availability of post-exposure rabies vaccination, respectively. Each rabies death should prompt an enquiry to identify preventable system failures. Confidential enquiries into maternal deaths, infant deaths, peri-operative deaths, and malaria and cholera deaths have proven immensely valuable for correcting system weaknesses. Identifying the system root causes of deaths would allow targeting of resources to limit the likelihood of recurrence. Governments may argue that this will come at an opportunity cost as there are many competing priorities for finances. Sustainable development requires investment in many areas including clean water, adequate sanitation, quality education and secure food supply. However, immunisation is a wonderful public good, in that a single intervention can provide long-lived benefits to the individual. Further, dog rabies vaccination programs are a basic indicator of the coverage and quality of veterinary public health initiatives. Recent case studies from Bhutan and Tanzania demonstrated the effectiveness of programs implemented to achieve at least 70% canine coverage [7].

A health service that does not effectively reach communities with education messages about stray canine and wild life avoidance, and appropriate first aid measures, or cannot provide a reliable timely supply of potent rabies vaccine, will likely be failing to provide other primary health care services. Both dog rabies vaccination and human pre-exposure (in certain high risk settings) and post-exposure rabies vaccination have been shown to be cost-effective in developing countries, and for canine rabies vaccination, cost-saving (if the estimated \$2.7 billion wasted with post-exposure prophylaxis annually is included), so it is time for individual governments to demonstrate appropriate accountability and deliver on their human rights commitment [5,7].

A true commitment to the rights of children demands that every country’s government implements carefully monitored strategies for guaranteeing all children protection against rabies through equal access to effective vaccines based on local rabies epidemiology [8].

But impoverished endemic countries should not shoulder this responsibility alone. Article 24 (Health and Health Services) states: “Children have the right to good quality health care—the best health care possible—to safe drinking water, nutritious food, a clean and safe environment, and information to help them stay healthy. Rich countries should help poorer countries achieve this”. This places a specific legal obligation on developed countries to support less-developed countries to ensure social justice in the delivery of preventive and curative health care, including rabies prevention. These developed countries have already experienced the benefit of eradication of canine rabies through well organized and funded veterinary vaccination campaigns. GAVI, the global alliance for vaccines and immunization, has provided a dependable and accountable mechanism for raising and administering funds from donors and wealthy countries for critical vaccine introductions and immunisation program strengthening in developing countries. It is time that GAVI carefully reviewed the evidence supporting expanding access to rabies vaccine and immunoglobulin, including the childhood mortality burden, the cost-effectiveness data and the opportunity for strengthening immunisation programs.

3. Principle of Beneficence

This legally binding prerogative for eliminating childhood rabies deaths is supported by important ethical considerations. The principle of beneficence is succinctly summarised by the Golden Rule: “Do unto others, as you would have them do unto you”. This ethical principle dictates that national governments and the international community have a duty of care to ensure that all children enjoy the protection offered by effective vaccines [9]. The strength of the duty of care depends on the availability of effective and affordable measures [10]. This requirement is clearly satisfied by rabies vaccines, which enjoy a proven track record if administered correctly without delay.

4. Rule of Rescue

The rule of rescue places a compelling obligation on those that are able, in this case governments and health personnel, to “rescue identifiable individuals facing avoidable death” if personal sacrifice is not excessive [11]. This duty is influenced by the urgency of the situation, the consequences of doing nothing, the feasibility of preventing serious consequences and the sacrifice required [12]. Rabies vaccination for all children who are at high risk of exposure or have been exposed to a bite or scratch from an infected animal easily meets each one of these criteria: “urgency”—delayed vaccination can result in preventable death; “consequence of doing nothing”—death is almost inevitable once clinical symptoms have occurred [2]; “feasibility of preventing serious consequences”—vaccine and immunoglobulin are highly effective and cost-effective in preventing disease; and “sacrifice required”—surely the opportunity costs of providing vaccine in these circumstances are morally defensible! [13]

5. Conclusions

The principle of Justice obligates those who are better off to assist those who are worse off and to allocate resources accordingly [14]. A global Convention considers the lives of children as precious and demands that governments ensure child health and survival. We have effective tools to rescue children from agonising preventable deaths due to lyssavirus 1. Rabies deaths in children are a true measure of our generation’s commitment to children’s rights and social justice.

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MDPI/BOOKS



Communication

The Formation of the Eastern Africa Rabies Network: A Sub-Regional Approach to Rabies Elimination

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Abstract: International rabies networks have been formed in many of the canine-rabies endemic regions around the world to create unified and directed regional approaches towards elimination. The aim of the first sub-regional Eastern Africa rabies network meeting, which included Kenya, Ethiopia, Tanzania, Rwanda, and Uganda, was to discuss how individual country strategies could be coordinated to address the unique challenges that are faced within the network. The Stepwise Approach towards Rabies Elimination and the Global Dog Rabies Elimination Pathway tool were used to stimulate discussion and planning to achieve the elimination of canine-mediated human rabies by 2030. Our analysis estimated a total dog population of 18.3 million dogs in the Eastern Africa region. The current dog vaccination coverage was estimated to be approximately 5% (915,000 dogs), with an estimated 4910 vaccinators available. Assuming that every vaccinator performs rabies vaccination, this equated to each vaccinator currently vaccinating 186 dogs per year, whilst the target would be to vaccinate 2609 dogs every year for the community to reach 70% coverage. In order to achieve the World Health Organization-recommended 70% vaccination coverage, an additional 11 million dogs need to be vaccinated each year, pointing to an average annual shortfall of \$ 23 million USD in current spending to achieve elimination by 2030 across the region. Improved vaccination efficiency within the region could be achieved by improving logistics and/or incorporating multiple vaccination methods to increase vaccinator efficiency, and could serve to reduce the financial burden associated with rabies elimination. Regional approaches to rabies control are of value, as neighboring countries can share their unique challenges while, at the same time, common approaches can be developed and resource-saving strategies can be implemented.

Keywords: rabies surveillance; rabies network; Africa; rabies elimination

1. Introduction

Rabies is a neglected disease that kills an estimated 59,000 people every year, with more than 21,000 of those deaths (36%) occurring in Africa [1,2]. As rabies is preventable, every death should be considered a public health failure. However, one human still dies from rabies every 9 minutes, with the majority of these deaths occurring in low- and middle-income countries (LMICs) in Africa and Asia [1].

In line with the Sustainable Development Goals (SDGs) [3], a preponderance of African governments seeks to emulate other continents that have drastically reduced the number of human deaths from rabies and controlled or eliminated the disease from the domestic dog, e.g., the Americas and Europe [4,5]. The vast majority of human rabies deaths are attributed to bites from rabid dogs, with children and individuals in poor communities being disproportionately affected [1,2,5,6]. Despite effective preventive measures and available post-exposure prophylaxis in humans, the most cost-effective control measure to eliminate canine-mediated human rabies remains the routine vaccination of dogs [4,7]. Across Africa, however, canine mass vaccination efforts are primarily still in the developmental stages. The global community, led by the World Health Organization (WHO), World Organisation for Animal Health (OIE), the Food and Agricultural Organization (FAO), and the Global Alliance for Rabies Control (GARC), has set a globally agreed upon goal to eliminate canine-mediated human rabies deaths by 2030 [8].

International rabies networks have been formed in many of the canine-rabies endemic regions to create unified and directed approaches towards elimination within the given regions. The Meeting of Directors of Rabies Programs in the Americas (REDIPRA) structure acted as a regional network, encompassing 27 countries in the Americas [9]. Additionally, smaller multi-national strategic planning groups were implemented to drive rabies elimination efforts; for example the North American Rabies Management Plan (NARMP) was created with a focus on wildlife rabies elimination between Canada, Mexico, and the United States [10]. In Africa, the Pan-African Rabies Control Network (PARACON), under the secretariat of GARC, was recently established as the regional network for sub-Saharan African countries [11], but smaller, community-based, sub-regional planning structures have not been implemented. For this reason, the United States Centers for Disease Control and Prevention (CDC) and GARC worked with regional leaders to establish an Eastern African rabies control planning commission. The aim of this group is to discuss how individual country strategies could be coordinated to address the unique challenges that are faced in terms of rabies control efforts within the region.

The first Eastern African regional rabies control group meeting was held from 7–9 February 2017 in Nairobi, Kenya, and was hosted collaboratively by GARC, CDC, and the Kenya Zoonotic Disease Unit (Kenya ZDU). Representatives from four Eastern African countries, along with regional and international rabies partners, attended the meeting to assess the sub-region's current rabies control strategies, develop sub-regional targets for the future, and showcase a needs assessment analysis to approximate the cost of rabies elimination within the sub-regional network. One Eastern African country was unable to attend, but provided its data the following week. All five countries' data were included in the model for projecting resource needs for canine rabies vaccination.

2. The Stepwise Approach towards Rabies Elimination

In a detailed workshop focused on the Stepwise Approach towards Rabies Elimination (SARE), and its linkage to the Rabies Blueprint (<http://rabiesblueprint.org/>), country representatives identified the current status of rabies control within their country. The SARE assessment provided countries with measurable steps to progress from canine rabies endemic to a canine rabies free status [12]. Representatives outlined these steps to prioritize short- and medium-term activities for each country

towards a dynamic and ongoing development and implementation of national rabies control plans. Common priority activities from each of the countries were compiled into a sub-regional assessment to provide a basis for the development of a comprehensive sub-regional roadmap for the network. The development of this roadmap is focused on facilitating partnerships and coordination between countries in an effort to address the transboundary nature of canine rabies.

3. Regional Business Plan and Estimated Needs for Rabies Mass Vaccination

Country representatives used the Global Dog Rabies Elimination Pathway (GDREP) model for projecting resource needs for canine rabies vaccination (e.g., vaccines, vaccinator resources, and funds) [13]. The GDREP model accounts for resources needed to achieve elimination of canine-mediated human rabies, accommodating varied inputs for cost of dog vaccination, availability of vaccine, and existing workforce available for vaccinating animals to generate outputs of estimated resources needed to achieve vaccination goals. The analysis estimated a total dog population of 18.3 million dogs, based upon the Human to Dog Ratio (HDR) method [6,14–16], for the region (Kenya, Ethiopia, Tanzania, Rwanda, and Uganda). The current dog vaccination coverage in the region was estimated to be approximately 5% (915,000 dogs), with an estimated 4910 vaccinators available to be mobilized for vaccination campaigns [1]. This is likely to be sufficient personnel to achieve 70% vaccination of dog populations in the region, based on projections made by the GDREP tool. The estimated cost of the current 2017 level of 5% vaccination coverage (915,000 dogs) was approximately \$ 3.2 million USD per year, equating to an average of \$ 3.50 USD per vaccinated dog. In order to achieve the recommended 70% vaccination coverage necessary to eliminate canine rabies [5], an additional 11 million dogs will need to be vaccinated each year, equating to an approximately \$ 23 million USD annual shortfall in current spending across the region.

The total cost of vaccinating a single dog encompasses supplies and personnel, not just the cost of the vaccine alone. Improving the efficiency of vaccination programs by improving logistics and/or incorporating multiple vaccination methods (e.g., central point vaccination, capture-vaccinate-release), could reduce overall costs [13]. Considering the range of vaccination costs reported in the GDREP [13], from \$ 1.50/dog to \$ 7.00/dog, the estimated average annual shortfall in current spending is likely between \$ 8.5 million USD and \$ 49.8 million USD. Improvements in the planning and efficiency of national canine rabies vaccination campaigns coupled with frequent refinements of national strategies will be critical to ensure that the expected shortfall is reduced and additional funds can be secured. Sharing of resources and technical experience between regional countries will help to facilitate these improvements and more efficiently identify and secure support from national and international partners.

4. Data Sharing Within the Eastern African Rabies Region

The PARACON epidemiological bulletin, a web-based surveillance database, is a useful tool for the collection, collation, analysis, and dissemination of rabies surveillance data [17]. To encourage the use of the bulletin, sub-regional country-specific data were collected from meeting participants, and collated data were summarized on a single viewable page. This 'Eastern Africa Dashboard' was made available to all of the participating countries within the network and is envisaged to encourage international collaboration and communication. With increased transparency, a stronger case can be made for transboundary control effort by the participating countries, drawing global interest and raising awareness and advocacy for rabies control within the region. It is also important to engage other Eastern African countries (Burundi and South Sudan) in the Eastern Africa rabies network to achieve a unified regional approach.

5. Conclusions

Rabies prevention should be considered a free public good globally. Despite the presence of an effective vaccine for the prevention of rabies for more than a hundred years, rabies continues to

kill thousands of people across the globe each year [1,18]. Some countries have achieved success in eliminating rabies through effective, organized, and coordinated control efforts, but many LMICs remain rabies endemic.

Across Africa, rabies control and elimination efforts are primarily still in the developmental stages. A concerted and carefully conceived approach will need to be created and undertaken to ensure that the goal of elimination by 2030 is reached. The overarching PARACON network guides African countries and provides exposure to new and innovative tools and approaches, while facilitating the development and revision of their national control strategies [11]. Although each country within the PARACON network has access to the same tools, the implementation and use of these tools will often need to be adapted to sub-regions that experience different or additional challenges to those faced by Africa as a whole. Thus, the establishment of the Eastern Africa rabies network has addressed this issue by providing a close-knit working group within the PARACON network for strategic planning and cooperation.

These tools, in conjunction with the increased transparency between the participating countries, and leveraging on existing regional economic and technical bodies promoting the One Health approach, will enable this network to develop their own sustainable roadmap towards rabies control and elimination, with a focus on addressing the transboundary nature of the disease throughout the region. It is envisaged that this roadmap will provide detailed priority activities, steps, and plans directed to the mobilization and unification of the community towards the common goal of canine-mediated human rabies elimination. For the Eastern Africa rabies network, Kenya was nominated to act as the chair on a two-year rotational basis and will be the leading country in the development of such a roadmap for presentation, review, and discussion at the next PARACON meeting (2017). By sharing the roadmap and presenting the concept of the smaller focused communities within the PARACON network, the Eastern Africa rabies network will act as a flagship for other communities with common challenges to unite and tailor the foundational basis of the roadmap to their specific needs.

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Article

Epidemiology of Rabies in Lesotho: The Importance of Routine Surveillance and Virus Characterization

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Abstract: Rabies is widespread throughout Africa and Asia, despite the fact that the control and elimination of this disease has been proven to be feasible. Lesotho, a small landlocked country surrounded by South Africa, has been known to be endemic for rabies since the 1980s but the epidemiology of the disease remains poorly understood due to limited sample submission, constrained diagnostic capabilities, and a lack of molecular epidemiological data. Considering the existing challenges experienced in Lesotho, we aimed to evaluate the direct, rapid immunohistochemical test (DRIT) as an alternative to the direct fluorescent antibody (DFA) test for rabies diagnosis in Lesotho. Towards this aim, extensive training on the implementation and interpretation of the DRIT was hosted in Lesotho in April 2016 before both tests were applied to all samples subjected to routine rabies diagnosis at the Central Veterinary Laboratory (CVL). We found agreement between the DFA and DRIT assays in 90/96 samples (93.75%). The samples that produced inconsistent results ($n = 6$) were re-tested a further two times with both assays before being subjected to a real-time qPCR to confirm the diagnosis. Additionally, a statistically significant three-fold increase in the average number of samples submitted per month was observed after the DRIT implementation started, following continuous rabies awareness initiatives amongst the animal health professionals in the country over a 12-month period ($p = 0.0279$). Partial G-L intergenetic regions of selected rabies-positive samples ($n = 21$) were amplified, sequenced, and subjected to phylogenetic analyses. Molecular epidemiological analyses, which included viruses from neighbouring provinces in South Africa, suggested that at least three independent rabies cycles within Lesotho were implicated in instances of cross-border transmission. This study has evaluated alternative methods for diagnosing and improving rabies surveillance in Lesotho, as well as providing new information that would be of importance in the planning of future disease intervention campaigns, not only in Lesotho, but also in neighbouring South Africa.

Keywords: surveillance; rabies; diagnosis; southern Africa

1. Introduction

Canine-mediated rabies, caused by rabies virus (RABV), is a neglected tropical disease that has the highest case-fatality rate of any known infectious disease, accounting for an estimated

59,000 human deaths every year globally [1]. The burden associated with the disease is highest in developing countries and is typically a scourge on the poorest people in low and middle income countries (LMICs) [1–3]. On the African continent, rabies causes an estimated 21,000 (36% of the global canine-mediated human rabies cases) deaths annually, with the number of infected animals being several magnitudes higher [1]. While the availability of post-exposure prophylaxis (PEP) prevents the onset of human rabies after an exposure, the most cost-effective control measure is the routine vaccination of the domestic dog populations [3]. Unfortunately, various social, economic, and political factors contribute to the inadequate control of canine-mediated rabies in endemic areas, with human rabies cases occurring as a result thereof [1,3,4].

Rabies was first recorded in Lesotho in the 1980s when the disease was introduced into the northeastern part of the country from the KwaZulu-Natal (KZN) province of South Africa [5]. Subsequent to the introduction, rabies spread throughout the country within two years and has remained endemic ever since [5–8]. Despite annual dog vaccination campaigns undertaken throughout the country, the control and elimination of rabies in the country is complicated by a low estimated vaccination coverage [8] and the fact that Lesotho is bordered by three rabies-endemic South African provinces, viz. the Free State (FS), Eastern Cape (EC), and KZN provinces. These provinces have historically been shown to contribute to the cross-border transmission of rabies between the two countries [5,9–11].

The transboundary spread of rabies between South Africa and Lesotho was first confirmed during a molecular epidemiological investigation of canine rabies in the FS province [9]. During that study, 13 sequences originating from Lesotho were included in the molecular epidemiological analyses. It was not only shown that the FS province and Lesotho shared the same epidemiological cycle, but historical surveillance data also indicated that canine rabies was most likely introduced into the FS province after crossing the border from Lesotho [9]. Since then, no further molecular epidemiological studies had been performed in either the FS province or Lesotho, limiting the contemporary information needed to guide disease intervention campaigns within the two countries.

One of the main limitations to the improved epidemiological understanding of rabies in Lesotho is limited surveillance data. Although rabies is a notifiable disease in Lesotho, the surveillance network has historically been constrained by a lack of samples subjected to routine rabies diagnosis at the only laboratory capable of diagnosing rabies in the country, the Central Veterinary Laboratory (CVL) in Maseru (Figure 1). The limited surveillance data and molecular epidemiological information directly contributed to the underestimated burden of the disease [8], as well as the subsequently low prioritisation and lack of rabies control activities.

In an effort to enhance the rabies diagnostic capabilities and capacity in Lesotho, we implemented the direct, rapid immunohistochemical test (DRIT) in a twinning approach to the gold standard direct fluorescent antibody (DFA) test at the CVL in Maseru. Throughout this project, the routine submission of samples was continuously encouraged amongst the animal health professionals operating within the country. The awareness activities not only facilitated the submission of samples to be tested with the DFA and DRIT assays, but also enabled us to investigate whether stimulating the members of the expert network into collecting and submitting samples would result in a statistically-significant increase in sample submission over a 12-month period. To improve molecular epidemiological information within Lesotho, a sub-set of the samples—including viruses from the neighbouring provinces of South Africa—was analysed by targeting the G-L intergenic region of RABV cDNA.

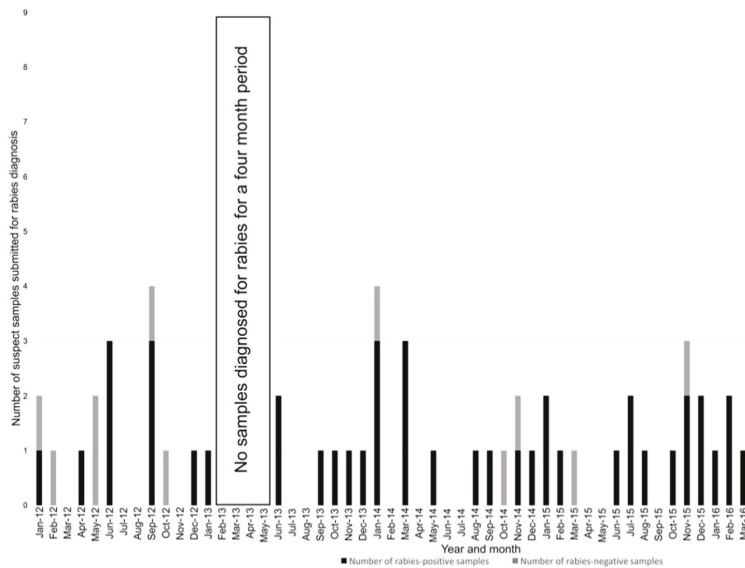


Figure 1. The number of positive and negative samples submitted per month for rabies diagnosis at the Central Veterinary Laboratory in Maseru, Lesotho (2012–2016). The number of samples subjected to rabies diagnosis per month between January 2012 and March 2017 are depicted as vertical bars. The number of rabies-positive samples are depicted as the black-filled bars, while the number of rabies-negative samples are depicted as the grey-filled bars. Months where no vertical bars are present indicate a month where no samples were subjected to rabies diagnosis. The vertical dashed line represents the start of the twelve-month study period during which the DRIT was actively promoted in the country.

2. Materials and Methods

2.1. Diagnostic Training and Sample Cohort

Before the routine implementation of the DRIT assay in Lesotho, a workshop on the implementation and interpretation of the assay was presented in April 2016 by a South African diagnostician experienced in the use and interpretation of the assay [12]. Over the course of the five-day workshop held at the CVL in Maseru, four local diagnosticians were introduced to the DRIT assay and its use in a diagnostic setting by applying the assay blindly to a cohort of archival samples ($n = 57$) that had been found to be either rabies-positive or -negative with the gold standard DFA test. The DFA test applied at the CVL in Lesotho relied on a FITC-labelled polyclonal antibody (PAb) preparation prepared by the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI, Rabies Division) prior to the samples being stored in a 50% glycerol-saline solution at ambient temperature (Table 1) [13,14]. While all of the diagnosticians were familiar with the DFA, the samples in this study were the first DRIT-diagnosed samples that the local diagnosticians had interpreted.

After the diagnostic training programme had been completed in April 2016, the DRIT and DFA assays were routinely applied concurrently to newly-submitted brain tissue samples ($n = 39$) that were collected by the trained technicians without further supervision from the DRIT trainer (Table 1). Additionally, the expert network of trained animal technicians and veterinarians (both state and private) operating within Lesotho were continuously contacted via short messages service (SMS), emails or telephonic calls and encouraged to collect samples for diagnostic confirmation within their communities. The aforementioned messages were sent on a monthly basis to the animal health

technicians responsible for each of the ten districts, who subsequently disseminated the information to all of relevant stakeholders operating in the specific resource centres. Suspect samples were subsequently collected, submitted, and diagnosed in support of the project which, in turn, increased the level of surveillance data generated for the country.

Table 1. Neuronal tissue sample cohort from Lesotho depicting the initial diagnostic results from the CVL in Maseru, Lesotho, the diagnostic discrepancies and their independent molecular confirmation at the laboratory in South Africa.

Sample Number	Species	Town	DRIT Result	DFA Result	Real-Time PCR Result	
1 *	15/09/11	Bovine	Maseru	Positive	Positive	---
2 *	254/09/11	Bovine	Mokhotlong	Negative	Negative	---
3 *	11/01/12	Bovine	Berea	Positive	Positive	---
4 *	24/01/12	Bovine	Maseru	Negative	Negative	---
5 *	04/02/12	Bovine	Maseru	Negative	Negative	---
6 *	106/04/12	Bovine	Maseru	Negative	Negative	---
7 *	113/05/12	Bovine	Maseru	Negative	Negative	---
8 *	123/05/12	Canine	Maseru	Negative	Negative	---
9 *	14/06/12	Canine	Maseru	Positive	Positive	---
10 *#	145/06/12	Bovine	Maseru	Positive	Positive	---
11 *#	136/06/12	Canine	Maseru	Positive	Positive	---
12 *	12/09/12	Bovine	Maseru	Negative	Negative	---
13 *#	151/09/12	Bovine	Quthing	Positive	Positive	---
14 *#	190/09/12	Canine	Maseru	Positive	Positive	---
15 *#	201/09/12	Caprine	Maseru	Positive	Positive	---
16 *	211/10/12	Canine	Maseru	Positive	Positive	---
17 *	276/12/12	Bovine	Maseru	Positive	Positive	---
18 *#	07/01/13	Bovine	Mokhotlong	Positive	Positive	---
19 *#	136/06/13	Bovine	Maseru	Positive	Positive	---
20 *#	137/06/13	Canine	Qacha's Nek	Positive	Positive	---
21 *#	164/09/13	Canine	Maseru	Positive	Positive	---
22 *	10/10/13	Bovine	Berea	Positive	Positive	---
23 *	05/11/13	Bovine	Berea	Positive	Positive	---
24 *#	194/12/13	Canine	Maseru	Positive	Positive	---
25 *#	08/01/14	Bovine	Maseru	Positive	Positive	---
26 *	10/01/14	Bovine	Maseru	Negative	Negative	---
27 *#	13/01/14	Bovine	Maseru	Positive	Positive	---
28 *	23/01/14	Canine	Maseru	Positive	Positive	---
29 *	20/03/14	Bovine	Maseru	Positive	Positive	---
30 *#	45a/03/14	Bovine	Maseru	Positive	Positive	---
31 *#	45b/03/14	Bovine	Maseru	Positive	Positive	---
32 *#	60/05/14	Canine	Qacha's Nek	Positive	Positive	---
33 *	26/08/14	Bovine	Maseru	Positive	Positive	---
34 *#	99/09/14	Bovine	Maseru	Positive	Positive	---
35 *	21/10/14	Canine	Maseru	Negative	Negative	---
36 *	192/11/14	Bovine	Berea	Negative	Negative	---
37 *	193/11/14	Canine	Qacha's Nek	Positive	Positive	---
38 *	10/12/14	Equine	Berea	Negative °	Positive °	Negative
39 *	07/01/15	Canine	Maseru	Positive	Positive	---
40 *#	30/01/15	Ovine	Maseru	Positive	Positive	---
41 *#	17/02/15	Canine	Maseru	Positive	Positive	---
42 *	18/03/15	Feline	Maseru	Negative	Negative	---
43 *	18/06/15	Ovine	Qacha's Nek	Positive	Positive	---
44 *	150/11/15	Canine	Maseru	Positive	Positive	---
45 *#	21/07/15	Bovine	Maseru	Positive	Positive	---
46 *	23/07/15	Bovine	Maseru	Positive	Positive	---
47 *#	24/08/15	Canine	Maseru	Positive	Positive	---
48 *	06/10/15	Canine	Maseru	Positive	Positive	---
49 *	10/11/15	Caprine	Maseru	Positive	Positive	---
50 *	26/11/15	Bovine	Mohales Hoek	Negative °	Positive °	Negative
51 *	149/11/15	Caprine	Maseru	Positive	Positive	---
52 *	29/12/15	Canine	Maseru	Negative °	Positive °	Negative
53 *	161/12/15	Canine	Berea	Positive	Positive	---
54 *	14/01/16	Canine	Maseru	Negative °	Positive °	Negative
55 *#	22/02/16	Bovine	Maseru	Positive	Positive	---
56 *	26/02/16	Bovine	Berea	Negative °	Positive °	Negative
57 *	03/03/16	Canine	Maseru	Negative °	Positive °	Negative
58 +	95/04/16	Bovine	Berea	Positive	Positive	---
59 +	100/05/2016	Canine	Maseru	Negative	Negative	---
60 +	105/05/2016	Canine	Berea	Negative	Negative	---

Table 1. Cont.

	Sample Number	Species	Town	DRIT Result	DFA Result	Real-Time PCR Result
61 +	109/05/2016	Canine	Maseru	Positive	Positive	---
62 +	110/05/2016	Canine	Berea	Positive	Positive	---
63 +	113/05/2016	Ovine	Berea	Negative	Negative	---
64 +	115/05/2016	Bovine	Berea	Positive	Positive	---
65 +	123/6/2016	Canine	Maseru	Positive	Positive	---
66 +	125/6/2016	Ovine	Maseru	Positive	Positive	---
67 +	127/06/2016	Porcine	Mohales Hoek	Negative	Negative	---
68 +	128/06/2016	Canine	Mohales Hoek	Positive	Positive	---
69 +	131/07/2016	Canine	Berea	Positive	Positive	---
70 +	132/07/2016	Canine	Maseru	Positive	Positive	---
71 +	133/07/2016	Canine	Maseru	Positive	Positive	---
72 +	136/07/2016	equine	Maseru	Negative	Negative	---
73 +	137/07/2016	Canine	Mohales Hoek	Positive	Positive	---
74 +	138/07/2016	Bovine	Mohales Hoek	Positive	Positive	---
75 +	163/09/2016	Ovine	Maseru	Positive	Positive	---
76 +	179/09/2016	Bovine	Maseru	Positive	Positive	---
77 +	182/09/2016	Canine	Mohales Hoek	Positive	Positive	---
78 +	184/09/2016	Canine	Maseru	Positive	Positive	---
79 +	185/09/2016	Canine	Maseru	Positive	Positive	---
80 +	196/10/2016	Canine	Berea	Positive	Positive	---
81 +	198/10/2016	Ovine	Maseru	Negative	Negative	---
82 +	199/10/2016	Bovine	Maseru	Positive	Positive	---
83 +	210/11/2016	Canine	Berea	Positive	Positive	---
84 +	234/12/2016	Canine	Maseru	Positive	Positive	---
85 +	30/01/2017	Bovine	Mafeteng	Positive	Positive	---
86 +	62/02/2017	Bovine	Maseru	Positive	Positive	---
87 +	63/02/2017	Canine	Maseru	Positive	Positive	---
88 +	71/02/2017	Bovine	Quthing	Negative	Negative	---
89 +	63/03/2017	Ovine	Maseru	Positive	Positive	---
90 +	69/03/2017	Bovine	Maseru	Positive	Positive	---
91 +	70/03/2017	Equine	Maseru	Positive	Positive	---
92 +	72/03/2017	Canine	Maseru	Positive	Positive	---
93 +	88/03/2017	Canine	Mokhotlong	Positive	Positive	---
94 +	89/03/2017	Bovine	Mokhotlong	Positive	Positive	---
95 +	90/03/2017	Canine	Mokhotlong	Positive	Positive	---
96 +	91/03/2017	Ovine	Mokhotlong	Positive	Positive	---

* denotes archival samples subjected to DRIT diagnosis during the diagnostic training programme; + denotes samples subjected to routine rabies diagnosis by both the DFA and DRIT assays; # denotes samples included in the molecular epidemiological analysis; The DFA and DRIT assays were repeated twice on samples that produced inconsistent results and the diagnostic outcomes of the two assays remained unchanged; --- Real-time PCR not performed on samples without diagnostic incongruities.

2.2. Direct, Rapid Immunohistochemical Test (DRIT)

All of the suspect rabies samples diagnosed in this study ($n = 96$) were subjected to the DRIT diagnostic assay according to the standard operating procedure [15,16]. The DRIT assay used a biotinylated anti-ribonucleoprotein PAB preparation prepared by the ARC-OVI, Rabies Division, and both positive and negative controls, consisting of homogenized brain material that had been confirmed as rabies-positive and -negative, respectively, by the OIE Rabies Reference Laboratory in South Africa, were included in every run.

Briefly, the brain material was homogenised prior to diagnostic confirmation to prevent viral tissue tropism from influencing the outcome of the DRIT test. A single touch impression was made from the brain tissues by placing a small amount of homogenised material on clean tissue paper. Touch impressions of the samples were allowed to air dry for five minutes before being submerged in 10% neutral buffered formalin (Sigma-Aldrich, Missouri, MO, USA) for ten minutes. After fixation, the touch impressions were re-hydrated by dip-rinsing the slides in TRIS phosphate-buffered saline (TPBS) buffer (phosphate-buffered saline (PBS), pH of 7.5 (Whitehead Scientific, Stikland, South Africa) containing 1% Tween 80 (Merck, Kenilworth, NJ, USA) and, thereafter, were submerged in 3% hydrogen peroxide (Merck) for ten minutes at room temperature in order to halt all endogenous peroxidase activity. Subsequent to the hydrogen peroxide flooding, the slides were dip-rinsed in fresh TPBS buffer and the excess buffer was shaken from the slides. The areas surrounding the smear impressions were

blotted using a fresh paper towel. Anti-ribonucleoprotein biotinylated polyclonal antibody (PAb) preparation (1:300 working concentration) was applied dropwise until the impression was completely covered. After the application of the antibody, the slides were placed in a humidity chamber and incubated at room temperature for ten minutes, and subsequently dip-rinsed in fresh TPBS buffer. The excess buffer was shaken from the slides, and the areas surrounding the smear impressions were blotted using a fresh paper towel. All of the touch impressions were covered in a ready-to-use solution of 2 µg/mL streptavidin-peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), after which the slides were transferred to a humidity chamber. The humidity chamber was incubated at room temperature for ten minutes, and the slides were dip-rinsed in fresh TPBS buffer. The excess buffer was shaken from the slides and the areas surrounding the smear impressions were blotted using a fresh paper towel. The impressions were covered in a working solution of the 3-amino-9-ethylcarbazole (AEC) chromogen (Sigma-Aldrich), and the slides were transferred to a humidity chamber and incubated at room temperature for five minutes. After sufficient staining had occurred, the slides were submerged in distilled water. The touch impressions were counterstained with a 1:2 dilution of Gill's formulation #2 (Sigma-Aldrich) for two minutes before they were dip-rinsed in distilled water in order to wash away the residual counterstain. Finally, the slides were mounted with a water-soluble mounting medium (1×PBS (Whitehead Scientific)/glycerol (Sigma-Aldrich) prepared 1:1) and examined by light microscopy (Olympus, CX21) at both 200× and 400× magnification in order to score the respective immunoreactivity based on both the presence and staining intensity of the visible red inclusions present on the blue cellular background [16].

2.3. Direct Fluorescent Antibody (DFA) Test

The DFA diagnostic assay was repeated on all the archival samples that produced discrepant results from those obtained after performing the DRIT assay during the training programme and, thereafter, in parallel to the DRIT assay on all of the samples that arrived for routine rabies diagnosis. The DFA test was performed according to the standard operating procedure [13,17] and relied on treating homogenised tissue impressions with a 1:1000 working dilution of FITC-labelled anti-ribonucleoprotein PAb preparation (ARC-OVI, Rabies Division, Pretoria, South Africa) in order to confirm any false results. Two microscopists based at the CVL interpreted the results on a Zeiss Axiovert 25 (Axiolab) fluorescent microscope at a magnification of 400× (excitation: 490 nm; Emission: 525 nm) in a blind reading in order to eliminate reader bias.

2.4. Resolving Diagnostic Incongruities

Samples that produced diagnostic incongruities observed between the DFA and DRIT assays ($n = 6$) were first re-tested a further two times with both assays at the CVL to confirm the relevant diagnostic results. Continued discrepancies were resolved by the attempted amplification of viral nucleic acid from the total RNA extracted from each sample (Table 1). Briefly, the total RNA of the homogenised brain tissue samples ($n = 6$) was extracted using the Direct-Zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) as per the manufacturer's instructions. An established "one-step" quantification real-time PCR (qRT-PCR) assay targeting the partial nucleoprotein gene was applied to amplify any lyssavirus RNA present in the brain material [18].

2.5. Data Analysis

The determination of the diagnostic sensitivity, specificity and respective confidence intervals of the DFA and DRIT diagnostic assays was determined using an exact binomial distribution (MedCalc 12.2.1.0, Ostend, Belgium). In order to determine whether the increase in sample submissions observed during the study period was statistically significant, a one-way analysis of variation (ANOVA) analysis was performed using the Epi Info™ software (version 7.2). The one-way ANOVA analysis was used across differences in mean sample submissions per month prior to the onset of the study (January 2012–March 2016) and within the study period (April 2016–March 2017).

2.6. Molecular Epidemiology of Rabies in LESOTHO

2.6.1. Sample Cohort

Twenty-one rabies-positive brain samples, collected and stored at the CVL in Maseru, were selected and used for molecular epidemiological characterization and phylogenetic analysis (Table S1). The representative panel of samples from across the country was chosen based on both the location where the samples were originally collected within Lesotho and the amount of available brain material stored at the CVL.

2.6.2. Viral RNA Extractions, PCR, and Sequencing

The total RNA of all of the brain tissue samples ($n = 21$) was extracted using the Direct-Zol™ RNA MiniPrep Kit (Zymo Research). A reverse transcription PCR was performed on the rabies-positive samples using the G(+) and L(-) primers [19,20], which amplifies the cytoplasmic domain of the glycoprotein gene and the adjacent G-L intergenic region of the RABV genome.

The PCR-positive products obtained from the 21 samples were electrophoresed on a standard 1% agarose gel and subsequently gel-extracted and purified using the Zymocelan™ Gel DNA Recovery kit per the manufacturer's instructions (Zymo Research). Both the forward and reverse strands of the purified PCR amplicons were sequenced using the respective PCR primers and the BigDye® Terminator v3.1 sequencing reaction kit per the manufacturer's instructions (Applied Biosystems). The Sanger sequencing was performed using an ABI 3100 automated capillary sequencer situated at the University of Pretoria, RSA. Consensus sequences were generated and trimmed to 592 nucleotides (nt), representing the cytoplasmic domain of the glycoprotein gene and the adjacent G-L intergenic region of the RABV genome using the CLC Main Workbench software (CLC Bio, Version 7.7.2). The final sequences were subsequently submitted to the NCBI GenBank and allocated unique accession numbers (MF197287–MF197307) (Table S1).

2.6.3. Phylogenetic Analysis

The phylogenetic analysis included a total of 100 G-L intergenic region sequences obtained from Lesotho and selected neighbouring South African provinces (Table S1). An alignment of the collection of sequences was created using the ClustalW subroutine of the BioEdit software [21] and the phylogenetic analysis was performed using a Bayesian Markov Chain Monte Carlo (MCMC) method in the BEAST software package (version 1.8.1) [22]. The best fitting DNA substitution model (TVMef+G) was selected using the Akaike's information criterion (AIC) determined using the JModel software (version 2.1.3). For the purpose of the phylogenetic analysis, three independent Markov chains were sampled for 50 million states and a sampling frequency of 50,000 was combined after discarding at least 10 per cent burn. The posterior distributions were inspected using Tracer (version 1.6) to ensure adequate mixing and convergence. The associated statistics were summarised as a maximum clade credibility tree and visualised using the FigTree software (version 1.4.2).

3. Results

3.1. Statistical Analysis of DRIT Diagnostic Efficacy

The number of true-positive ($n = 72$) and negative ($n = 24$) samples (determined by either agreeing DRIT and DFA assays or qRT-PCR amplification of nucleic acid) were used to determine the diagnostic efficacy of the DFA and DRIT assays (Table 2). Based on the confirmatory results provided by the "one-step" quantification qRT-PCR assay, the DFA test had produced an inaccurate result in six archival brain samples (DFA-positive but DRIT- and qRT-PCR-negative), indicating a reduced diagnostic specificity of 75% (Table 2). In contrast, the DRIT and qRT-PCR produced identical results for the samples in question and the DRIT was considered to have an overall diagnostic sensitivity and specificity of 100% (Table 2).

Table 2. Diagnostic sensitivity, specificity of the direct rapid immunohistochemical test applied to a cohort of samples stored at the central veterinary laboratory in Lesotho.

	True Positive	False Positive	True Negative	False Negative	Diagnostic Sensitivity *	Diagnostic Specificity *
DFA	72	6	18	0	100% (95.01–100%)	75.00% (53.29–90.23%)
DRIT	72	0	24	0	100% (95.01–100%)	100% (85.75–100%)

* Values in brackets represented the 95% confidence interval (CI).

3.2. Increased Sample Submission and Distribution Analysis

Over a 51-month period prior to the start of the DRIT implementation in Lesotho (January 2012–March 2016), the CVL had intermittently received and diagnosed an average of one sample per month. Over the same 51-month period, a total of 17 months (33%) had passed without any samples being submitted for rabies diagnosis (Figure 1). During the first 12 months of DRIT implementation in Lesotho (April 2016–March 2017), the average number of samples had increased to three samples per month, primarily due to the continued encouragement of the trained animal health professionals, resulting in a statistically significant three-fold increase in the monthly sample submission for rabies diagnosis ($p = 0.0279$, 95% CI: 0.2449 to 3.4556) (Figure 1). The significance of the increase is further appreciated when considering that samples were submitted regularly for every month of the year, with the exception of August 2016.

3.3. Molecular Epidemiology

The RABV sequences included in this study could, phylogenetically, be divided into three clades (Clade A–C), with each clade comprising separate lineages with posterior probability scores of 0.99, 0.96 and 1, respectively, (Figure 2). Clade A consisted largely of canid rabies viruses ($n = 79$) from Lesotho and two provinces of South Africa (the FS province and the northern region of the EC province) (Figure 3). Clade B consisted primarily of rabies-positive samples collected from the north-eastern region of the KZN province where a sylvatic outbreak is currently ongoing (2012–2017) [23] (Figure 3). The sequences that form part of Clade B clustered independently from Clade A as the cross-border spread of rabies between Lesotho and the KZN province is limited to a large extent by geographical barriers such as the Drakensberg and Maloti mountain ranges, which limit the movement of people and animals between Lesotho and the neighbouring South African province to the south [9]. Clade C was comprised of rabies-positive samples collected from the southeastern and central regions of the EC province bordering the KZN province (Figure 3).

Clade A could be further divided into three separate sub-clades (sub-clade AI–AIII) with each sub-clade representing independent endemic cycles that are both geographically defined and shared with its immediate regional neighbour (Figure 2). Sub-clade AI consisted exclusively of RABV sequences obtained from the FS province and Lesotho (Figure 3). The clustering observed in this sub-clade is similar to the cross-border spread that was observed in an earlier study investigating the increased number of dog-rabies cases in the FS province [9]. The branching clusters observed within the sub-clade illustrated that the viruses in this clade were closely related, which suggests that a single RABV strain historically circulated between Lesotho and the FS province. This finding was also in line with the previous findings where the authors concluded that canine rabies most likely spread from Lesotho to the FS prior to becoming an established active cycle spanning the entire region [9].

Sub-clade AII comprised of a single RABV sequence (146/98) collected from a dog in the FS province in 1998 and RABV sequences obtained from Lesotho and the northern region of the EC province (Figure 3). Evidence of an endemic cycle between the FS and northern region of the EC province had previously been suggested, but our study provides evidence that rabies virus-positive samples, identified in Lesotho between 2012 and 2013, can also be linked to this cycle [9].

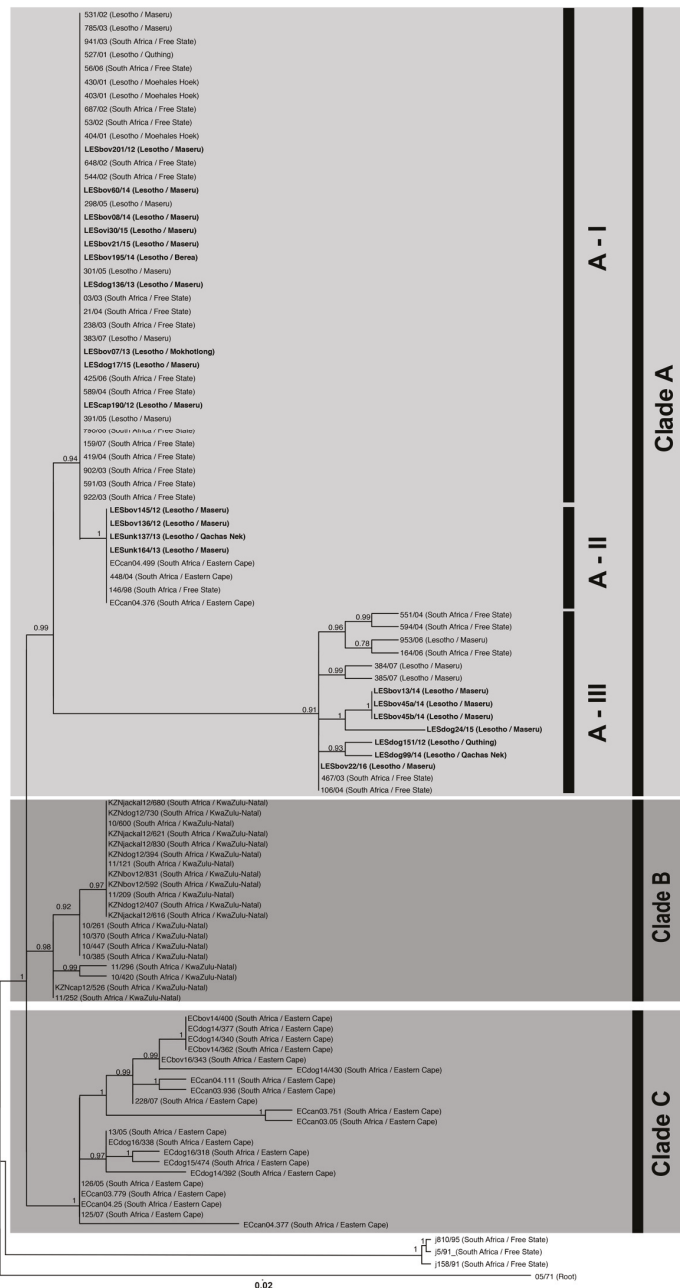


Figure 2. Maximum clade credibility tree of the cytoplasmic domain of the G-L intergenic region of RABV sequences originating from selected sub-Saharan African countries (Table S1). The horizontal branch lengths are proportional to the similarity of the sequences within and between groups and all branches with a posterior probability of 0.75 or less were collapsed. A bat-eared fox sequence from the Western Cape Province (isolate 05/71) was used to root the tree. The new sequences generated in this study have been indicated in a bold font (Table S1).

The third clade, sub-clade AIII, highlighted genetic relatedness between isolates originating from the capital, Maseru, the rural areas in the southernmost district of Lesotho (Quthing) and the FS province of South Africa (Figure 3). To our knowledge, this is the first report of an independent phylogenetic relationship between rabies viruses from the FS province and this specific region of Lesotho.

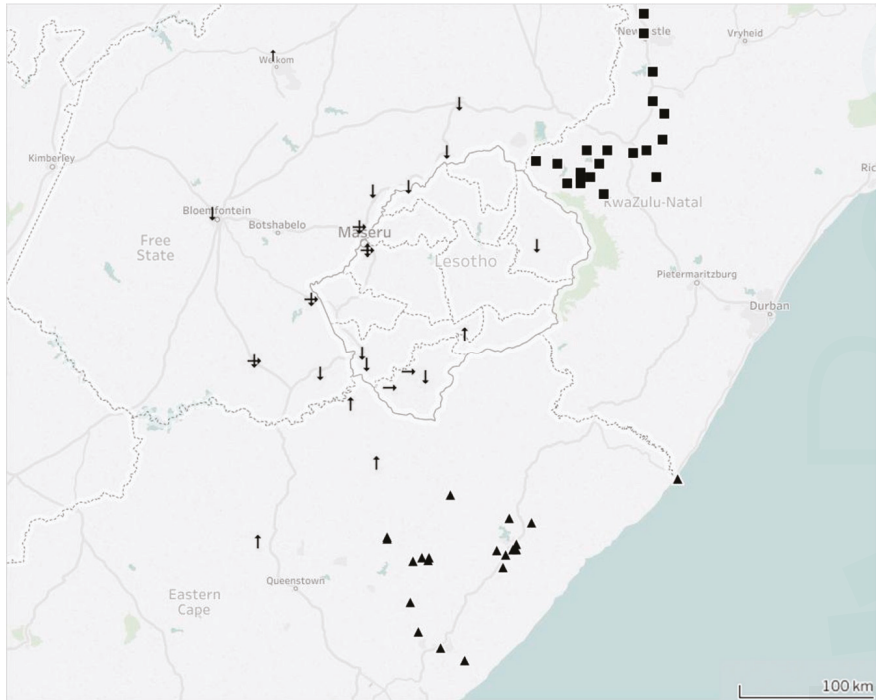


Figure 3. Illustrated map showing the inferred cross-border spread of endemic dog rabies between Lesotho and South Africa. Samples forming part of Clade A have been indicated with arrows, Clade B with squares, and Clade C with triangles. The three sub-clades have been indicated as follows: arrows facing downwards (sub-clade AI), arrows facing upwards (sub-clade AII) and arrows facing to the right (sub-clade AIII).

4. Discussion

In this study, we analysed 96 samples and found that 93.75% had correctly been diagnosed as either rabies-positive or -negative using the DFA assay when compared with qRT-PCR results. Several reasons could explain the occurrence of the false-positive results associated solely with the archival samples, especially when considering that the DFA test had been implemented in a resource-limited laboratory, where the maintenance of equipment is often lacking [24,25]. The most common possible reasons for incongruent results, viz. antigen degradation/putrefaction and the misinterpretation of fluorescence when performing the DFA test, were all considered [12,26–30]. However, sample degradation should be discussed in more detail. During our investigation, the archival samples had been placed in 50% glycerol-saline solution upon collection, before being stored at ambient temperature for extended periods of times (collected between 2011 and 2016). Despite relying on the glycerol-saline solution to preserve the samples, some of the samples had visible and olfactory signs of putrefaction (varying between samples) when tested with the DRIT assay.

Samples that have undergone putrefaction due to inadequate sample storage could contain bacterial or fungal contamination, which could, in turn, produce a large amount of non-specific fluorescence [27]. The presence of non-specific fluorescence could be misinterpreted as positive signals in the hands of an inexperienced reader or a reader relying on an improperly calibrated fluorescence microscope, leading to false-positive results being recorded [12]. The use of sub-optimal equipment while relying on an inadequate infrastructure in developing countries, is commonplace and a genuine concern [12]. Regardless of the cause of the incorrect diagnostic outcomes, the results reported here do not reflect negatively on the competency of the CVL staff. Instead, the technical challenges and difficulties associated with equipment, cold storage and the shipment of samples in developing countries, are highlighted.

This study adds to the realization of the versatility of the DRIT assay. In our view, this test should be considered for routine rabies diagnosis as it has been shown to be advantageous to resource-limited settings such as the one described here. Apart from having a diagnostic efficacy that is comparable to that of the DFA test, the DRIT is less sensitive to potential misinterpretations of results by not relying on fluorescent signals [12,24,31–33]. By reducing the visible interfering background staining (especially in samples that have undergone putrefaction), and simplifying the differentiation between a positive and negative result, the DRIT provides accurate diagnostic results by adequately-trained personnel.

In this study, 62/96 (65%) of the samples diagnosed as rabies-positive originated from the Maseru district where the CVL is located, with the number of samples dissipating as the districts locate further away from the CVL (Table 1). The positive correlation between the number of samples submitted for routine rabies diagnosis and the distance from the CVL indicates that the surveillance network could be enhanced even further by decreasing the distance samples must travel before reaching a laboratory. Considering the financial benefits associated with starting and routinely implementing the DRIT test at new diagnostic facilities, the assay could be used for decentralised rabies diagnosis without incurring a large capital investment [15]. In recognition of this fact, two decentralised diagnostic facilities in the Mokhotlong and Moehale's Hoek districts of Lesotho have already been identified as possible locations for decentralised routine rabies diagnosis in the future.

The submission and diagnosis of suspect samples provide a basic burden indicator for rabies, but in situations as described here, the resolution of the surveillance network remains very limited. Molecular epidemiological analyses are helpful in advancing our understanding of virus cycles and the transboundary movement of viruses in the larger region. The phylogenetic component of this study represents the first application of molecular epidemiology to specifically investigate the occurrence of endemic rabies within Lesotho. The results of our study were helpful to demonstrate that host species in Lesotho maintain three independent rabies cycles, which could impact negatively on any rabies intervention initiatives that are implemented in either of the two countries as animals can move freely between the two countries due to the porous nature of the borders on the African continent [12,34].

Although the presence of cross-border spread of rabies between Lesotho and the FS province is not a new observation [9], the sequence similarity observed for samples originating from Lesotho and the FS province (sub-clade AI) indicates that cross-border spread has been ongoing since the last investigation was performed in 2009 (Figure 2). As such, it could be speculated that human movement (along with their livestock and companion animals) is responsible for the ongoing exchange of rabies between two geographically-separated dog populations. This supposition is further supported by the observation that the Maseru district, which is approximately 11 km from the nearest border post between South Africa and Lesotho, is a potential rabies hotspot where all the various known genetic cycles of rabies occur (sub-clade AI–AIII) (Figure 3).

The sequence similarity observed for samples forming part of sub-clade AII indicates that cross-border spread occurs between Lesotho and two neighbouring provinces of South Africa (EC and FS provinces) (Figure 2). To our knowledge, the involvement of Lesotho in this active cycle is a novel observation and contradicts previous speculations that the sequence similarity between samples collected in the FS and EC province was due to a single spill-over event [9].

Sub-clade AIII demonstrates the value of undertaking continuous molecular investigations while relying on contemporary samples. Prior to this study, no molecular epidemiological information was available for this rural area in the southern highland districts of Lesotho. By including samples recently collected within Lesotho, it has become evident that rabies is not only present in this region, but that the Lesotho samples in this sub-clade have a higher level of sequence diversity to those in the other sub-clades (Figure 2). Our conjecture is that the endemic cycle observed in sub-clade AIII might be maintained by sylvatic species such as the black-backed jackal rather than dog populations. This observation is based on the sequence diversity, high number of rabies-positive livestock cases, comparatively low number of rabies-positive dog cases from the area, and the presence of jackal species in the surrounding highlands areas of Lesotho [35–37]. However, the lack of samples originating from sylvatic species in Lesotho prevents any firm conclusion in this regard.

5. Conclusions

The presence of multiple endemic cycles involved in the cross-border spread of rabies between Lesotho and South Africa highlight the importance of regional collaboration towards rabies control [38,39]. Without the regional collaboration of all the stakeholders, the success of disease intervention campaigns will not be sustainable or long-lasting as the re-introduction of immunologically naïve dog populations could result in a reduced vaccination coverage or negative impact on the sustainability of existing vaccination campaigns. By creating vaccination buffer zones on both sides of pre-determined national or regional borders [40], the re-introduction of rabies can be prevented and the vaccination coverage can be systematically increased and maintained within and beyond the buffer zones. As such, the collaboration between all interested stakeholders will not only prevent low vaccination coverage, but will also ensure that neighbouring countries work towards the same objective of being declared free of canine-mediated human rabies by 2030, an objective in line with the sustainable development goals [41].

Supplementary Materials: The following are available online at <http://www.mdpi.com/2414-6366/2/3/30/s1>, Table S1. Panel of rabies viruses from Lesotho and neighbouring South African provinces included in the phylogenetic analysis performed in this study.

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Article

The Importance of a Participatory and Integrated One Health Approach for Rabies Control: The Case of N'Djaména, Chad

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Abstract: This study compares data on animal rabies cases from the Chadian national rabies laboratory, hosted at the Institut de Recherche en Elevage pour le Développement (IRED), with bite case reporting from health facilities. The data collection accompanied a mass dog vaccination intervention over two years in N'Djaména, Chad. This allowed for a comparison of the dynamics of the incidence of animal rabies cases, human bite exposure incidence and post-exposure prophylaxis (PEP) demand during a dog rabies elimination attempt. Following the mass vaccination, the monthly animal rabies incidence dropped from 1.1/10,000 dogs, as observed prior to the campaign in 2012, to 0.061/10,000 dogs in 2014. However, the PEP demand was found to be largely unaffected. The suspicion of the rabies exposure as reported by health personnel in most cases did not reflect the status of the biting animal but rather the severity of the bite wound, resulting in inappropriate PEP recommendations. In addition, the levels of reporting dead or killed animals to the rabies laboratory was found to be very low. These results reveal a profound lack of communication between health facilities and veterinary structures and the absence of an integrated bite case management (IBCM) approach. Improved communication between human health and veterinary workers is imperative to prevent human rabies deaths through the appropriate use of PEP and to further translate success in animal rabies control into cost savings for the public health sector through a lower PEP demand. Improved training of health and veterinary personnel and the sensitisation of the public are needed to achieve good IBCM practice, to increase the rate of diagnostic testing, to provide adequate and timely PEP, and to reduce the wastage of scarce vaccine resources.

Keywords: rabies incidence; post-exposure prophylaxis; integrated bite case management (IBCM); One Health

1. Introduction

Within a One Health framework, rabies is likely the best documented example for the added value of closer collaboration of human and veterinary medicine for the control of zoonotic diseases [1]. With a few singular exceptions, humans only contract the disease through contact with an infected animal [2]. The highest disease burden is found in resource-poor settings where rabies is endemic in domestic dogs, which are the predominant species causing exposure in humans [3]. Poverty negatively impacts the access to post-exposure prophylaxis (PEP), which is urgently needed after a bite from

a suspected rabid animal. In most rabies-endemic countries in Africa, the cost for PEP exceeds the monthly income of people living below the poverty level [1,3,4]. In many remote areas, PEP is not available because of long distances to a health facility or the inefficiency of the health system [5]. In order to achieve the challenging goal of zero human deaths from dog-mediated rabies by 2030, as postulated by the World Health Organization (WHO) and partners [6], access to and the adequate use of PEP must be implemented. Because only a fraction of all bite cases are inflicted by a rabid animal [7,8] and because many countries face a shortage of the human vaccine for PEP, an integrated bite case management (IBCM) approach is required to guide treatment recommendations in order to save the highest percentage of human lives in the short term. The long-term sustainable control of rabies and the reduction of human fatalities can only be achieved through interventions that interrupt transmission in the reservoir species. Vaccination of dogs is the only control measure that will lead to the elimination of rabies in domestic animals and result in a reduction of the exposure risk in humans by more than 90% [3]. Dog vaccination reduces the need for PEP and considerably reduces the burden of premature deaths from rabies, averting a high number of years of life lost (YLL) [9,10]. Therefore, investment in dog vaccination, especially mass vaccination, although potentially more expensive than prevention in humans in the short term, is advantageous in the long term, with higher cost-efficiency compared to the cumulative costs of PEP alone [11–13]. However, reduction of the dog rabies incidence does not necessarily translate directly into reduced demand for PEP. Rabies control can lead to even higher PEP demand in the face of decreasing the exposure risk, which can be explained by heightened rabies awareness in the community [14–16]. To maximise the beneficial financial effects, dog vaccination should be carried out in conjunction with IBCM to prevent the overuse of PEP. The identification of bite victims who are not exposed to a suspected animal can be achieved through closer communication of medical staff with veterinary workers who are informed of the status of the respective animal. Ultimately, IBCM would also improve surveillance for the validation (proof of the absence of dog-mediated human rabies deaths) and verification (proof of the absence of dog rabies) of the 2030 goal.

The present study describes a lack of communication between the human and animal health sectors in the absence of IBCM, as shown by the comparison of laboratory and health center data. The data collection was performed during an epidemiological follow up study on the dynamics of the dog rabies incidence, the human dog-bite incidence and the PEP demand during a rabies elimination program in N'Djaména, Chad.

2. Methods

2.1. Background

In 2000, research on rabies control was initiated in N'Djaména, the capital city of Chad. Prior to the intervention, rabies was endemic in the local dog population, circulating at a low and stable level with an effective reproductive ratio (R_e) of just over 1 [12]. Pilot vaccination campaigns in 2003 and 2006 validated the feasibility of dog vaccination in the city, showing good participation by dog owners, provided the vaccine was offered without charge [17,18].

Given the promising initial results, the Swiss Tropical and Public Health Institute (Swiss TPH) implemented a large-scale mass dog vaccination intervention, together with two local partners, the Institut de Recherche en Elevage pour le Développement (IREDE) and the Centre de Support en Santé Internationale (CSSI). In May 2012, community awareness was emphasised to obtain accurate incidence data before the planned vaccination campaigns. Posters in French and Arabic illustrating the best practices after a bite incident were distributed to health centers, hospitals, pharmacies and veterinary facilities throughout N'Djaména. Drawings were used to accompany the text, because of the high illiteracy rate in Chad. The information included the importance of washing wounds after a bite, the need to seek medical treatment, and the importance of contacting a veterinarian (in the case of a live animal) or bringing the body to the IREDE (in the case of animal death). Fixed-post

parenteral vaccination campaigns took place across the entire city from October to December in 2012 and 2013. The organisational details and results of the vaccination campaigns are explained in detail elsewhere [13,19,20]. Both campaigns reached consecutive vaccination coverage of above 70% leading to the short-term elimination of the rabies virus from N'Djaména [20]. However, the PEP demand remained high even when there were no animal rabies cases (January–October 2014) [13]. To investigate why successful rabies control in the animal sector did not translate to beneficial effects in the human health sector through a lower demand for PEP, we investigated the available epidemiological follow-up data at the laboratory and health facility level.

2.2. Laboratory Data on Suspected and Confirmed Animal Rabies Cases

The IRED is the only rabies laboratory in Chad equipped to perform the standard fluorescent antibody test (FAT). In addition to the FAT, samples at the IRED were analysed with the Rapid Immunodiagnostic Test (RIDT) [21]. All positive samples were sent to the Pasteur Institute in Paris for virus isolation by polymerase chain reaction (PCR), to confirm the test results. Rabies surveillance in Chad is based on passive reporting; thus, animals are brought to the IRED on a voluntary basis with no active contact tracing. In most cases, the animal dies or is killed before its submission to the IRED. When an animal is still alive, the rabies laboratory refers the owner to the nearby public veterinary clinic for observation. The IRED charges the owner of the animal 5000 FCFA (8 USD) for rabies diagnostic testing, and there is no charge for feral dogs. The rabies surveillance in N'Djaména continued before, during and after the vaccination campaigns. Some samples from areas outside of N'Djaména were also sent to the IRED for a rabies diagnosis. The present analysis includes cases reported within the time period from June 2012 to end of December 2014, to mirror the data collection period at the health facility level. Information on the animal (vaccination status, location, symptoms observed, and outcome), on the bite victims (number, age, sex, and bite location and severity) and on the history of the bite (time, place, and circumstances) were routinely collected on the diagnostic request sheet. If the test result was positive, the victims were advised to initiate PEP at the Mother and Child Hospital (Hôpital Mère et Enfant), where the vaccine was available free of charge to women and children. Adult male victims were referred to the Central National Reference Hospital (Hôpital Centrale de Référence Nationale).

2.3. Health Facility Data on Animal Bite Victims

Data collection on bite cases was performed as in a previous study estimating human deaths from animal bite injuries in N'Djaména [22]. The health facilities for inclusion were identified using the same list as the previous study performed in 2008 [22]. However, not all facilities were still operating, and some new structures were identified during exploratory visits to the districts. In total, 91 facilities were contacted and included in the awareness campaign, representing all the public health centers in N'Djaména, the most frequented private health structures (medical practice) and hospitals (for profit and non-profit), and major pharmacies that had the capability to store the rabies vaccine. The largest private veterinary facility (veterinary practice) was also included. A questionnaire, developed for the study in 2008 [22], was distributed to the facilities, and personnel were asked to complete a form for every bite case presented. The information collected included demographic information about the victim (residence, age, and sex), the nature of the bite wound (severity, number and location), the circumstances of the bite incident (place, provoked/unprovoked, and other victims), the background of the animal (owner status, vaccination status, location, and outcome) and contact with other human health or veterinary structures (referrals from or to other facilities, including the IRED). The source of information related to the biting animal was sometimes the dog owner but could also be the victim or their representative (especially in the case of feral dogs). The decisions on the actions to be taken regarding the animal were primarily made by the owner, but they could also be made by the victim, in the cases of unowned dogs. The questionnaires were collected biweekly by study personnel, with an incentive of 300 FCFA (0.5 EUR) per completed questionnaire provided to the participating health structure. Health facilities were informed about the study in May 2012, and the questionnaires

were collected from June 2012 until the end of December 2014. Sixty-one facilities responded with at least one questionnaire (mean of 19.7, median of 4, and range of 1–143; Table 1). This number represented about 30% of the health facilities identified during the study in 2008 [22].

Table 1. Number of responding health facilities and questionnaires collected.

Facility Type	Health Facilities		Questionnaires		Quest/Facility
	Count	Percent	Count	Percent	
Pharmacy	33	54%	729	61%	22
Veterinary practice	1	2%	69	6%	69
Hospital (public)	6	10%	210	18%	35
Medical practice	6	10%	33	3%	6
Health center (public)	15	25%	154	13%	10
Missing information	N/A	N/A	4	0%	N/A
Total	61	100%	1199	100%	N/A

2.4. PEP Use and Cost

The WHO recommends including rabies immunoglobulin (RIG) in the PEP protocol for cases with a category III exposure (transdermal injuries or contact of saliva with mucosa) [23]. However, RIG is not available in Chad, and therefore PEP only includes active vaccination with cell culture vaccine (CCV) given according to the intramuscular five-dose Essen regimen [24]. The price of one dose of the human rabies vaccine ranges between 9000 and 12,000 FCFA in N'Djamena, such that a full course of PEP costs 45,000–60,000 CFA (80–100 USD). Adding the costs for wound treatment (antiseptic, antibiotics, or tetanus vaccine), the private costs of lost work time and the transportation to a health facility, the full PEP and bite treatment costs are estimated to be over 90,000 FCFA (160 USD) per case [13]. Our study did not include any follow up of the bite victims; thus, data on the completion rate and the outcome for rabies-exposed people is not available.

2.5. Data Analysis

The questionnaire data was double entered and compared using Epi Info, and was then transferred to an Access (Microsoft, Redmond, DS, USA) database. Data collected at the rabies laboratory were entered continuously into an Excel spreadsheet. For both data sets, the analysis was performed with Stata/IC 14. The dog rabies incidence was calculated on the basis of the number of positive dog rabies cases observed in N'Djaména over the study period and the dog population estimates for the city obtained during the two vaccination campaigns, ranging from 24,547 in 2012 to 30,074 in 2013 [19]. The national human population census of 2009 provided by the Chadian national statistical institute for economic and demographic studies (Institut National de la Statistique des Etudes Economiques et Démographiques; INSEED) served as the basis for the calculation of the bite exposure and PEP incidence. Only bite cases reported from N'Djaména (1143) were included for this calculation. To statistically evaluate differences in the incidences (dog rabies cases, dog bites, and PEP use) before and after the mass vaccination intervention, paired *t*-tests were performed. The respective monthly incidences observed from June to December 2012 were compared to the monthly incidences of the period of June to December 2013.

For the analysis of the health facility data, the vaccination status of the biting animals was categorised as “vaccinated” (date of vaccination reported and less than one year before reported bite incident), “vaccination unconfirmed” (missing vaccination date or more than one year before bite incident) or “unvaccinated” (vaccination status reported as unvaccinated or unknown). In addition, each bite case was attributed a rabies-exposure risk variable on the basis of the status and outcome of the animal as drawn from the information in the questionnaire: “high exposure risk” was attributed to animals reported to have disappeared or have been killed after the bite attack; “moderate exposure risk” was attributed to animals with a negative, unclear or out-dated vaccination status, regardless of

being under observation or not. Vaccinated animals that bit more than two people were also defined as having a moderate exposure risk because data from the laboratory level showed that the number of victims by case was related to a positive test result. In the cases of no known owner for an animal that was reported to be under observation, the exposure was also considered to be moderate. “No exposure risk” was attributed when the animal had a confirmed vaccination status, had bitten no more than two people, and was placed under observation. The rabies-exposure risk categories, derived from the animal’s status as described above, were compared to the rabies suspicion as noted by the health personnel in the questionnaire (“yes”, “no”, or “do not know”). To evaluate the parameters influencing the PEP recommendation, the respective explanatory variables were coded into categories: the severity of the bite was coded as WHO category II exposure/WHO category III exposure (WHO category I was not observed in this data) [23]; age was coded as adults (>15 years)/children (≤15 years); the number of bites was coded as single bite/multiples bites; the number of victims was coded as single victim/multiple victims. The categories per parameter were then compared by risk ratio (RR) analysis with calculation of respective confidence interval (CI). A statistical comparison of the PEP recommendations per respective categories was performed by an odds ratio (OR) calculation. Regarding the risk of a dog being killed after having inflicted a bite, the 10 districts of N’Djaména were assigned an observed predominant cultural background (Christian/Muslim) and the differences between the two categories were evaluated by a calculation of the RR.

2.6. Ethical Consideration

The dog rabies mass vaccination intervention was approved and co-funded by the government of Chad. The data on rabid animals and human bite exposure was collected on a routine basis by the rabies laboratory at the IRED. This study was approved by the ministry for higher education in Chad (Letter N°012/PR/PM/MES/SG/DGESRSFP/DRST/012; Date: 31 May 2012). Meetings were held with the mayor of N’Djaména and the district and quarter chiefs in each administrative area who granted permission prior to beginning the study.

3. Results

3.1. Rabies Diagnostic Results and Respective Case Histories

The awareness campaign before the mass vaccination led to a rapid and considerable increase in the number of rabies-suspicious animals reported to the IRED, from a monthly mean of 1.2 diagnostic requests observed from January to May 2012 (prior to the study period) to 3.6 observed from June to December 2012 (during the study period). Throughout the study period (June 2012–December 2014), a total of 60 rabies-suspect animals were sent to the IRED, of which 46 originated from N’Djaména, 9 originated from other areas in Chad (mostly located very close to N’Djaména), and 2 originated from Cameroon (which borders N’Djaména). In three cases, the sample origin was unknown. Overall, 32 samples tested positive, 25 were negative and 3 were not testable because of a poor sample quality. Table 2 shows the distribution of the test results by species.

Table 2. Summary of results of samples received for rabies diagnosis at the Institut de Recherche en Elevage pour le Développement (IRED), by species.

Species	Negative	Positive	No Result	Total
Dog	13	30	2	45
Cat	3	2	1	6
Monkey	6	0	0	6
Sheep	2	0	0	2
Shrew	1	0	0	1
Total	25	32	3	60

In total, 30 (67%) of the submitted dog samples and 2 (33%) of the cat samples were positive. No positive cases were observed in other species. Amongst all animals sent to the IRED, only 10% (five dogs and one cat) were initially put under observation prior to death and subsequent submission to the IRED. In two of those six cases, the animals tested positive for rabies. In most cases, the animal was killed immediately after a bite rather than put under observation (43 cases; 72%). The percentage of positive cases among killed animals was 67% (29 out of 43). The percentage of confirmed rabid animals that were found dead or that died during the observation period was 22% (two out of nine). This observed difference was found to be significant, but with a large confidence interval due to limited sample size (RR 3.7; 95% CI : 1–13.2; p = 0.043). In eight cases, the circumstances of death were not specified.

The majority of animals brought to the IRED were owned (72%), but only 2 out of 32 (6%) owned dogs had a valid vaccination (vaccination <1 year), which was confirmed by a certificate. In one of these two cases, the dog nonetheless tested positive. In six cases, the vaccination status was unconfirmed or out of date and five of these dogs tested positive. Animals other than dogs were all unvaccinated. The most commonly observed symptoms were aggression (88% of cases) and a sudden change of behaviour (40% of cases).

On average, two (min: one; max: six) human bite victims were observed per rabid animal. The proportion of children among the bite victims of confirmed rabies cases was 42% (25 of 59). For rabies-negative cases, the proportion of children among all victims was only 27% (3 out of 11). However, risk ratio analysis showed that this difference was not statistically significant (RR 1.4; 95% CI : 0.5–3.8; p = 0.5)

3.2. Reported Bite Cases and Related Animal History at the Health Facility Level

In total, 1203 questionnaires were collected from health facilities during the survey. Three questionnaires were excluded from the data set because the biting animal was a snake and one questionnaire was not completed. The vast majority of the remaining 1199 bite cases were those inflicted by dogs (936 cases; 78%), followed by cats (58 cases; 5%) and monkeys (15 cases; 1.5%). For the remaining 16% (190 cases), information on the animal was missing. A high number of reported victims were children \leq 13 years of age (42%).

More than 58% of bite exposures took place at the victim's home, while an additional 36% occurred very close to the place of residence. Table 3 shows the distribution of reported bite cases and health facilities by district and inhabitants on the basis of the population census of 2009 (INSEED). District 10 was very sparsely represented; only one health facility participated and only 11 bite cases were reported from the entire district (Table 3). There was a low representation of health facilities in this district because of its remote location on the periphery of the town. Additionally, a very low dog population density was reported from this district during the vaccination campaigns [19]. In contrast, districts 6 and 7 had the highest rate of questionnaires per inhabitants (Table 3). This was in accordance with the high density of health facilities and the very high dog-to-human ratio found in these two districts (Figure 1).

Table 3. Number of questionnaires and participating health facilities per district and inhabitants on the basis of the population census of 2009 (INSEED).

District Number	Questionnaires (Q)		Health Facilities (HS)		Population (P) 2009		HS/1000P	Q/1000P
	Count	Percent	Count	Percent	Count	Percent		
1	54	5%	8	13%	72,742	8%	0.11	0.73
2	9	1%	3	5%	36,450	4%	0.08	0.25
3	22	2%	6	10%	38,101	4%	0.16	0.58
4	14	1%	3	5%	72,954	8%	0.04	0.19
5	39	3%	3	5%	102,169	11%	0.03	0.38
6	122	11%	6	10%	43,948	5%	0.14	2.64
7	700	61%	17	28%	221,811	23%	0.08	3.08
8	106	9%	12	20%	185,065	20%	0.06	0.53
9	66	6%	2	3%	75,893	8%	0.03	0.86
10	11	1%	1	2%	98,982	10%	0.01	0.10
Total (N'Djaména)	1143	100%	61	100%	948,115	100%	0.06	1.17

District 9 had a similar dog density to district 7, however, like district 10, it was an area at the periphery of the town, where health facilities were not numerous. Figure 1 also shows the monthly incidences of dog rabies and bite exposures by district reported before and during the first vaccination round in 2012 (June–December).

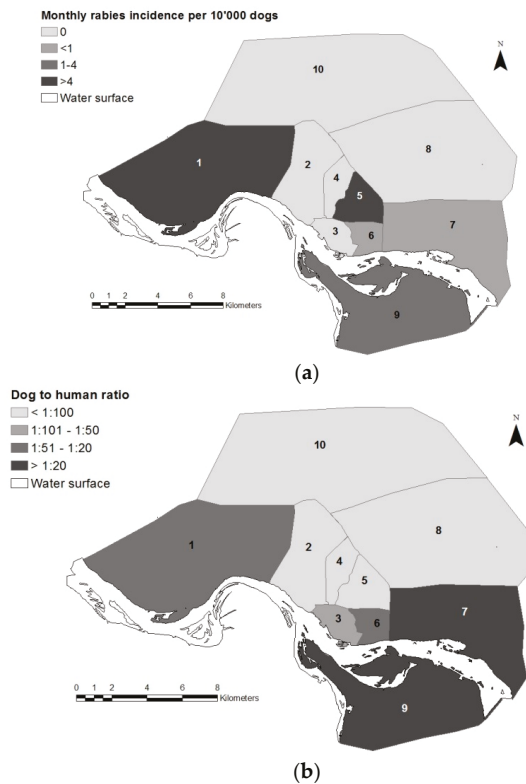


Figure 1. Cont.

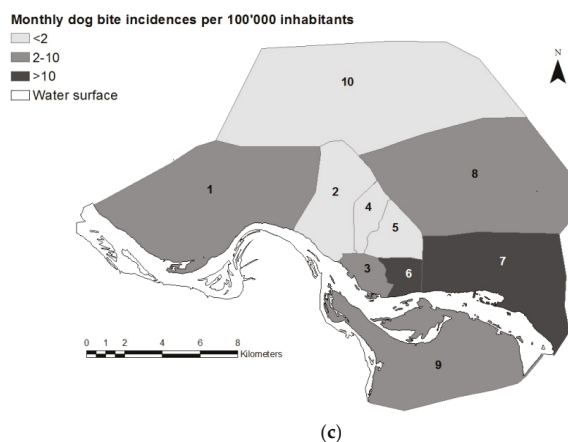


Figure 1. Maps of N'Djaména depicting monthly dog rabies incidences (a); dog-to-human ratios (b) and monthly dog bite incidences (c) observed from June to December 2012. Dog population estimates are based on the results of the vaccination coverage analysis in 2012 published previously [19]. The human population by district is derived from the population census of 2009 (INSEED). Numbers on the maps indicate the district number.

In over 70% of the bite cases, the animal was reported to be put under observation, but in less than one in four of these cases (23.5%) was it noted that the animal was taken to a veterinary facility. A total of 144 cases (15%) stated that the animal was brought to the rabies laboratory at the IRED. However, this number does not correspond to the actual number of animals submitted to the rabies laboratory within the same time period; see above. Moreover, in only 2 out of 72 cases for which the animal was killed and in 1 out of 4 cases for which the animal died was it reported that the carcass was sent to the rabies laboratory. Six dogs were killed, despite a reported confirmed vaccination status. For bite cases reported from districts with a predominantly Muslim background, the animal was 10 times more likely to be killed, compared to those of bite cases occurring in districts with a predominantly Christian background (RR: 10.5; 95% CI: 6.37–17.36; $p < 0.0001$). The mean number of victims per biting animal observed during the health facility survey was 1.5 (max: 13). In the majority of cases (82%), only one victim was reported.

The highest number of bite victims was reported by pharmacies. Compared to the other types of health facilities involved, hospitals had the highest mean number of reported bite cases, followed by pharmacies and health centers (Table 1).

In total, 161 victims were referred to another facility (Figure 2), and pharmacies were the facilities referring the highest numbers of people to another facility. In 64% of the referrals, the patients were sent to another human health facility, the majority to hospitals (54%), likely because of a shortage of the vaccine or for further wound treatment. In only 36% of referrals were the victims sent to a veterinary facility (28.5% to veterinarians and 7.5% to the IRED). Hospitals and health centers were the facilities that referred the highest numbers of cases to veterinary facilities or to the rabies laboratory at IRED (83% of overall referred cases). The single veterinary facility that participated referred 74% of their bite victims to a hospital or a health center, assumedly because a veterinary facility would not provide the human vaccine.



Figure 2. Cases of referral of victims to another health facility.

3.3. PEP Treatment Decisions on the Health Facility Level

The PEP treatment recommendation was not always consistent with the rabies suspicion status reported by the health personnel (Figure 3). In 27% of the bite cases, it was noted that the animal was not suspected for rabies but PEP was recommended. In contrast, 56 patients (0.05%) were not recommended to undergo PEP although the animal was reported as a rabies suspect (Figure 3).

When comparing the rabies exposure risk status defined during analysis (as based on the information available regarding the biting animal), with the suspicion status reported by the health personnel, we found 40 cases in which no suspicion was reported but a high exposure risk was assigned on the basis of the case history. Similarly, when the exposure risk was absent, as assessed by the case history, 54 cases were nonetheless identified by the health personnel as suspected for exposure to a rabid animal.

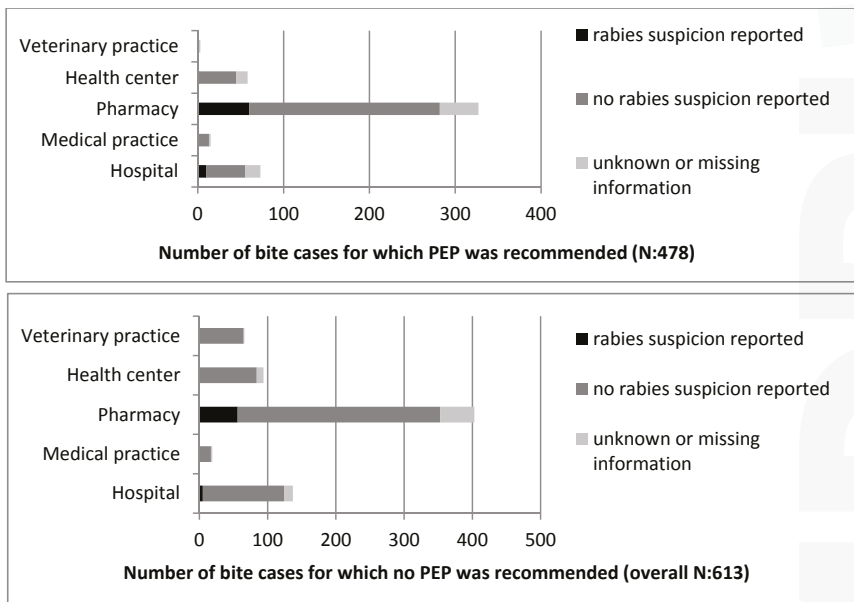


Figure 3. Comparison of post-exposure prophylaxis (PEP) recommendation and reported rabies suspicion by facility type.

The comparison of the rabies exposure risk status with the PEP recommendation illustrated even higher discrepancies. Of all the bite cases, including those reported from outside N'Djamé na, with no indication of suspected rabies exposure, 36% (208 out of 577) were recommended to undergo PEP treatment. In 38% (189 out of 487) of the cases for which PEP was recommended, the exposure risk was moderate. Finally, in only 17% (81 out of 487) of the cases for which PEP was recommended did the history of the animal indicate a high exposure risk. Most alarmingly, in 62% (312 out of 501) of all the cases judged to be of moderate exposure risk and in 33% (40 out of 121) of those judged as high exposure risk, the bite victims were not advised to undergo PEP treatment. Detailed results on the PEP recommendations by facility type and the comparison to the rabies exposure risk are presented in Figure 4.

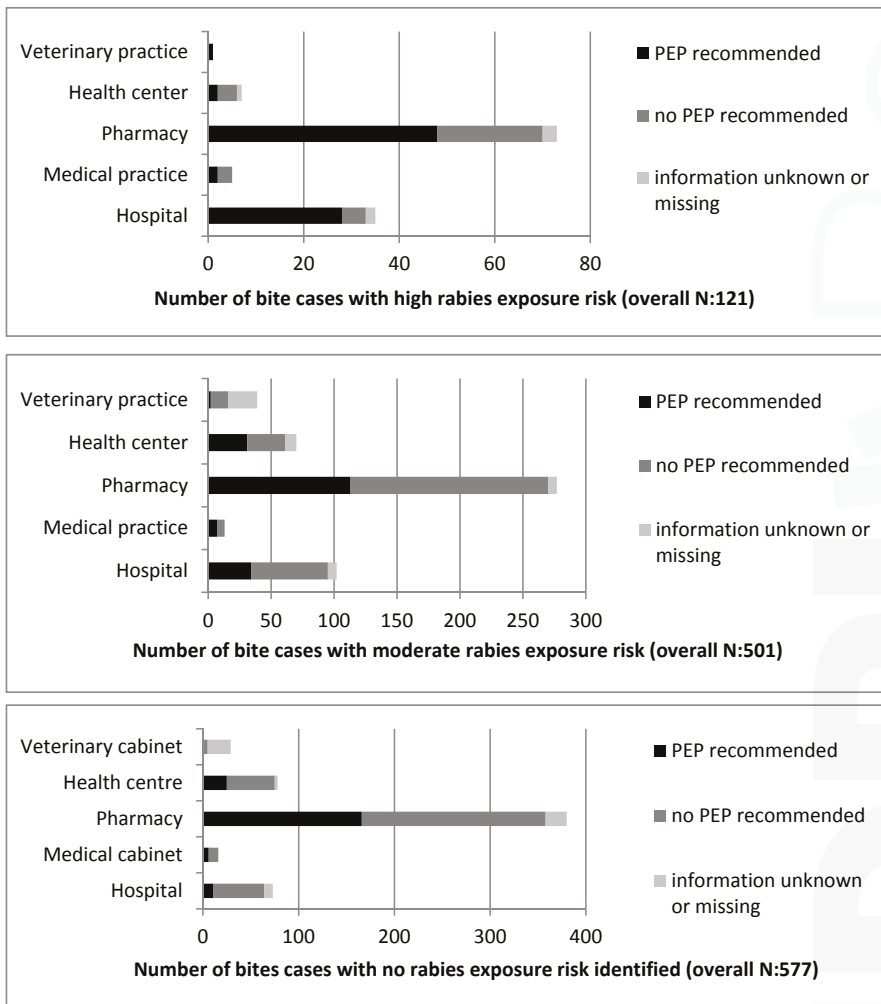


Figure 4. Comparison of post-exposure prophylaxis (PEP) recommendations made by facility type and rabies exposure risk category (as attributed to bite cases on the basis of the animal status).

The inconsistencies in the PEP recommendations can be explained by the fact that health personnel were more likely to judge the rabies suspicion according to the severity of the bite inflicted rather than on the vaccination status of the animal. When the wound was deep or the skin was clearly broken, one in five cases was declared as suspicious, and in 50% of these cases, PEP was recommended. In contrast, when the wound was reported as superficial or only a minor scratch, less than 1 in 20 were reported suspicious and 28% of these patients were recommended to undergo PEP. PEP was found to be more likely recommended for WHO category III than category II exposures (OR: 0.36; 95% CI: 0.28–0.46; $p < 0.0001$). No difference was observed for the PEP recommendation between children and adults (OR 1.0065; 95% CI: 0.79–1.28; $p = 0.96$) or the number of bites inflicted (single or multiple; OR: 1.17, 95% CI: 0.9–1.52; $p = 0.22$). There was also no relation observed between the number of victims reported per animal (single or multiple) and the rabies suspicion status defined by the health personnel (OR: 0.81; 95% CI: 0.51–1.3; $p = 0.4$).

3.4. Impact of Mass Dog Vaccination on Dog Rabies Incidence, Animal Bite Incidence and PEP Demand

After the beginning of the vaccination intervention in October 2012, rabies reports from N'Djaména as observed at the IRED dropped steadily (Figure 5). Following the mass vaccination, the monthly animal rabies incidence dropped from 1.1/10,000 dogs, observed prior to the campaign in 2012, to 0.12/10,000 dogs in 2013, and only 0.061/10,000 dogs in 2014. This translates as a reduction from one rabid dog per week in 2012 to only two rabid dogs throughout the whole year in 2014. During the same period of time, the reporting of rabies cases from areas outside of N'Djaména was steady but remained low (only 11 cases over the study period), mainly due to lower public awareness and logistical challenges. Before the vaccination intervention, most rabies cases were reported from the seventh district (Figure 1). After the mass vaccination, rabies cases were absent from districts north of the Chari River for well over a year (February 2013 to October 2014); the only cases observed during this period came from district 9, which lay south of the Chari River.

Figure 5 shows that the incidence of PEP treatment did not decline with the decline of the animal rabies incidence but was instead closely linked to the overall bite incidence rates observed from the health facility data over the study period. For the dog rabies incidence, the paired t -test showed a significant difference between the two periods from June to December in 2012 and 2013 ($p = 0.0063$; $t = 3.8$). The difference in the monthly mean PEP incidences over the same period of time was not significant ($p = 0.1$; $t = 1.4$), nor was the difference observed between the monthly mean dog bite incidences ($p = 0.1$; $t = 1.5$).

The proportion of confirmed vaccinated animals among all the bite-inflicting animals observed on the health facility level only increased by 15%, from 47%, observed prior to the start of the vaccination campaign, to 62% by the end of 2014. The percentage of unvaccinated animals decreased from 23% to 11% and cases with an unconfirmed, out-of-date or unknown vaccination status remained stable at around 30%.

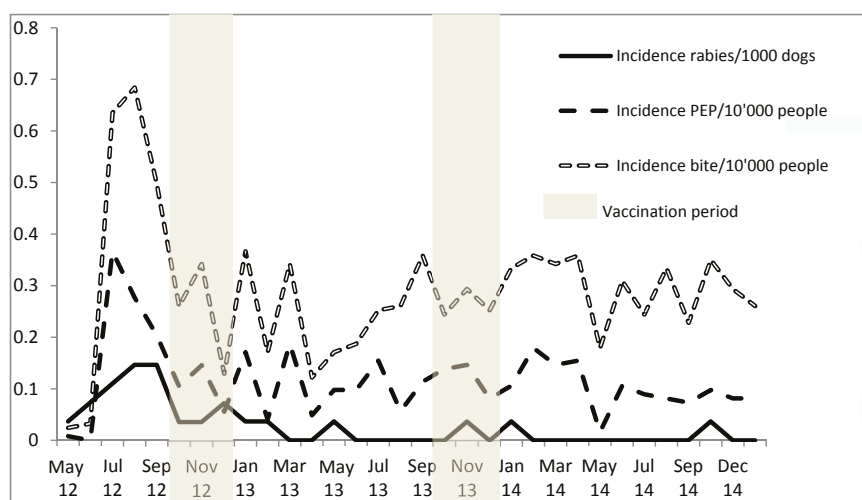


Figure 5. Dynamics of monthly animal rabies, human bite exposure and post-exposure prophylaxis (PEP) demand incidence rates following the dog mass vaccination intervention in N'Djaména.

4. Discussion

The multiple studies undertaken in N'Djaména since 2000 serve as a good example for investigation on the feasibility and description of barriers and shortcomings for rabies control in Sub-Saharan Africa. The most recent work that included a citywide dog mass vaccination and a follow-up of the epidemiological impact has demonstrated the interruption of rabies transmission [20], but it highlights the need for better communication between the veterinary and human health sectors to translate success in dog vaccination to beneficial economic effects in public health [13]. In the absence of dog vaccination, the current PEP use in N'Djaména is inadequate to prevent all dog-mediated human deaths [13]. In this paper, we describe underlying problems that contribute and that should hypothetically be similar in other countries. These factors constitute barriers to the goal of achieving zero human deaths due to dog mediated rabies and they should be addressed early towards the 2030 agenda. The challenges crystallise around the implementation of an integrated, community-based One Health approach for efficient rabies surveillance and control. This can only be achieved through high public participation in control measures (vaccination and surveillance), compulsory dog registration and IBCM training for health and veterinary personnel to ensure the best practice use of PEP.

Our data illustrate the evident fact that the elimination of dog-mediated rabies will not lead to considerably lower bite case incidences, as only a very small proportion of bites are caused by rabid animals. For example, in the present study, the number of bite victims of truly rabid animals recorded at the laboratory constituted only 5% of the overall observed bites in the health facility survey during the same period of time. Therefore, it would be an unnecessary burden on the public health sector to recommend PEP for every bite case. Such extensive use of PEP would even be counterproductive given the current shortage of the vaccine, as it would lead to insufficient PEP for actual rabies-exposed victims.

Our reported numbers for the bite incidence are conservative. Not all victims present to a health facility, as many use traditional forms of treatment [25]. In addition, this study only covered about one-third of the facilities in N'Djaména. Therefore, the actual numbers of bite exposures are believed to have been considerably higher. In general, there were fewer health facilities per inhabitant observed in districts at the periphery of the town compared to the central districts, which could have led to

an underrepresentation of cases from the peripheral districts. The socio-economic background also differed between the central (wealthier areas) and peripheral (underprivileged areas) districts; poorer communities might have been less represented in this study. However, N'Djaména is a relatively small town (292 km²) with adequate road access and public transportation; therefore, the geographical distance to a respective health facility should have a minor influence on health seeking and PEP accessibility. Most dogs in N'Djaména are owned but are free roaming, which results in high contact between humans and dogs. Nonetheless, notable differences in the bite exposure incidences were observed between different districts. N'Djaména reflects the diverse socio-cultural and socio-economic context of Chad. This background has a significant influence on dog ownership [19]. Districts with a predominantly Muslim background (2nd, 4th and 10th district) have a much lower dog-to-human ratio than areas with a Christian context (1st, 7th and 9th district). Similarly, the dog density is extremely low in the wealthy neighborhoods found in the 2nd district as compared to the very high densities found in the slum areas at the periphery of the town (9th district). Because of this diverse context, dog rabies cases and dog bite exposures are likewise heterogeneously distributed. As the dog population estimates were derived from extensive surveys during the mass vaccination campaigns, and because the number of health facilities per district did not depend on the district cultural background, we are confident that these differences were not a result of underreporting in predominantly Muslim areas.

The study did not include a follow-up of bite patients, and therefore we do not know if victims completed all the PEP doses required. Additionally, we do not know how many cases of human rabies occurred during the study period in N'Djaména, as the disease is not notifiable in Chad. In 2008/2009, the annual number of human rabies cases in N'Djaména was estimated to be seven, and an extrapolation of the mean number of victims per rabid dog registered revealed a huge proportion of possibly exposed people who did not seek PEP [22].

Another study limitation was the lack of follow-up on the observation of biting animals, as the collection of such data on the veterinary level was not included. Regional governmental veterinary institutions in Chad do maintain registries on animal observations, but the results are not regularly reported to higher national levels. In our study, we observed that although, after most exposures, the biting animal was reported to be put under observation, a veterinarian was rarely contacted. This indicates that the owner judged his animal to be alive and well, but that no action was taken to check the animal for signs of rabies by a veterinarian. No legislation on rabies control exists in Chad, other than a short paragraph from 1961 that recommends culling stray dogs. Therefore, the 10 day observation period for an animal that has bitten a person cannot be enforced by law. In our study, animals that died were less likely to be tested positive for rabies at the laboratory level. This has important implications for animal welfare; for instance, dogs accustomed to free-roaming were reported by owners to have died of strangulation while kept on a leash.

The high number of rabies-positive cases among animals with an unconfirmed or out-of-date vaccination status highlights the importance of confirming the owner-reported vaccination status by a certificate. This issue is also highlighted when comparing the proportion of biting animals reported as vaccinated by the health facility survey prior to the mass vaccination campaign, which was 47%, with data from a household survey conducted in 2001, for which the proportion of vaccinated dogs was only 19% [26]. As the negative financial implications (cost of PEP for all bite victims) and social consequences (responsibility for a human death) for owners of a rabid dog are unbearable, some may not accurately represent the vaccination status of their animal. On the other hand, the small increase in the proportion of confirmed vaccinated animals observed over all the reported bite cases during the dog vaccination intervention was concerning, and could have been related to the high likelihood of losing paper-based vaccination certificates [19]. Therefore, to establish an effective IBCM, a unique identification and registration system for all dogs is necessary.

The positive result found in a dog that was certified as vaccinated highlights the need for an observation period even for vaccinated animals. Vaccine failure due to inappropriate storage conditions

or the inadequate immune response of the animal can never be excluded. Rabies antibody detection and titration is not yet possible in Chad.

Our findings at the laboratory level indicate that the symptoms of rabies and the abnormal behavior of animals are generally well interpreted. When there was a suspicion of rabies, especially for aggressive dogs, the animal was killed quickly. However, the focus on aggression could mean that the paralytic form of rabies remains undetected in most cases. The IRED is regularly contacted by bite victims seeking PEP treatment who have already killed and disposed of the biting animal. The extremely low number of animals brought to the IRED after being killed or found dead shows that the importance of testing is not adequately perceived. One explanation could be the cost of the diagnostic fee, which is borne by the dog owner. Interaction with victims at the IRED indicated that they usually had little doubt about the symptoms observed in the biting animal and whether they had been truly exposed. This could explain a perception that additional laboratory testing is not necessary.

In addition to cases in which animals that died or were killed were not submitted for testing, there were several cases in which the animal disappeared. This could indicate that the actual number of rabies cases was significantly higher than reported in this study. The observed difference in killing rates between different cultural backgrounds could further point towards an underrepresentation of rabies cases from specific areas of town. However, even when simulating different case detection rates using a rabies transmission meta-population model established for N'Djaména, the outcome of the model, which suggested an interruption of rabies transmission, remained robust [27].

Despite the observed drop in the rabies incidence and the absence of animal rabies cases for nine months following the mass vaccination intervention in N'Djaména, the PEP demand remained largely unaffected by this epidemiological change. The PEP demand was clearly correlated to the overall number of bite exposures observed. The assessment of the rabies risk by health personnel was found to be based on the severity of the bite inflicted. However, scratches are listed as WHO category II exposures and they would therefore also warrant PEP treatment [23]. The influence of the animal status on the rabies risk rating was secondary. Moreover, very few patients were referred by health facilities to a veterinarian for animal observation/advice. All these findings clearly indicate that exchange between health personnel and veterinary workers remains inadequate for IBCM, and should be improved using inter-disciplinary training and communication platforms.

We only included one veterinary facility and this data was insufficient to compare the performance of veterinary facilities with human health facilities. However, the 69 cases of animal bite victims who sought help at the private veterinary practice likely indicate an understanding amongst the public for the link between human and animal health, which provides a basis for the scaling-up of knowledge.

Similarly to other studies, we report a significant number of children among the bite victims [28–30]. Also, we found that a high number of bite incidents occurred at home. School-based education and the sensitisation of dog owners regarding rabies and the prevention of dog bites should lead to a decline in bite cases. Raising awareness and knowledge about the rabies risk in communities is an important element to prevent the excessive demand of PEP [31,32] and the unnecessary killing of suspected dogs [33,34] resulting from a fear of rabies. Therefore, a third pillar to establish effective IBCM, along with dog registration and interdisciplinary training, includes community engagement and culturally sensitive education.

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Article

Retrospective Cohort Study to Assess the Risk of Rabies in Biting Dogs, 2013–2015, Republic of Haiti

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Abstract: Background: In canine rabies endemic countries the World Health Organization recommends post-exposure prophylaxis (PEP) be initiated immediately after exposure to an animal suspected to have rabies. Limited capacity in low and middle income countries to assess biting animals for rabies may result in the over prescription of rabies biologics. Few guidelines exist to determine the risk of whether a dog that has bitten someone is rabid. Given PEP cost and access limitations in many countries, accurate and timely assessment of dogs that have bitten people may reduce unwarranted PEP use and improve healthcare seeking behaviors. Methods: Haiti's animal rabies surveillance program utilizes veterinary professionals to conduct rabies assessments on reported biting dogs and records characteristics of the dog, health outcomes, and laboratory results in a national database. Characteristics of rabid dogs were assessed through a retrospective cohort study of biting dogs investigated during the period from January 2013–December 2015. 1409 biting dogs were analyzed; 1361 dogs that were determined to not have rabies were compared to 48 laboratory-confirmed rabid dogs. Rate ratios, sensitivity, specificity, positive predictive values, negative predictive values, likelihood ratios, quarantine survival of biting dogs, and a risk matrix were developed. Findings: The assessor's determination that the animal likely had rabies was the most significant predictive factor for a rabid dog (RR = 413.4, 95% CI 57.33–2985, Sn = 79.17, Sp = 91.92). Clinical factors significantly associated with rabid dogs included hypersalivation, paralysis, and lethargy (RR = 31.2, 19.7, 15.4, respectively). Rabid dogs were 23.2 times more likely to be found dead at the time of the investigation compared to case negative dogs (95% CI 14.0–38.6). Rabid dogs were also significantly more likely to lack a history of rabies vaccination or be unowned (RR = 10.3 95% CI 2.5–42.3 and RR = 4.5 95% CI 2.0–10.1, respectively). Rabid dogs were four times more likely to have bitten multiple people (RR = 4.0 95% CI 1.9–8.3). Most rabid dogs died or were killed before quarantine (75%) and all died by day 3 of quarantine, compared to <1% of quarantined case-negatives. The greatest risk of death was predicted to be for persons bitten on the head or neck from symptomatic dogs. Bites from dogs deemed healthy by veterinary assessors and which were available for quarantine presented less than a 0.05% risk of rabies death to the victim. Conclusions: Vaccination of all persons exposed to a suspected rabid dog is a highly effective approach to minimize human rabies deaths. However, this may place undue financial burden on bite victims that have had a low-risk exposure and over-prescription may contribute to regional supply shortages. The results here indicate that in a low-resource country such as Haiti, a well-trained veterinary assessor can provide an accurate risk assessment of biting dogs based on a standard case investigation protocol. In canine rabies endemic countries with limited access to PEP, or where PEP costs may cause undue burden on bite victims, structured risk assessments by trained professionals may be a reliable method of triaging PEP for bite victims. Evaluating rabies risk through a matrix of bite location and risk

factor in the dog presents a clear delineation of high and low risk encounters and should be used to develop data-derived PEP recommendations.

Keywords: rabies; post-exposure-prophylaxis (PEP); surveillance; risk assessment; dog bite; Haiti; retrospective cohort

1. Introduction

Rabies is a zoonotic disease responsible for at least 2.5 million human rabies deaths over the past century [1]. The overwhelming majority of human cases are the result of a bite from a rabid dog [1,2]. Rabies is vaccine-preventable; however, in the absence of appropriate post-exposure prophylaxis (PEP) the rabies virus induces an acute neurologic illness followed by inevitable death. Canine rabies is endemic in the Republic of Haiti, where an estimated 130 human rabies deaths occur annually [1,3]. Persistent human rabies deaths are due to a culmination of factors often seen in low and middle income countries: low dog vaccination coverage, low awareness, and lack of access to PEP in rural areas, all of which are barriers encountered in Haiti [3].

Until the rabies virus is eliminated in the reservoir population, humans will remain at risk of exposure. In rabies endemic countries, appropriate and timely wound management and PEP are critical for preventing human rabies deaths [4]. The World Health Organization (WHO) provides recommendations for rabies post-exposure prophylaxis after a dog bite in a canine rabies-endemic country but the recommendations leave ambiguity in interpretation [5]. These recommendations state that a risk assessment should be performed when considering initiating PEP [6]. However, they also recommend that “prophylaxis should be instituted immediately” and “continued while awaiting laboratory results or during the observation period” [6]. Information regarding how to assess rabies risk in a biting animal, and when assessment results should impact initiation of PEP, are not explicit in this WHO document.

While Haiti is considered endemic for rabies, results from a multi-year surveillance program revealed that only 1%–5% of biting dogs had rabies [3]. Considering current WHO recommendations, this combination of a low rabies prevalence in biting dogs and a risk-averse, conservative recommendation for PEP initiation may result in over-prescription of rabies biologics [7]. In low and middle income countries, the cost of PEP in relation to income can be quite burdensome to households [8]. As many as five rabies vaccine doses over 4 weeks are required, placing a burden on those who cannot afford the travel or time off of work, let alone the cost of biologics [6]. Furthermore, there is an unmet need in many countries for safe and accessible PEP [5]. This warrants investigation into how limited and expensive rabies biologics can be used as efficiently as possible while ensuring that truly exposed bite victims seek medical care for prevention of this 100% fatal disease.

The need to characterize low-risk rabies exposures has become an increasingly important issue in Haiti, where a government-operated post-bite dog investigation program resulted in an 85% increase in the detection of persons with dog bites [3]. As Haiti has adopted the WHO rabies exposure recommendations, this increase in bite detection significantly increased PEP utilization and costs for bite victims [9,10]. Undurraga et al. reported in 2017 that delaying PEP when the risk of rabies was low, and when the dog was available for quarantine, could reduce PEP costs without imposing undue risk to bite victims in Haiti [9].

This community based animal rabies surveillance program, Haiti Animal Rabies Surveillance Program (HARSP), employs veterinary professionals to investigate biting dogs, conduct a rabies assessment, quarantine or euthanize, and provide rabies counseling to bite victims [3]. Under this type of structured program, the possibility for standardized risk assessment has become possible, but there is an unmet need to specify what WHO considers to be a low-risk exposure. This study provides a descriptive analysis of the characteristics of rabid versus non-rabid dogs that have

bitten humans. The purpose of this study was to evaluate whether a risk assessment conducted by veterinary professionals in a program such as HARSP could reliably approximate the risk of rabies in a biting dog, and to generate evidence for the classification of low-risk exposures in the canine rabies endemic setting.

2. Methods

2.1. Data Set and Cohort Selection

Data analyzed for this study were made available from the Haiti Ministry of Agriculture from the national HARSP database (2013–2015). HARSP assessors are trained veterinary agents from the Ministry of Agriculture, Natural Resources, and Rural Development (MARNDNR) and undergo a one-week field training program conducted by the CDC and the Christian Veterinary Mission, and must pass the Global Alliance for Rabies Control's Rabies Educator Certification program [11,12]. HARSP has standard investigation protocols and investigation forms (see Supplementary Materials) for which veterinary agents must display proficiency. New assessors conduct job shadowing for 2–4 weeks. Each department has one lead technician who typically has two years of agriculture-focused education at the University level and is responsible for compiling reports for central collection.

Assessors receive reports of animals that are suspected to have rabies from the Ministry of Health, hospitals, veterinarians, and directly from community members. Assessors respond within 24 h to assess the animal, make a determination as to whether to pursue a 14-day quarantine or euthanasia, and to counsel the bite victim on appropriate post-bite wound care. Animals considered symptomatic for rabies are immediately euthanized and submitted to the national laboratory for testing. All samples are confirmed by the direct fluorescent antibody test. Results are validated bi-annually at the United States Centers for Disease Control and Prevention (CDC) to ensure diagnostic proficiency. Standardized data are collected and entered into a national database (Microsoft Access). At the conclusion of an investigation, each dog is classified as 1-confirmed case, 2-probable case (clinical case definition), 3-suspect case (unavailable for assessment), 4-case negative (14-day quarantine or negative direct fluorescent antibody result) [3]. All investigation forms and case determinations are reviewed by a program manager weekly.

Data for the time period from 1 January 2013 to 31 December 2015 were used for analysis. The analysis was limited to dogs involved in a human bite event for which diagnostic test results or quarantine results were recorded (classifications 1 and 4). Dogs classified as probable or suspect rabies cases were excluded. The final cohort analyzed comprised 48 rabies positive dogs and 1361 dogs for which rabies was ruled out (total $n = 1409$).

2.2. Evaluation of Single Variables

HARSP surveillance data contained twelve stratified and binary variables that were considered in this study to represent various demographics and clinical signs present in rabid dogs: entity reporting the bite, ownership status of the dog, the number of individuals bitten, dog's sex, dog's age, presence of aggression, hypersalivation, paralysis, lethargy, vaccination status of the dog, condition of the dog upon location, and the decision of the trained veterinary agent (also referred to as the assessor). Further details on each variable are described in Table 1. Risk ratio (RR) and corresponding 95% confidence interval (CI), sensitivity (S_n), specificity (S_p), positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), and negative likelihood ratio (NLR^{-1}) of each variable were calculated, using both Microsoft Excel and CDC Epi Info software. Risk ratios were calculated for variables with greater than 2 strata against an assigned reference group within that variable.

2.3. Evaluation of Mortality during Quarantine

All case negatives ($n = 1361$) were compared to confirmed rabies cases ($n = 48$) to determine how many died before and during quarantine. The time period between the date of report and the date of death—regardless of quarantine status—was used to calculate the variable corresponding to the number of days until a dog died. The date of death is the date that the dog was reported to have died, was found dead by a veterinary agent, was killed (by the public or in quarantine for humane purposes), or died by natural causes (rabies or other disease). The percent of biting dogs which were alive was calculated on a daily basis for a total of 14 days, the quarantine duration used for biting dogs in the Republic of Haiti.

Table 1. Summary of variables.

Variable	Strata	Specifics
Entity Reporting the Dog Bite Incident	All Health Sectors Veterinary Sectors Public	Ministry of Health, Local Health Department, Hospitals Veterinarians and Veterinary Agents Notifications originating directly from a community member
Ownership Status	Owned Stray/owner not identified	
Number of People Bitten	1 person bitten 2 people bitten 3 or more people bitten	
Age	Puppy Junior Adult Unknown	<6 months 6 months–1 year >1 year No age reported
Sex	Female Male Unknown	
Aggression	Present or Absent	Aggression is determined by the rabies assessor
Hypersalivation	Present or Absent	Hypersalivation is determined by the rabies assessor
Paralysis	Present or Absent	Paralysis is determined by the rabies assessor
Lethargy	Present or Absent	Lethargy is determined by the rabies assessor
Vaccination Status	Vaccinated Not Vaccinated	Owner-reported that dog was vaccinated at least once Includes unvaccinated and unknown vaccination status
Status of Dog at the Time of Investigation	Alive Dead	Hit by car, killed, died of natural causes
Rabies Assessor's Decision	Probably not Rabies Probably Rabies Dead/Not Assessed	

2.4. Risk Matrix

A risk matrix was created to assess the probability of dying from rabies based on the physical location of the dog bite and the characteristics of the biting dog. The probability of rabies in dogs was calculated for selected high and low-risk variables assessed in this study. The probability of rabies death was obtained by the product of the two variables: probability of rabies in the biting dog and probability of death based on location of exposure. Probability of death by exposure was obtained from Babes et al. [13].

3. Results

1409 animals were eligible for this retrospective cohort analysis. Rabies was confirmed in 48 animals and ruled out in 1361. Suspect and probable cases were excluded from the study, as no definitive case determination could be assigned (probable cases, $n = 42$ and suspect cases, $n = 265$). Of the rabies-positive dogs included in the study, 29 (60.4%) were dead at the time of investigation, 7 (14.6%) were euthanized on the day of the investigation, and 12 (25%) were placed into quarantine. Table 2 and Figure 1 show risk ratios and 95% confidence intervals. Table 3 and Figure 2 show the Sn, Sp, PPV, NPV, PLR and NLR^{-1} of all variables.

Table 2. Risk ratios of the 12 dog bite-related variables.

Variable	Test Group		Rabies Positive n = 48		Case Negative n = 1361		Risk Ratio and 95% CI
	n	%	n	%	n	%	
Entity Reporting the Dog Bite Incident	All Health Sectors ¹	19	39.6%	1033	75.9%	REF	
	Veterinary Sectors ² Public	22	45.8%	252	18.5%	4.45 (2.44–8.09)	
Ownership Status	Owned	42	87.50%	1324	97.28%	REF	
	Stray or Unknown	6	12.50%	37	2.72%	4.54 (2.04–10.10)	
Number of People Bitten	Bit 1	36	75.00%	1260	92.58%	REF	
	Bit 2	8	16.67%	64	4.70%	4.00 (1.93–8.29)	
	Bit ≥ 3	4	8.33%	37	2.72%	3.51 (1.31–9.41)	
Sex	Female	14	29.17%	456	33.50%	REF	
	Male	17	35.42%	759	55.77%	0.74 (0.37–1.48)	
	Unknown	17	35.42%	146	10.73%	3.50 (1.77–6.94)	
Age	Adult	16	33.33%	803	59.00%	REF	
	Puppy	7	14.58%	37	2.72%	8.14 (3.53–18.76)	
	Junior	17	35.42%	475	34.90%	1.77 (0.90–3.47)	
	Unknown	8	16.67%	46	3.38%	7.58 (3.40–22.38)	
Aggression	Non-aggressive	9	18.75%	87	6.39%	REF	
	Aggressive	39	81.25%	1274	93.61%	0.32 (0.16–0.63)	
Salivation	Normal Salivation	29	60.42%	1351	99.27%	REF	
	Hypersalivation	19	39.58%	10	0.73%	31.18 (19.95–48.73)	
Paralysis	Non-paralytic	37	77.08%	1351	99.65%	REF	
	Paralytic	11	22.92%	10	0.35%	19.65 (11.72–32.95)	
Lethargy	Non-lethargic	44	91.67%	1357	99.71%	REF	
	Lethargic	4	8.33%	4	0.29%	15.92 (7.51–33.75)	
Vaccination Status	History of Rabies Vaccination	2	4.17%	434	31.89%	REF	
	Not vaccinated or unknown history	46	95.83%	927	68.11%	10.31 (2.51–42.26)	
Status of Dog at Time of Investigation	Found Alive	23	47.92%	1323	97.21%	REF	
	Found Dead	25	52.08%	38	2.79%	23.22 (13.99–38.55)	
Assessor's Decision	Dog not showing signs of Rabies	1	2.08%	1251	91.92%	REF	
	Dog likely to be rabid	38	79.17%	77	5.66%	413.4 (57.33–2985)	
	Dead/Not Assessed	9	18.75%	33	2.42%	268.1 (34.79–2069)	

¹ Ministry of Health, Local Health Department, Hospitals. ² Veterinarians and Veterinary Agents.

Table 3. Sensitivity, specificity, positive predictive value, negative predictive value, negative likelihood ratio, and positive likelihood ratio of the 12 dog bite-related variables.

Variable	Test Group	SENS	SPEC	PPV	NPV	NLR ⁻¹	PLR
Entity Reporting the Dog Bite Incident	All Health Sectors	39.58%	24.10%	1.81%	91.88%	0.399	0.522
	Veterinary Sectors	45.83%	81.48%	8.03%	97.71%	1.504	2.475
	Public	14.58%	94.42%	8.43%	96.91%	1.105	2.612
Ownership Status	Owned	87.50%	2.72%	3.07%	86.05%	0.217	0.899
	Stray or Unknown	12.50%	97.28%	13.95%	96.93%	1.112	4.598
Number of People Bitten	Bit 1	75.00%	7.42%	2.86%	89.38%	0.297	0.810
	Bit 2	16.67%	95.30%	12.50%	97.01%	1.144	3.544
	Bit ≥ 3	8.33%	97.28%	10.81%	96.78%	1.061	3.065
Sex	Female	29.17%	66.50%	2.98%	96.38%	0.939	0.871
	Male	35.42%	44.23%	2.19%	95.10%	0.685	0.635
	Unknown	35.42%	89.27%	10.43%	97.51%	1.382	3.302
Age	Adult	33.33%	41.00%	1.95%	94.58%	0.615	0.565
	Puppy	14.58%	97.28%	15.91%	97.00%	1.139	5.364
	Junior	35.42%	65.10%	3.46%	96.62%	1.008	1.015
	Unknown	16.67%	96.62%	14.81%	97.05%	1.159	4.931
Aggression	Non-aggressive	18.75%	93.61%	9.38%	97.03%	1.152	2.933
	Aggressive	81.25%	6.39%	97.03%	90.63%	0.341	0.868
Salivation	Normal Salivation	60.42%	0.73%	2.10%	34.48%	0.019	0.609
	Hypersalivation	39.58%	99.27%	65.52%	97.90%	1.643	53.873
Paralysis	Non-paralytic	77.08%	0.73%	2.67%	47.62%	0.008	0.027
	Paralytic	22.92%	99.27%	52.38%	97.33%	1.288	31.190
Lethargy	Non-lethargic	91.67%	0.29%	3.14%	50.00%	0.035	0.919
	Lethargic	8.33%	99.71%	50.00%	96.86%	1.088	28.354
Vaccination Status	History of Vaccination	4.17%	79.96%	0.46%	95.74%	0.834	0.208
	Not vaccinated or unknown history	95.83%	57.20%	4.73%	97.41%	13.718	2.239
Status of Dog at Time of Investigation	Found Alive	47.92%	2.36%	1.71%	58.18%	0.045	0.491
	Found Dead	52.08%	97.21%	39.68%	98.29%	2.029	18.654
Assessor's Decision	Dog not showing signs of Rabies	2.08%	8.08%	0.08%	99.86%	1.020	14.496
	Dog likely to be rabid	79.17%	91.92%	33.04%	99.23%	4.764	102.446
	Dead/Not Assessed	18.75%	97.58%	21.43%	97.15%	1.196	6.572

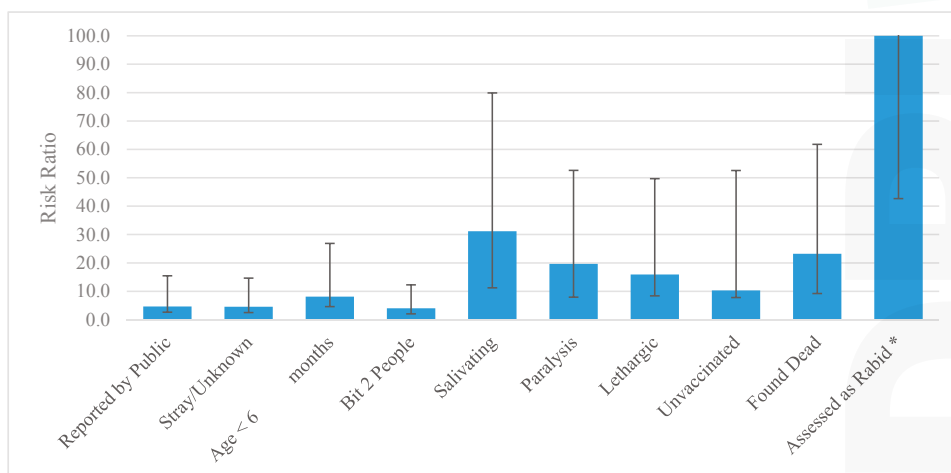


Figure 1. Risk ratio of significant variables and their 95% CI. * “Assessed as rabid” has a RR = 413 and 95% CI upper limit = 2985. For ease of comparison to other variables in this figure, it is limited to RR = 100 and no upper limit is shown.

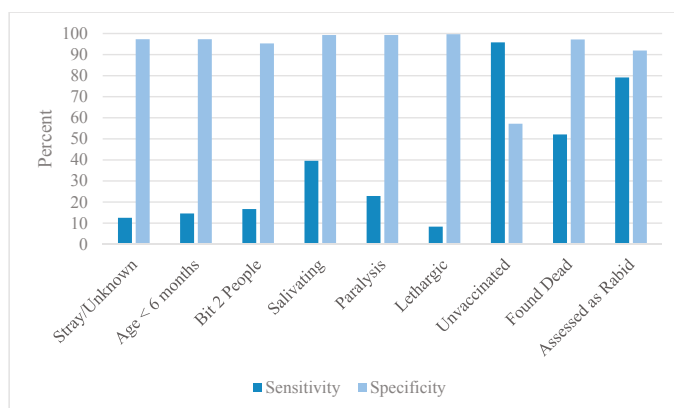


Figure 2. Sensitivity and specificity of the relevant variables.

3.1. Single Variable Results

The assessor's determination that the animal likely had rabies was the most significant predictive factor for a rabid dog (RR = 413.4, 95% CI 57.33–2985). Clinical factors significantly associated with rabid dogs included hypersalivation, paralysis, and lethargy (RR = 31.2 95% CI 20.0–48.7, 19.7 95% CI 11.7–33.0, and 15.9 95% CI 7.5–33.8, respectively). Dogs that were dead at the time the assessor arrived for investigation were 23.2 times more likely to have rabies compared to those that were alive (95% CI 14.0–38.6). Rabid dogs were 8.1 times more likely to be puppies than adult dogs (95% CI 3.5–18.8). Dogs lacking a history of rabies vaccination and unowned dogs were significantly more likely to be rabies positive (RR = 10.3 95% CI 2.5–42.3 and RR = 4.5 95% CI 2.0–10.1, respectively). Rabid dogs were four times more likely to have bitten multiple people (RR = 4.0 95% CI 1.9–8.3). Sex was the only variable assessed in this study that did not display an association with rabid dogs. Aggressive behavior was protective (RR < 1). However, this dataset is limited to biting dogs, therefore this should be interpreted cautiously.

When analyzed independently, the sensitivity of most variables was low. Of those that were above 50%, a lack of rabies vaccination was the highest (Sn = 97.8%). Other notable variables with good sensitivity were the rabies determination of the assessor and when the dog was found dead at the time of investigation (Sn = 79.2% and 52.1%, respectively). In contrast, most variables had a specificity >90%. The notably low specificities were the vaccination status (57.2%) and the presence of aggression (6.4%).

Likelihood ratios indicate the probability that a biting dog with the presence of a particular variable is rabies positive, compared to a biting dog that does not have the presence of that variable [14]. Likelihood ratios were interpreted as follows: >4 indicated a 25% increase in probability of an outcome (moderate), and those >10 indicated a 45% increase in probability of an outcome (large) [15,16]. The NLR^{-1} results show a moderate increase in the probability that a rabid dog will be classified as rabid by a veterinary agent compared to case negatives (4.8), and a large increase in the likelihood that a biting dog is rabid if it has an unknown or no vaccination history (13.7). The PLR results indicate a moderate increase in the probability of rabies for stray/unknown dogs (4.6) and puppies (5.4), and a large increase for hypersalivation (53.8), paralysis (31.2), lethargy (28.4), and being found dead (18.7). A large increase in probability was found in the PLR for both assessor decisions of a dog that has rabies (102.4) and does not have rabies (14.5).

3.2. Mortality and Quarantine Results

Twenty-four of the total cohort died during the quarantine period, of which 12 tested positive and 12 tested negative for the rabies virus. The majority of rabid dogs were dead at the time of

investigation ($n = 36$, 75%), and of the 12 dogs quarantined, 100% were dead by the third day of quarantine. The average duration until death for a rabid dog that was placed in quarantine was 1.75 days (Figure 3). Forty-two of the case-negatives died before a quarantine was issued (3.1%). Assessors placed 1319 of the case-negatives into quarantine, of which 1307 (99.1%) were healthy after 14 days, and 12 died (0.9%). The average duration until death for a case-negative dog that was placed into quarantine was 3.67 days, 2.1 times longer than for rabid dogs (See Supplementary Materials for the table of values that correspond to Figure 3).

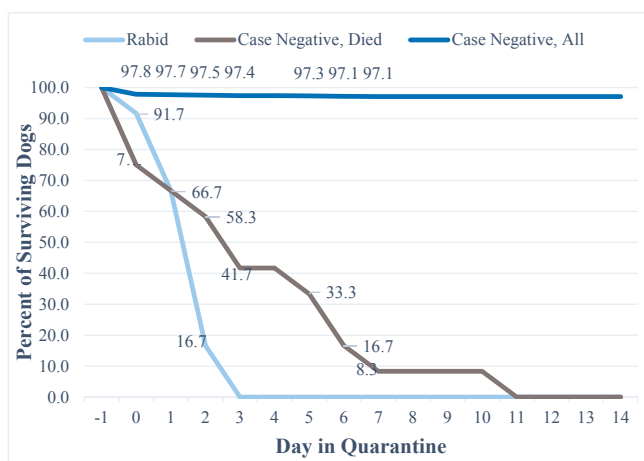


Figure 3. Proportion of surviving dogs during a 14-day rabies quarantine.

4. Discussion

4.1. Objective of the Study

Although untreated rabies infections are 100% fatal, vaccination of all persons bitten by a dog in a canine rabies endemic country is a highly conservative approach to minimize rabies cases, and it may place an undue burden on low-risk exposure bite victims while also contributing to regional vaccine supply shortages [7,9,10]. Few studies describe the risk of rabies in biting dogs in low and middle income countries, and global recommendations provide little guidance on how biting animals should be assessed. A 2005 study by Tepsunmethanon et al. in Thailand looked at six criteria in living dogs to determine rabies status, with all but one of the variables focusing on clinical signs and disease course [17]. This algorithm was reported to have high Sn and Sp ($Sn = 90.6\%$, $Sp = 96.0\%$), establishing the precedent that risk assessments may be a reliable method for determining PEP recommendations. However, the Tepsunmethanon evaluation must be completed over a 10-day period, making the results incompatible for determining risk for the purpose of PEP initiation [5,17]. This study included rabies positive and case negative dogs, but excluded dogs that were defined as suspect or probable. This selection bias was essential, because determining defining characteristics of rabid versus non-rabid dogs requires a definitive diagnosis. In addition, there is likely a contingent of dogs who are not reported, and therefore the data set is limited to only those biting dogs that have been assessed by veterinary agents. It is possible that this data set does not fully represent the biting dog population, although it is not feasible to say for certain how excluding these dogs would affect the results. The data presented here account the characteristics of over 1400 biting dogs and show that some factors collected as part of a post-bite rabies risk assessment are highly predictive for rabies and could be used to inform decisions to initiate a rabies PEP series.

4.2. Characteristics of Rabid Dogs

While it is commonly assumed that biting dogs in canine-rabies endemic countries present a high risk for rabies, this study of a post-bite surveillance system found that only 3.4% of biting dogs are actually affected with rabies. Haiti is largely considered to have one of the highest rates of canine rabies, globally. Therefore, this should not be interpreted as evidence of a low rate of enzootic rabies transmission, rather this is likely a reflection of the frequent and continuous bite exposures that occur in countries that value dog ownership, which obscures the lower rate of continuous bite exposures from rabid dogs. This low frequency of rabies among biting dogs in Haiti supports the need to develop risk assessment criteria that can reliably identify low-risk situations in which it would present little to no risk to delay PEP during a quarantine period.

This is the largest evaluation of characteristics of biting dogs in a canine-rabies endemic country, and establishes common characteristics that increase the likelihood that a biting dog is affected with the rabies virus. To little surprise, dogs with symptoms consistent with rabies were at higher risk of being confirmed rabid (hypersalivation, paralysis and lethargy, RR = 31.2, 19.7, 15.9 respectively). Even more significant predictors that a biting dog would have rabies were a lack of previous rabies vaccination and when the dog was dead at the time of the post-bite investigation. However, the greatest risk factor for predicting whether a biting dog was or was not rabid, was not a clinical sign, but rather the subjective opinion of the assessor. An assessor declaring an animal as 'rabid' increased the likelihood of rabies 400-fold. This variable has no concrete, reproducible definition, rather it is the assessor's overall judgment after considering a combination of clinical factors, vaccination history, and the circumstances surrounding the bite event. Other variables such as age, number of people bitten, and ownership status were significantly associated with rabies among biting animals, but to a lesser degree. The findings presented here confirm that it is possible to identify rabid animals, with high confidence, based on a basic health evaluation by trained veterinary professionals.

4.3. Decision Making Based on the Variables

Sensitivity refers to a test's ability to identify positive cases, and a high sensitivity would result in few rabid dogs being misclassified as case-negative [18]. Rabies is an invariably fatal disease if PEP is not initiated in a timely manner, therefore it is more prudent to consider the sensitivity of variables in a risk assessment for PEP determination. Relying on variables with low sensitivity for PEP recommendations could result in a proportion of bite victims being incorrectly told that rabies was not a risk, PEP would incorrectly be delayed or not given, and these bite victims would risk death. Relying on variables with low specificity for PEP recommendations could result in a proportion of bite victims being incorrectly told that they had a rabies exposure, PEP would be given unnecessarily, and these bite victims would have undergone an unnecessary expense. Clearly, when considering the lethality of rabies, variables with high sensitivity should be prioritized during a risk assessment.

Considering that rapid and reliable rabies diagnostic capacity is lacking in most rabies endemic countries, risk assessments and quarantine periods may be the only tools available to guide PEP recommendations [19]. For this analysis we considered the variables collected in HARSF's risk assessment as 'diagnostic evaluations' and compared them to a rabies outcome (i.e., rabid vs. case-negative). In this respect, the diagnostic sensitivity of most risk assessment variables was fairly low (on average, between 30% and 50%). Many variables, when considered alone, would not be reliable for determining rabies risk and influencing PEP decisions. Only vaccination status and assessor decision had relatively high sensitivities (95.8% and 79.2%, respectively) when compared to the Tepsu-methanon criteria (91%) [17]. The ideal variable for determining rabies PEP need would be 100% sensitive, which no single variable achieved. However, the objective of this study was to determine situations that present low-risk, not no-risk, and to identify scenarios in which PEP could be safely delayed. Given that all rabid animals died within 3 days of quarantine, reduced sensitivity of risk assessment variables may be tolerable when the dogs are available for quarantine. Certain variables stand out as having multiple significant diagnostic properties. Unowned dogs and puppies were

moderately diagnostic, with a fairly high RR, Sp, and PPV. Stronger still were the three clinical signs of lethargy, paralysis, and hypersalivation, which had high RR, Sp, PPV, and PLR. Dogs that were dead at the time of the bite investigation, and those without a documented rabies vaccination history, also displayed good diagnostic performance. However, the most important diagnostic test considered in this analysis was the rabies determination made by the assessor, which displayed consistent prognostic value across the single variables. (Sn = 79.2%, Sp = 91.9).

Consideration of the interactions of multiple variables may help further clarify a classification scheme to gauge low-risk exposures, as has been done for other diseases that require treatment before laboratory diagnosis is possible [15,20,21]. In the case of rabies, this would mean identifying a combination of easily assessable variables at the time of the bite incident for the purpose of immediate PEP decisions. There are also benefits to quick assessment of high risk exposures, as this leads to prompt healthcare seeking behaviors as well as increases adherence to treatment recommendations.

In addition to aiding risk assessment decisions, some variables may provide critical data for monitoring rabies programs. For example, in Haiti a large proportion of rabid dogs were unowned, which may influence policy makers to promote responsible dog ownership. While some variables have a clear impact on risk assessments, others may act as programmatic and policy indicators.

4.4. Evaluation of Mortality and Quarantine Data

During this study period, HARSP veterinary agents only misclassified one rabid dog as probably not having rabies at the time of the risk assessment (2.1%). In this instance the dog was quarantined and died 3 days later, suggesting that the quarantine could serve as a secondary measure to gauge rabies risk [4,22]. It is not surprising that all the confirmed rabid animals died in quarantine, but it is interesting and of relevance that 75% died before the quarantine was instituted, and that the remaining dogs died within three days of placement in quarantine. Death took over a week for the majority of case-negative dogs that died in quarantine. Of all the case negative dogs that entered quarantine, over 99% were still alive by day 14. This study evaluated factors that could lead to an immediate risk assessment determination to influence PEP decisions. This data would indicate that even if a risk assessment were misclassified after a bite, rabid dogs are likely to die early during the quarantine period and this early death event should trigger a re-assessment of rabies risk.

It has been well established that a dog that is shedding the virus will show signs of illness within a 10-day time period [2,22–25]. This finding was supported by the results of this study; all rabid dogs died within 3 days of quarantine. Given both the historical findings, and findings from this study, a 10-day post-bite quarantine is supported in dogs. Programs, such as in Haiti, utilizing longer quarantine periods may be inefficiently utilizing resources that could be diverted to post-bite follow up of dogs or dog vaccination.

4.5. Risk Matrix

The risk of developing rabies from a dog bite is dependent on multiple factors including the epidemiology of rabies in the country, the type of exposure, and the probability the biting animal was infectious. By systematically collecting surveillance data through Haiti's Integrated Bite Case Management program, the risk of developing rabies can be calculated and presented as a matrix to visualize situations of high and low probability of rabies death (see Table 4). Regardless of the assessment of the biting dog, nearly all head/neck bites were associated with a high probability of death (28–0.04%). Likewise, regardless of the location of exposure, nearly all bites from dogs with symptoms of rabies were associated with the high probability of death (28–0.6%). Contrasting these high-risk settings, the probability of death was very low for most situations of non-penetrating bite exposures and all situations in which the animal was assessed as 'healthy and available for quarantine'. Tolerance for 'risk' is subjective, therefore interpretation of the risk matrix may vary. However, this presents an objective method for beginning to develop algorithms for PEP determination. The risk matrix presented here utilizes data specific to biting dogs in Haiti and may not be representative of all cultural and epidemiologic situations.

Table 4. Risk matrix ¹.

Exposure Consideration	Objective Risk Matrix														
	Probability of Rabies Based on Clinical Categorization of Bite					Limited Surveillance Program Capacity					Advanced Surveillance Program Capacity				
	Dog Symptomatic	Dog Dead At Follow-up	Dog Bite Was Not Provoked	Stray Dog	Dog Bit Multiple People	Dog Not Vaccinated	Dog Healthy and Available for Quarantine	Dog Healthy 10 Days Post-Bite	Tested Negative						
Bite to head/neck	27.99%	17.87%	6.75%	6.26%	4.77%	2.12%	0.04%	0.00%	0.00%						
Multiple severe bite wounds	17.11%	10.92%	4.13%	3.82%	2.92%	1.29%	0.02%	0.00%	0.00%						
Bites to young children	17.11%	10.92%	4.13%	3.82%	2.92%	1.29%	0.02%	0.00%	0.00%						
Bites to extremities	3.11%	1.99%	0.75%	0.70%	0.53%	0.24%	0.00%	0.00%	0.00%						
Minor bites (no break in skin)	0.62%	0.40%	0.15%	0.14%	0.11%	0.05%	0.00%	0.00%	0.00%						
Category II	62.20%	39.70%	15.00%	13.90%	10.60%	4.70%	0.08%	0.00%	0.00%						
Medley et al. probability of rabies ²															

¹ Risk levels illustrated by Green (Low), Yellow/Orange (Moderate), and Red (High). ² Probability of rabies as calculated from the data sets used in this study. "Dog Bite Was Not Provoked" was a best guess estimate at 15%.

5. Conclusions

HARSP is a unique integrated bite case management program for low-resource settings that employs para-veterinary professionals dedicated to assessing biting animals. The risk factors identified in this study will act to inform veterinary agents in Haiti as they conduct in the field assessments of biting dogs. These assessors operate under a defined protocol and training regimen. It is unclear whether similar results could be reproduced in other programs where assessors might receive different training or follow different protocols. Therefore, the generalizability of these findings to other country's programs should be undertaken with attention to these differences. In many developed nations, existing infrastructure allows public health systems to delay PEP in low-risk scenarios while diagnostic testing is pursued [4]. However, in low-resource settings, reliable and timely diagnostic capacity is often not available [3,8,26]. As a consequence, the WHO's conservative vaccination policy, although prudent given rabies infection has nearly 100% mortality, may result in the over-prescription of PEP. The results here indicate that in a low-resource country, such as Haiti, a well-trained assessor can provide a highly accurate estimate of the rabies risk from a biting dog for the purposes of recommending no treatment or advising high risk victims of the need for timely treatment. Policy makers who wish to divert resources from reactive, PEP-based, prevention to pro-active, dog-vaccination oriented prevention may wish to consider conducting similar risk analyses.

Supplementary Materials: The following are available online at www.mdpi.com/2414-6366/2/2/14/s1, Supplementary S1: HARSP Surveillance Form and Investigation Checklist. Supplementary S2: Proportion of Surviving Dogs during 14-Day Rabies Quarantine.

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Conflicts of Interest: The authors declare no conflict of interest.

Disclaimer: The views expressed here are those of the authors and do not necessarily represent policy of the federal government.

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Review

The History of Rabies in Trinidad: Epidemiology and Control Measures

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Abstract: Vampire bat-transmitted rabies was first recognized in Trinidad during a major outbreak reported in 1925. Trinidad is the only Caribbean island with vampire bat-transmitted rabies. We conducted a literature review to describe the changing epidemiology of rabies in Trinidad and give a historical perspective to rabies prevention and control measures on the island. The last human case of rabies occurred in 1937 and although no case of canine-transmitted rabies was reported since 1914, sporadic outbreaks of bat-transmitted rabies still occur in livestock to date. Over the last century, seven notable epidemics were recorded in Trinidad with the loss of over 3000 animals. During the 1950s, several measures were effectively adopted for the prevention and control of the disease which led to a significant reduction in the number of cases. These measures include: vampire bat population control, livestock vaccination, and animal surveillance. However, due to lapses in these measures over the years (e.g., periods of limited vampire control and incomplete herd vaccination), epidemics have occurred. In light of the significant negative impact of rabies on animal production and human health, rabies surveillance in Trinidad should be enhanced and cases evaluated towards the design and implementation of more evidence-based prevention and control programs.

Keywords: rabies; Trinidad; Caribbean; public health; vampire bat; *Desmodus rotundus*; bat-transmitted rabies; epidemic; zoonosis; One Health

1. Introduction

Rabies is a neglected viral zoonosis of major public health and veterinary importance, present in more than 100 countries and territories worldwide [1–4]. Although vaccine-preventable, this disease is estimated to cause in excess of 60,000 annual human deaths worldwide, with the vast majority in Africa and Asia [5]. Rabies viruses are maintained and transmitted by several mammalian hosts, primarily carnivores and bats [6]. In the Americas, bats are significant reservoirs of rabies viruses, and although rabies virus can affect any species of bat, vampire bats are considered an especially effective vector due to their haematophagous nature [1,7,8]. Vampire bat-transmitted rabies is on the increase in the tropical Americas [9], where it is considered a limiting factor to livestock production [10,11], causing the death of more than 100,000 cattle annually at costs likely exceeding US \$30 million dollars [5].

Bovine rabies outbreaks (only later attributed to vampire bats) were first reported in the Americas during the late 16th century in Guatemala [12]. Similar incidents were subsequently observed in Ecuador, Brazil, and Trinidad [13,14]. In 1931, the scientific correlation between bats and human rabies was demonstrated in Trinidad, during a historical multi-species rabies outbreak [15–17]. Subsequent to this, the disease was diagnosed on the South American mainland with increasing prevalence, and was noted to have affected at least 18 countries by 1968 [1,11,16,18–21].

The twin island republic of Trinidad and Tobago is located off the northeastern coast of South America, lying approximately 12 km from Venezuela [22]. Trinidad is the only Caribbean island with vampire bat-transmitted rabies. To date, Tobago (which lies 42 km to the northeast of Trinidad [23]), remains free of vampire bats [22], hence, bat-transmitted rabies has not been reported on this island [24]. Given its importance to the natural history of bat-transmitted rabies, the objective of this study was to present a descriptive summary of the epidemiology and control of rabies in Trinidad and to provide a historical perspective to the current measures for prevention and control of the disease on the island. Specifically, we aimed to describe how the overall burden of the disease has changed over the last century in relation to the control and prevention measures implemented and use this to inform future prevention activities for this zoonosis.

2. Methodology

A literature review was conducted on rabies in Trinidad from the first reported case of the disease onwards. We leveraged the use of various well-established electronic databases, which included PubMed, WHOLIS (World Health Organization Library Database), SciELO, ScienceDirect, and the AFPMB (Armed Forces Pest Management Board). Key words included “rabies”, “epidemiology”, “rabies epidemic”, “rabies epizootic”, “Trinidad”, “bat rabies”, “bat-transmitted rabies”, “*Desmodus rotundus*”, and “paralytic rabies”. Multiple references also were sourced from the West Indiana Special Collection of the Alma Jordon Library, University of the West Indies, St. Augustine Campus, and the Library Collection at the Caribbean Public Health Agency (formerly the Caribbean Epidemiology Centre), Port of Spain, Trinidad. Supplemental case information, livestock vaccination and human post-exposure prophylaxis data were also collected from personal accounts of field investigations of contemporary animal cases, where possible.

3. Epizootiology and Epidemiology in Susceptible Hosts

3.1. Terrestrial Rabies in Trinidad

Historically, rigid quarantine laws were employed in Trinidad whereby all dogs and cats entering the British colony (apart from those from the United Kingdom) were subjected to at least six months quarantine and inspection with subsequent certification by a Government Veterinarian [25]. The last confirmed case of canine-transmitted human rabies (classical hydrophobia) in Trinidad was in 1912. No case of this type of rabies in dogs or other carnivores has been reported since 1914 [15,26–28]. In contrast, up to 1993, canine rabies was still a significant problem in the western region of Venezuela, where a minimum of 468 cases of canine-transmitted rabies occurred between 1989 and 1993 [29].

Despite the prevalence of rabies in the mongoose population in Grenada and other Caribbean islands (Puerto Rico, Cuba, and Hispaniola), to date, more than a century after the introduction of the small Indian mongoose (*Herpestes auropunctatus*) into Trinidad (1870s), this invasive species still remains on record as rabies-free [30]. In 1955, Dr. Malaga-Alba, a World Health Organization (WHO) rabies expert, detected Negri bodies in a mongoose carcass during his visit to Trinidad [31]. However, island-wide surveys conducted during the same year, and later in 1957 to assess the likelihood of the mongoose as vector for rabies in Trinidad, did not yield any evidence of rabies virus in this species [31,32]. Therefore, it is possible that either the earlier (1955) case may have represented a false positive or the sample size of the second study was not sufficient to detect the virus in the population.

3.2. Bat-Transmitted Rabies in Trinidad

The first documented human outbreak of bat-transmitted rabies occurred in Trinidad, during the first half of the 20th century. At the time the first human case was diagnosed, in 1929, the disease was already occurring in the cattle population, but was misdiagnosed [17,27]. A historical multispecies vampire bat-transmitted outbreak followed (1925–1937) that recorded the deaths of approximately 73 humans and thousands of livestock [24,26,33]. Although animals deaths attributed to poisoning were recorded from 1923 in the northwest and southwest of the island, animal deaths ascribed to an infectious agent began during July 1925 with cattle dying on the northwestern Government pastures around the capital of Port of Spain, recording a cumulative herd mortality of approximately 20% [16,17,26]. The disease spread further within the colony for the next four years until 1929, when there was a sudden spike in livestock mortality in the two southern villages of Siparia and Fyzabad [16]. In July of that year, the first documented case of bat-transmitted human rabies occurred in Siparia. The disease advanced within the human population and by the end of 1929 there was a total of 13 human cases [17].

During the period of 1929–1931, when the disease was first recognized, over 1000 animals per year (mainly cattle) were estimated to have died from rabies [15]. However, the actual mortality figures (based on clinical signs) that were recorded were substantially lower [16]. Laboratory confirmation was employed for animal cases from 1931 onwards. Animal cases occurred throughout the island, but were most prevalent in the southern districts until 1933 when a shift occurred in the geographical distribution of cases, from south to north [34]. Human case distribution followed a similar trend until 1935, when disease prevalence peaked with cases occurring principally in the northern community of Santa Cruz [16,17,34], as illustrated in Figure 1. During the human epidemic the highest numbers of cases were reported in Siparia (9), Santa Cruz (10), and Biche (12), and the disease progressed from southwest to northeast within the island [35]. The last human rabies case was in 1937 [24,28,36]. At this time, there was a notable decline in rabies prevalence in the animal population, with approximately 74% fewer cases than the previous year [34]. The clinical disease in humans was typically acute myelitis with spreading (flaccid) paralysis of the lower or upper limbs depending on the bite site [17,33]. Incubation was usually 3–6 weeks with paresthesia (numbness and tingling) and paresis preceding paralysis [17,33]. Other common symptoms included pyrexia (99–104 °C), urine retention, constipation, and profuse salivation and perspiration [17]. Disease duration was generally 6–10 days resulting in death and laboratory confirmation was made by the visualization of Negri bodies in brain tissue [17].

After 1937, a reduced prevalence of rabies was observed in the resident animal population, with sporadic outbreaks every few years [16,24,34]. As illustrated in Figure 2, large outbreaks were noted during 1944, 1951, 1954–1955, 1997–1998, and 2010, with smaller events occurring in 1953, 1958, 1960, 1974, 2000, and 2012–2013 [16,24,34,37]. During 1944, 73 bovine cases were documented in the central and northeastern regions of the island followed by a shift to a southern geographic distribution with a similar sized outbreak in 1951, a smaller outbreak in 1953 (41 cases) and a total of 328 rabies cases (89% bovine) between 1954 and 1955 during a major epizootic event [31,34]. During the smaller 1958 event (18 cases), in addition to cattle, cases also occurred in goats, donkeys, and even pigs [38]. In contrast, a comparable sized outbreak two years later (1960) only affected the bovine population [38]. Figure 3a illustrates the distribution of animal cases from the first observation of the disease until 1962 around which time there was an apparent decrease in the geographic range of cases. Figure 3b illustrates the distribution of animal cases from 1971–2015 which, when compared to Figure 3a, demonstrates the decrease in the geographic range of cases.

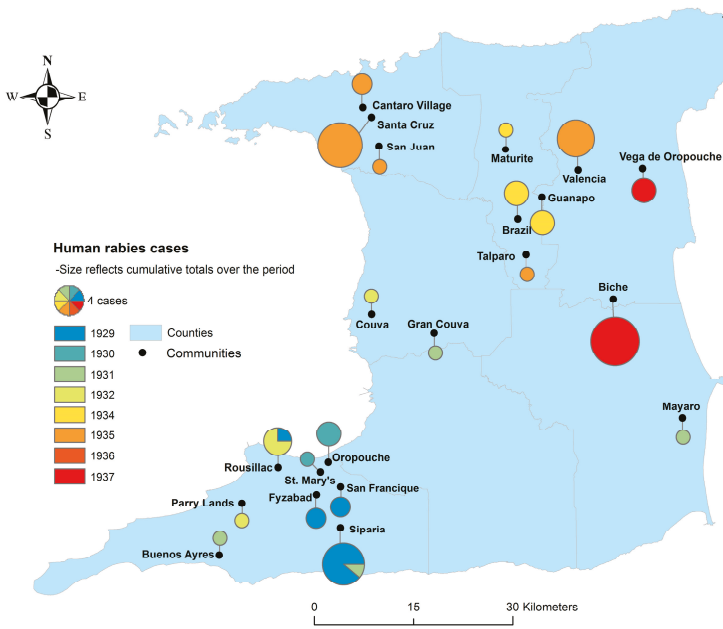


Figure 1. Geographic locations and number of cases by year for bat-transmitted human rabies cases in Trinidad during the period of 1929–1937.

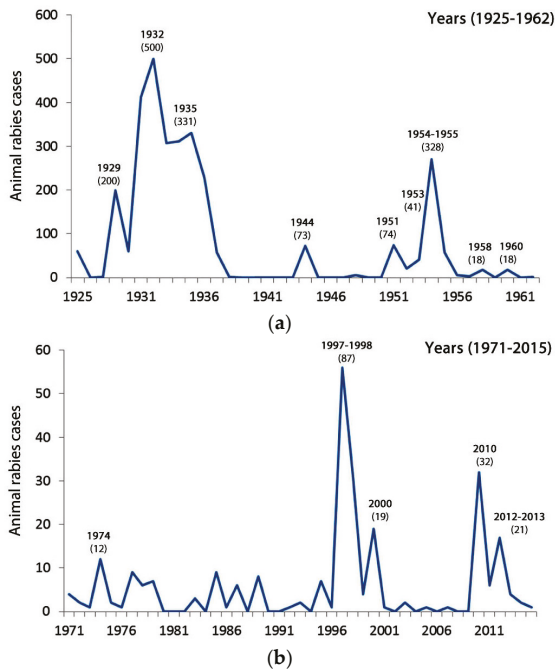
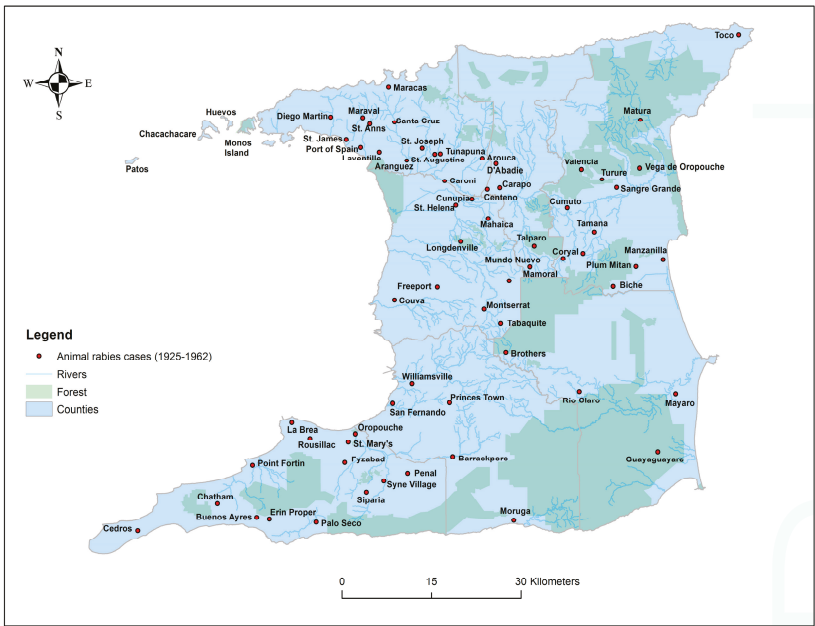
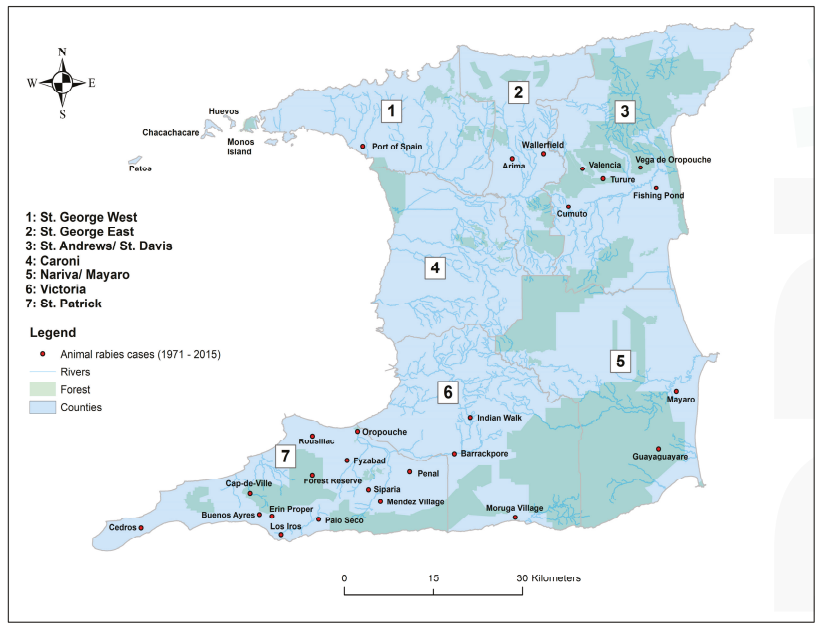


Figure 2. Animal rabies cases reported in Trinidad during the periods (a) 1925–1962 and (b) 1971–2015.



(a)



(b)

Figure 3. Geographic locations from which animal rabies cases were reported in Trinidad during the periods (a) 1925–1962 and (b) 1971–2015.

After a single case of bovine rabies in 1962 [39], limited information was available on cases until 1971. One study reported two cases of rabies from Trinidad in 1965 [40], and another noted that there was a low sporadic incidence of the disease on the island from 1968 to 1985 [21]. In 1971, four bovine cases were diagnosed [37] by the Veterinary Diagnostic Laboratory (VDL) of the Ministry of Agriculture, which assumed responsibility for rabies diagnostics in 1956 [39]. Initially, diagnostic testing in Trinidad was conducted by histopathological examination of brain tissue for Negri bodies, and intracerebral inoculation of mice with the brain tissue homogenate [15,26,41,42]. However, these methods were phased out and replaced by the direct fluorescent antibody (DFA) test, which was implemented at the VDL in the 1970s [43].

The number of rabies cases decreased proportionally until 1974 when there was an abrupt spike of 12 ruminant cases [37]. Thereafter, cases occurred sporadically, primarily in the southwestern area of the island, until 1997 [37]. An epizootic spike occurred during 1997–1998 consisting of 87 cases (90% bovine), mainly from the northeast region of the country, particularly Wallerfield (76%) [37]. In 1999, the disease again appeared in the south with four cases (including one bat) confirmed from Guayaguayare in the southeast [37]. In 2000, 19 bovine cases were diagnosed [37], mostly from the southern villages of Fyzabad and Mayaro. Isolated cases of bovine rabies occurred from 2001 to 2007 in the northeast (Valencia and Fishing Pond) and southwest (Barrackpore and Siparia) regions, until the most recent epizootics in 2010 (32 cases) and 2012–2013 (21 cases), which consisted of ruminant cases and one bat case in 2012, with a notable increase in the prevalence of small ruminant (caprine and ovine) cases, occurring primarily in the southwest particularly from the town of Penal [37].

During the 2010 epizootic, 91% (21 cases) of confirmed animal rabies cases for which age was recorded ($n = 23$ cases) were ≥ 4 months and eligible for rabies vaccination under the national rabies vaccination program [44]. Forty-nine percent (14 cases) of confirmed cases ($n = 29$) for which vaccination status was recorded were eligible for vaccination due to their age but not vaccinated [44]. For the 2012–2013 event, 85% (17 cases) of confirmed cases were eligible for vaccination, but only 15% (three of 20) were documented as being vaccinated [44]. Animal vaccination data was not available for the previous epizootics. The only epizootic event for which human rabies post-exposure prophylaxis (PEP) data was available was in 2010. During this event, 54 humans (82% male) received rabies PEP within the main outbreak area of county St. Patrick, the majority (41 persons) were between the ages of 22–63 years old [45]. Only 72% completed the full course of vaccination, while 28% did not complete PEP [44,45]. Rabies immune globulin was not administered, as it was neither warranted due to the categories of exposure nor available locally at that point in time [44]. No human rabies cases occurred.

4. Rabies Control and Prevention Strategies

Over the last century, bat-transmitted rabies in Trinidad has evolved from a mysterious syndrome causing significant loss of both human and animal life to a vaccine-controllable disease of ruminants. As a prototypic zoonosis, the prevention and control of rabies is mainly targeted at the animal host. In this light, historically, several strategies have been adopted to prevent rabies in Trinidad. These focus on surveillance, vampire bat control, and livestock vaccination strategies, and will be discussed herein within the context of the epidemiology, socio-cultural factors, and natural history of the disease in Trinidad.

4.1. Vampire Bat Population Control

Trinidad was the first country to administer a government program for the control of vampire bats. The Bat Control Unit was established during 1934 under the Medical Department, but due to the predominance of livestock cases after 1937 it has since been relocated to the Ministry of Agriculture, where it currently resides as the Anti-Rabies Unit [34,46]. Vampire bats are the primary focus of control efforts as their haematophagous feeding upon mammals favors viral transmission [15]. In Trinidad, two species of vampire bats are indigenous, *Diaemus youngi*, and the more common *Desmodus rotundus* [22]. As previously noted, no vampires have been reported in Tobago [22,24,47].

Methods of vampire bat control have been studied extensively and implemented in Trinidad, based on correlations between the ecology and behavior of these bats and rabies outbreaks [24,26,48–51]. The control method presently promoted is the topical application of an anticoagulant (most commonly warfarin) paste to the vampire bats, which then return to the roost where they contaminate others in the colony, leading to decimation of the roost population [52].

In Trinidad, culling resulted in an approximately 88% reduction in the annual numbers of *Desmodus* bats caught from 1936 to 1942 [34]. The average annual cull rate of 2000 *Desmodus* bats during this period was estimated to have saved 3720 gallons of livestock blood per year and presumably reduced rabies virus transmission [10]. In recent times the annual vampire bat cull rates have been more conservative (e.g., 812 bats caught in 2002 compared to 2136 in 1971) [44]. This could be the cumulative effect of years of successful vampire population control programs with decreased overall population size leading to fewer bats being caught and a reduction in trapping efforts as disease priority and resource allocation dropped. For example, since about 2004, night trapping and anticoagulant pasting of vampire bats have been limited and was further challenged in 2006 by a shortage of anticoagulant paste in Trinidad [44]. This, in turn, appears to have precipitated increased reports of vampire bat-biting in livestock (e.g., from 2007 to 2009 there was an increase of 1369 reported bat biting cases in livestock) [44] cumulating in the 2010 epizootic.

Reduced cull rates have been manifested by observations of increased numbers of vampires in known roosts and increasing bite rates in both animals and humans. While vampire vector control by culling may pose a challenge to the conservation of biodiversity and some studies have indicated a possible increase in circulating virus due to the elimination of virus immune adults [53], there are currently no known feasible alternatives. Therefore, vector control by culling is the practice currently applied by all countries with vampire bat-transmitted rabies. Although genetic evidence for male-biased dispersal of rabies virus [54] may allow for selective culling, more ecologically sustainable approaches should be actively sought for the long term. It is also recommended that personnel charged with conducting control measures be properly trained in bat identification to avoid decimation of other bat species essential to pollination, seed dispersal, and insect control [55]. Some potential avenues to explore include a topical oral vaccine delivered in a similar fashion to the anticoagulant paste, a vaccine introduced to livestock which inoculates the vampire bats during feeding and prey management strategies to selectively exclude populations from depredation [56].

4.2. Animal Vaccination

In 1932, mass animal inoculations against rabies were initiated in Trinidad utilizing a locally-produced carbolised brain tissue vaccine, which was eventually phased out and replaced by a commercial purified chicken embryo cell vaccine [34]. In 1956, the Paralytic Rabies Regulations was enacted, which made animal rabies vaccination mandatory and stipulated a penalty charge for contravention [57]. Rabies livestock vaccination is currently provided by the government free of charge and only one commercially-available (inactivated cell culture based) rabies vaccine formulation has been used for this purpose in Trinidad for over 20 years. Due to the feeding habits of the *D. rotundus* vampire bat, bovine rabies cases typically predominate on the island [7,37]. As such, the bovine population has been the traditional target for rabies immunization in Trinidad, although recent increases in case attack rates for the caprine population [37] would suggest value in routinely vaccinating other livestock species. Vaccination was also recently extended to exotic mammals from private zoo collections (e.g., camels, llamas, zebras) as vampire biting was observed on these animals. In light of increased bat biting, further consideration should also be given to include hunting dogs in the national rabies vaccination scheme.

Most of the animal rabies cases that have occurred in Trinidad were not vaccinated for the disease. However, in some instances, as noted in 2010 and 2012, cases were reported in apparently vaccinated animals. These may represent situations of vaccine failure due to improper storage and handling of the biological agents, improper vaccine administration, variability in host immune-competence, or late

administration of the vaccine during the course of natural infection. An example of the latter scenario was the single vaccinated case in 2012, in which the vaccine was administered less than one month prior to the development of clinical signs. In this case, the immune response would not have had sufficient time to produce adequate antibodies to combat natural infection considering the peak rabies viral neutralizing antibody response is typically 28 days post vaccination [58]. Vaccine efficacy may also be affected if vaccination regimes differ from the manufacturers' recommendations due to factors such as the interference of the immune response by maternal antibodies. All these factors must be individually examined to determine the causes of variations in vaccine efficacy and decide upon appropriate action (e.g., education on proper vaccine handling, storage and administration) to rectify gaps in coverage. In 2015 the rabies vaccine coverage for the bovine population was estimated to be 70% [59] but this level is recognized to vary on a year-to-year basis, depending on resources available to deploy the annual rabies vaccination program. Additionally, records document that the vaccine population coverage does not necessarily reflect the level of herd immunity, especially given the possibility of vaccine failure. For example, in 2009, only 2780 rabies vaccines were dispensed for livestock vaccination island-wide [44,60], despite a bovine population estimate of 19,088 animals [61]. This would have represented less than 50% coverage of the bovine population, and may have contributed to the 2010 epizootic event. Current estimates of bovine rabies vaccine coverage from the field are around 40–50% [62], which maybe sub-optimal to afford herd immunity.

In 2013, a decision was taken to abolish the quarantine of imported dogs and cats. Since then an import protocol for dogs and cats entering Trinidad from canine rabies-endemic countries, including mandatory rabies vaccination and serological testing (to prove a protective rabies titer response), is the main method of canine-rabies prevention by precluding entry of the virus. Import control in Trinidad and Tobago is aided by exclusive sea borders and legislatively supported under the Animal (Importation) Control Regulations under the Animal (Diseases and Importation) Act (1954) [63]. However, these measures do not protect against the introduction of rabies virus by bats.

4.3. Surveillance

4.3.1. General Rabies Surveillance Activities and Surveillance in Livestock

Rabies surveillance in Trinidad includes active and passive activities, both of which depend heavily on the effectiveness of reporting systems. Active surveillance is conducted in the *D. rotundus* bat population, while passive surveillance is conducted for all mammals with particular emphasis on livestock. Epidemiological surveillance in livestock entails the reporting of animals being attacked by vampire bats or that are clinically suspected as being rabid. The dependence of rabies surveillance on passive reporting systems may likely result in under-reporting and under-estimation of the actual number of animal cases in general. Other factors which may contribute to the under-reporting of animal rabies cases include: (i) farm inaccessibility to the veterinary services; (ii) limited farmer knowledge of disease etiology and reporting protocols; (iii) cases in small ruminants which are more easily lost or buried; (iv) misdiagnosis of cases (e.g., canine distemper in dogs and tick fever in ruminants); and (v) missed cases due to lack of surveillance in wildlife populations.

4.3.2. Surveillance in the Bat Population

Bat biting case reports allow for the identification of areas with high vampire bat activity and facilitates active bat sampling and the implementation of vampire bat control measures. Areas on the island where high vampire activity is usually noted include Penal and Barrackpore in the southwest, Valencia and Wallerfield in the northeast, and valleys along the foothills of the Northern Range, such as Maracas, Santa Cruz, and Maraval [34]. As a result, these areas have historically demonstrated high densities of rabies cases [34,37]. Earlier uncorroborated studies conducted in Trinidad, suggested that, although rabies causes aberrant behavior and death in bats, apparently healthy vampire bats could also harbor and transmit the virus for extended periods [26,41,42]. On this basis, active surveillance of

D. rotundus populations was established and is still conducted in parallel with vector control activities. However, virus isolation is rare. For example, during the period 1971–2015, only two of 4399 bats tested were diagnosed rabid by DFA testing [37]. This represents a slightly higher rabies positive rate (0.05%) than previous findings (0.03%), but less than earlier reports of up to 3.3% [41,64,65]. Local testing mainly targets apparently healthy vampire bats in areas of high bat activity (as guided by reports of bat biting cases). On the other hand, early passive surveillance studies conducted on non-vampire bats in the United States found viral isolation rates of 76% in sick bats [66], and approximately 5–6% positivity in all bats tested [66,67]. This may perhaps indicate lower viral circulation in vampire bats due to immunity, although, higher rates would be expected with passive surveillance as mainly sick or moribund bats would be tested. Other than the two vampire species, rabies virus has been isolated from seven other bat species in Trinidad (*Carollia perspicillata*, *Artibeus lituratus*, *Artibeus jamaicensis*, *Molossus molossus*, *Diclidurus albus*, *Pteronotus davyi*, and *Pteronotus parnellii*) [15,24,41,48,68,69] under earlier active surveillance programs. In a more recent study, *Desmodus* variant viruses were found to be the cause of Trinidadian rabies epizootics [64]. Other bat variants have not been documented and, therefore, transmission to other mammalian species from non-vampire bat species has also not been conclusively documented in Trinidad.

Routine monitoring of vampire bat populations for the presence of rabies virus could potentially provide an early warning of the risk of virus transmission to a susceptible host. Alternatively, since virus isolation in the bat population is often difficult, rabies antibody titers can be monitored for increases which may indicate increased virus exposure and risk of spill-over to livestock [37]. Epizootics of rabies in vampire populations have been suggested to occur at most every four years with variable viral levels at different stages of the epizootic event [70,71]. However, further studies need to be conducted in the Trinidadian vampire bat population to confirm the frequency of epizootics and the relationship between virus and antibody levels and risk of viral transmission.

4.3.3. Surveillance in the Canine Population

Trinidad remained a British colony until 1962 [72], so the early date of canine rabies elimination (1914), when compared to neighboring Latin America, may have been attributed to the elimination of canine rabies in Britain during 1903 [73], given the implementation of similar disease control measures. Additionally, although rabies has been enzootic on Trinidad since the early 20th century, despite reports of bat biting in dogs (particularly hunting dogs), no cases of rabies have been documented in this population since the 1930s [16]. However, no routine surveillance is focused presently on detecting rabies virus in the dog population of Trinidad. Recent reports of bat biting in dogs [74] underscores the importance of having protocols in place for dealing with a potential canine rabies case. Typically, when not available for testing, wild mammalian reservoirs, such as bats, are regarded as rabid [75] and the animals they bite are considered to be exposed to the virus. As the resident dog population is not routinely vaccinated against rabies, an exposed dog would likely be unvaccinated. Given that there are currently no biologics licensed for post-exposure prophylaxis of unvaccinated domestic animals, and since evidence suggests that vaccine alone is not reliable for prevention of disease in these animals, it has been recommended that unvaccinated exposed dogs should be euthanized [76]. Alternatively, the dog can be held under strict quarantine and observation for four months (with immediate post-exposure rabies vaccination) or six months (if vaccine administration is delayed past 96 h post-exposure) [75]. However, if signs suggestive of rabies develop while under quarantine, the animal should be immediately euthanized and the brain submitted for rabies testing [75]. Stray dog management protocols should also be enforced, particularly in rural forested areas where the risk of bat biting in dogs is higher. A serological approach may also be taken to monitor rabies exposure in this population, which would determine the risk of rabies virus transmission from bats to dogs and can inform preventative measures.

4.3.4. Surveillance in the Mongoose Population and Other Terrestrial Wildlife Populations

Unlike several other Caribbean islands [30], mongoose rabies has not been documented in Trinidad. Relative to these islands, Trinidad has a lower mongoose population density, which may have allowed the population to remain apparently disease-free [77]. Alternatively, cases could go unrecognized due to the small carcass size and rapid decomposition under the tropical conditions. In Latin America, an increased risk of rabies virus transmission from wildlife has been noted [78,79]. Additionally, in North America, the first case of natural infection of an armadillo with a skunk rabies virus variant and multiple spillover events from bat rabies viruses into foxes and skunks, demonstrates the possibility of rabies virus infection in non-conspecific mammalian taxa [80]. In light of these findings, local studies should be undertaken to determine if there are unrecognized terrestrial (wildlife) reservoirs for sylvatic rabies. The possibility of the initiation of a new virus-host relationship with sustained propagation in a species previously considered a dead-end mammalian host should not be precluded, and monitoring of various potential host species, (e.g., mongoose, ocelot, etc.) is recommended to identify the emergence of new viral reservoir hosts. In this regard, passive surveillance could be used to gather preliminary information which could then inform the development of more structured surveillance activities. As suggested above, serology may also be used in tandem with viral surveillance in the mongoose and other terrestrial wildlife populations to determine the extent of viral exposure and detect possible host shifts from bats.

4.4. Movement Control of Bats and Rabies Viruses

The geographic spread of rabies virus during epidemic events may be a result of the movement of the animal hosts or vectors. In the case of the former, animal movement control may curtail the spread of the disease. In Trinidad, there are regulations prohibiting the movement of potentially-rabid animals [57], but there is not enough manpower to enforce these regulations on a day-to-day basis so the appearance of livestock cases outside of the main outbreak areas usually represent human-mediated animal movement. On the other hand, viral spread facilitated by bat movement is much more difficult to control than with domestic species, as vampires from different districts visit communal feeding grounds and common livestock feeding may expedite district to district spread of the virus [26]. Nevertheless, spread of the virus during an outbreak event may be limited by the implementation of ring-vaccination of livestock around the index case(s). At present the maximum radius for ring-vaccination activities during outbreaks in Trinidad is eight miles around the index case [62]. Given a reported flight range of about 12 miles for the common vampire bat, the optimum range for ring-vaccination during an outbreak is recommended to be up to 12 miles around the last animal case [7,81]. A southwest–northeast pattern of intra-island virus progression has been observed for rabies epidemics in Trinidad [35,37]. As can be illustrated by Figure 3, over the years the geographic distribution of cases has narrowed to the southwestern regions of St. Patrick and Victoria and northwestern regions of St. Andrew/St. David and St. George East. These are areas with high-density livestock farming at forest fringes where numerous roosting sites may be found in hollow trees [37]. It is also possible that passive surveillance is more effective in these areas because their larger animal populations trigger more efficient case reporting.

Rabies virus importation to Trinidad from the South American mainland, was proposed by early researchers who also suggested that the virus was first introduced to Trinidad via this route around 1925 [42]. It is plausible that this initial introduction, considering the location of the first animal cases, occurred through the northwest peninsular by bats flying along the island chain (Patos, Chacahacare, Huevos and Monos Islands) between Venezuela and Trinidad (see Figure 3), with the first island only 2.5 miles from the mainland [24]. Subsequently, the northwest–southwest progression of animal rabies cases from 1925–1929 may have represented the initial flight path of infected bats possibly driven by the availability of food sources. Thereafter, human cases followed a southwest to northeast unidirectional pattern, previously hypothesized to be due to bats moving along the Earth's magnetic field [35]. More recently, a study on the phylogeography of Trinidad rabies viruses provided evidence

for at least three independent introductions of virus into Trinidad from the mainland during 1972, 1989, and 2004, and suggested the *D. rotundus* bat as the vector of introduction at the southwestern peninsular of the island with similar northeasterly progression of the virus during epizootics [64]. The flight range for the more common vampire (*D. rotundus*) is 20 km [82], so the distance between Trinidad and Tobago (42 km) [23] (in contrast to only 12 km between Trinidad and the mainland [22]) may preclude the movement of vampire bats between these islands, which would explain the absence of vampire bat rabies in Tobago. Further studies are currently underway to investigate the relationship between rabies virus spatiotemporal dynamics and vampire bat population ecology in Trinidad.

The lack of human rabies cases since 1937, may be attributed to the modernization of housing and associated infrastructure (including indoor and outdoor lighting), which may preclude the free entry of bats into human dwellings. Evidence of this effect may be illustrated by the fact that no rabies cases were reported in urban areas during the human outbreak in the 1930s where housing conditions were generally better than in rural areas, where cases occurred exclusively [35]. Recently, in some rural areas in Trinidad, human bat biting has noted to be increasing, possibly due to an increase in the vampire population. Furthermore, human population expansion into rural areas and the accompanying reduction in total forest cover over the last few decades [83] may result in increased human-bat contact and facilitate viral transmission to vulnerable groups.

4.5. Human Vaccination and Risk Communication Programs

In Trinidad, pre-exposure rabies vaccination is conducted for high-risk personnel (e.g., laboratory staff, veterinarians, and animal health staff), with biennial booster vaccinations. However, ideally, rabies virus neutralizing antibody levels should be monitored every six months or two years in these personnel depending on their risk of exposure, with booster doses if serum titer levels fall below 0.5 IU/mL [5]. The WHO-recommended Essen (five-dose) regimen is used for rabies post-exposure prophylaxis (PEP) of previously-unvaccinated individuals, with the rationale for PEP administration ideally based on the WHO guidelines [2,4]. Using these guidelines, the risk of rabies virus transmission by the handling of suspect ruminant cases is thought to be low, which is further supported by infrequent virus isolation from bovine saliva with bat-transmitted disease [84]. However, during 2006, an unvaccinated Brazilian veterinarian was infected via non-bite exposure while treating a rabid herbivore and died, highlighting the fact that the benefits of vaccination far outweigh the risks [85].

In Trinidad during 2010, the demographic of the cohort of humans administered PEP was consistent with that of small scale livestock farmers and butchers. The level of contact reported for farmers was primarily the handling and attempted treatment of suspect animals, which mainly involved manual (bare-handed) manipulation of the oral cavity of cattle during attempts to remove an assumed foreign body during suspected choking events. Even though the physical handling of a rabid animal on its own is not generally considered an exposure, the potential opportunity for scratches and abrasions from bovine teeth and oral cavity papillae complicated the risk assessment. The risk for butchers was based on possible splash exposure during the slaughtering of potentially-rabid animals. In most instances PEP was administered as a precaution due to the opportunity for exposure. In total, during the 2010 epizootic, 342 doses of human diploid cell vaccine (HDCV) were administered [44,45], which was projected to cost more than US\$75,500 using previously published HDCV cost estimates [86]. This figure would also have been higher if rabies immune globulin was needed and if all individuals adhered to recommendations for completion of the full-course of PEP. This emphasizes the relevance of risk communication to occupationally high-risk groups in an effort to reduce such exposures. Apart from the public health risks during animal production and slaughter, other risks are associated with consumption of raw or improperly-cooked meat. In fact, in New Mexico, 4% of cattle slaughtered for human consumption were reported to be infected with rabies, and although no human cases were documented to arise from the meat consumption, dogs which consumed the uncooked meat were noted to develop a paralytic disease [87]. In Trinidad, the carcasses of suspect rabid animals are buried on site

at the farm after samples have been taken for laboratory testing and in some instances (especially in cases of sudden death) the whole carcass is buried by the farmer without sampling. Dogs have been known to try digging up these carcasses, especially in cases of shallow burial. This poses a risk to dogs, as well as humans in contact with these animals.

Rabies educational programs, which are predominately reactive outbreak communications, can incite and scare the recipient population, with detrimental outcomes on the livestock market. Therefore, in Trinidad, where the disease is endemic, a recommended approach to risk communications would be routine rabies awareness programs with precaution advocacy in high-risk areas targeting high-risk groups. This may additionally increase compliance to vaccination programs and reduce the rabies prevalence in the animal population. Public education and awareness activities must also take into account the local cultural practices and provide information in a strategically-targeted, timely manner. For example, large-scale domestic slaughtering of livestock often conducted prior to specific cultural events in Trinidad provides an ideal opportunity for educating butchers on the potential risk of slaughtering rabid animals. Furthermore, the annual observation of World Rabies Day on 28 September presents a unique opportunity to conduct large-scale rabies awareness activities on a predictable basis.

5. Conclusions

Rabies poses major public health and veterinary health challenges in Trinidad. Given the epidemiology of rabies in Trinidad and existing prevention and control measures, rabies epizootics are likely due to gaps in preventative programs, such as prolonged periods of limited vampire bat population control, pockets of unvaccinated susceptible animals, and inadequate public awareness of the disease and the existing preventative measures. Therefore, actions taken to address these shortcomings should include sustained vampire control and vaccination activities (to afford herd immunity) particularly within high risk areas, as well as the implementation of routine public awareness programs. The age of primary vaccination, duration of immunity of vaccines, and the effective range of ring-vaccination efforts during outbreaks need to be evaluated in light of the available scientific data. These actions would not only result in the minimization of livestock losses, but also a substantive reduction in human healthcare costs. Furthermore, surveillance in Trinidad should be enhanced and the results examined actively to elucidate disease trends and risk factors.

Given the inter-related variable of the environmental, pathogen, public health, and veterinary concerns, future considerations should focus upon enhanced laboratory-based surveillance, epidemiologically sound livestock vaccination and risk-based prophylaxis of exposed humans, as well as novel methods for vampire bat management. In a One Health context, such information would aid in the reduction of rabies risks in both human and animal populations by facilitating the development of efficient evidence-based ecologically-sustainable approaches to disease prevention and control.

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Article

Spatial Association of Canine Rabies Outbreak and Ecological Urban Corridors, Arequipa, Peru

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Abstract: In the city of Arequipa, Peru, a rabid dog was detected in March 2015, marking the reintroduction of the rabies virus in the area; more rabid dogs have been detected since then. The presence of free-roaming dogs in Arequipa seems to be higher in dry water channels, which are widespread in the city. We created a geographic information system (GIS) with surveillance data on the location of rabid dogs detected during the first year of the outbreak, as well as the water channels. We conducted a spatial analysis using Monte Carlo simulations to determine if detected rabid dogs were closer to the water channels than expected. Thirty rabid dogs were detected during the first year of the outbreak, and they were statistically associated with the water channels (average distance to closest water channel = 334 m; p -value = 0.027). Water channels might play a role in the ecology of free-roaming dog populations, functioning as ecological corridors. Landscape ecology could assist in understanding the impact of these urban structures on control activities and the persistence of transmission.

Keywords: dogs; geographical information system; GIS; Monte Carlo method; rabies; spatial analysis; zoonosis

1. Introduction

The reintroduction of canine rabies to areas declared free of the virus is a rare event globally [1–4]. The outbreak of canine rabies in Arequipa, Peru in March 2015 is the first instance of canine rabies reintroduction in Latin America, where enormous advances have been achieved through dog vaccination [5]. Fortunately, no human cases have been detected in Arequipa to date. Genetic sequencing suggests that the rabies virus was introduced to Arequipa by dogs imported from Puno, a neighboring region in southern Peru that has reported sustained transmission of canine rabies for the last 15 years [6]. Interestingly, the urban landscape in Arequipa is characterized by the presence of large open water channels that are dry most of the time, where free-roaming dogs can be found.

In Arequipa, where 22% of owned dogs have free access to the street all the time, and 48% of owned dogs are unrestricted at some point during the day [7], it is unknown which proportion of dogs seen on the streets are actually stray dogs and which are owned. Upon detection of the first rabid dog,

Ministry of Health (MOH) authorities attempted containment by ring vaccination of an approximately five-block radius, in accordance with the Peruvian technical guidelines to control and prevent human rabies [8]. In addition, they conducted contact tracing: dogs that lived within that approximate five-block radius and that could be considered potentially exposed to the virus (unvaccinated and with relatively free access to the street or known history of contact with free-roaming dogs) were euthanized [8]. Humans potentially exposed to the virus were offered post-exposure prophylaxis [8]. Days later, a second case was detected in another district, over 1.25 kilometers from the original case (Figure 1); the same containment strategy was implemented. The next case appeared in still another district, and each subsequent case continued to be detected far beyond the containment area of the last (Figure 1). Four months after the first case was detected, mass dog vaccination campaigns were implemented by the MOH. Despite the initiation of this city-level approach in 2015, ring containment immediately following the detection of a case has also been practiced until 2017. The median vaccination coverage reported among the 29 districts of the province of Arequipa was 101% in the first year of the outbreak, with three districts reporting coverage above 200%. Similarly, the reported coverage in the ring vaccination activities was higher than 100% in all cases, suggesting a substantial underestimation of the dog population at that time. Additionally, culling of free-roaming dogs (without evidence in favor of this strategy [9–11]) has been conducted by some groups.

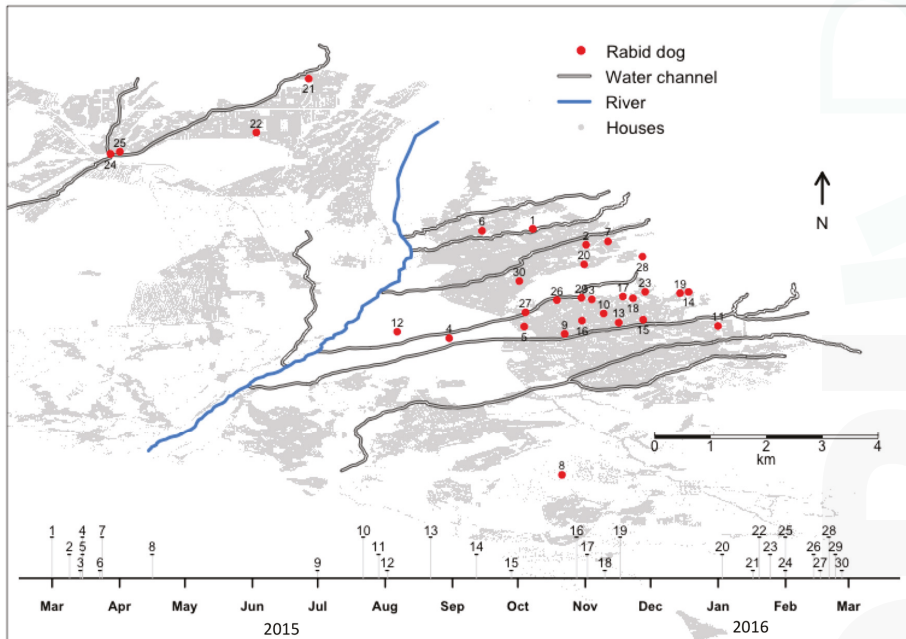


Figure 1. Spatio-temporal distribution of laboratory-confirmed rabid dogs and water channels system in the city of Arequipa, March 2015–March 2016 (different heights in timeline are used only to avoid overlap).

The reemergence of the virus transmission in Arequipa has been associated with the high density of free-roaming dogs in the city (i.e., stray and owned dogs that spend unsupervised time in the streets and water channels), and with low dog vaccination coverage [12]. The authorities report that water channels are central to the problem, because the density of dogs is higher in them, dogs feed on trash disposed in the channels, and large packs of dogs have been seen moving along these urban structures. In other parts of the world, urban structures similar to the dry water channels in

Arequipa are used by wild animals to move within city matrices. Foxes use ravines to move across urban areas of Toronto [13], and bobcats and coyotes use culverts and linear fragments of vegetation to circulate in southern California [14]. Here we considered the dry water channels of Arequipa as ecological corridors, following the definition given by Freemark for corridors [15]: “a physical linkage between habitat patches within a landscape that may serve as a pathway by which organisms move or interchange, or as a habitat in which organisms can feed or breed en route from one patch to another.” These urban ecological corridors and other physical aspects of urbanization in fast-growing cities such as Arequipa facilitate the emergence and persistence of zoonotic and emergent disease [16–18] and may complicate canine rabies control.

Despite the efforts to control the outbreak, transmission of the rabies virus continues in the city of Arequipa, placing the approximately 1 million inhabitants of the city at risk of infection. The main objective of our study was to assess the spatial association between detected rabid dogs and the most salient urban structures in the city of Arequipa—the dry water channels.

2. Materials and Methods

2.1. Study Setting

The city of Arequipa is located in the Andes at 2300 m above sea level; its temperature ranges from 10 °C to 29 °C. The Chili River runs through the city, and rainwater drains to it via uncovered semi-natural water channels. The rainy season lasts 8 weeks; the channels are dry the remainder of the year (Figure 2). The city of Arequipa has grown significantly during recent decades, reaching a population of approximately 998,000 people in 2015 and covering an urban area of over 101 square kilometers. On the outskirts of the city, urbanization creates peri-urban areas that, after decades, are absorbed by the continuous expansion of the city [18]. Arequipa is surrounded by desert, which has little wildlife that could sustain the rabies virus, nor resources (shelter, food) to support feral dog populations.



Figure 2. Dog in a dry water channel in the city of Arequipa.

2.2. Data

As part of routine surveillance activities, the MOH Reference Laboratory in Arequipa tested brain samples of suspected rabies cases using direct fluorescent antibody [19]. Additionally, mouse inoculation

testing [19] was conducted at the National Institute of Health Laboratory in Lima. Samples positive to either test were considered rabies positive. We created a geographic information system (GIS) with the coordinates of all the houses in the city of Arequipa, as well as those of the system of water channels. Over these geographic layers, we added the location of the canine rabies cases detected during the first year of the outbreak (March 2015 to March 2016). The rabies surveillance system in Peru only acquires location data of the health facility catchment where the sample was collected (areal data). Our team investigated the exact point location of positive samples. Some of these point locations were the house of the reporting owner, or the spot where a dead dog was found or an aggressive or disoriented dog was captured. We report the number of samples analyzed and why they were submitted for diagnosis.

2.3. Statistical Analysis

We conducted a spatial analysis to test the hypothesis that rabid dogs were detected closer to a water channel than would be expected by chance. We calculated the average shortest Euclidian distance between the location of rabid dogs and the closest water channel. The exact coordinates of negative dogs were not available. We used a Monte Carlo random-labeling simulation with 1000 trials to compare the average distance between observed rabid dogs and water channels to the distance from water channels of a randomly-generated spatial sample of households [20], matched on locality. We have found that the number of owned dogs per house can vary by locality [7], and we also wanted to take into account any unobserved difference in surveillance intensity at the locality level. Localities are informal district subdivisions which are widely used by the health inspectors to differentiate their catchment areas and organize their work. Under the null hypothesis, we assumed that all households are equally likely to have a rabid dog regardless of their distance to the water channels—on a door-to-door survey conducted in more than 4000 houses by our team, we did not find an association between the number of owned dogs per house and the water channels [7]. To determine whether the observed distance of rabid dogs to water channels was statistically different from the distance of the water channel to the random spatial sample, we compared the average observed distance to the distance distribution derived from the Monte Carlo trials. The proportion of distances in the Monte Carlo-generated random sample which were lower than the average observed distance was the estimate Monte Carlo *p*-value.

Separately, we assessed the spatial clustering of rabid dogs using the L statistic [20]. Briefly, spatial clustering is defined as a general tendency for point pattern events (here, detected rabid dogs) to occur more closely together than would be expected under complete spatial randomness (CSR) [21]. We also estimated the distance between each case and the subsequent case. Finally, we present if detected cases were owned dogs, and if it was reported that they bit any humans. The analyses were conducted in R [22].

3. Results

Out of 559 samples analyzed between 17 March 2015 and 16 March 2016, only 397 of the analyzed samples had some data about the case in the reporting form: 77 samples came from dogs hit by a car, 55 samples were collected during ring-containment activities, 43 forms reported that the dog was found dead or dying outdoors, 41 samples reportedly came from aggressive dogs, 37 samples came from dogs with neurological signs, 25 dogs were reported as “sick” or “sick and died”, 21 samples came from dogs that were killed or euthanized by their owners, 18 came from dogs found in the water channels, and 11 were samples or dogs sent by private veterinarians. Sixty-nine (69) forms state that the dog was found outside or was stray, but do not mention if the dog was dead when found. Thirty (30) dogs were diagnosed positive for rabies (5.37%) (Figure 1); 13 of the positive dogs were first suspected cases because they were aggressive, one was the contact of a positive dog, one was sent by a private veterinarian, one had clinical signs compatible with rabies, one was captured because it was a stray dog, and one was sent to the lab because it was “sick”.

The average distance between detected rabid dogs and water channels was 334 m. In only 27 of the 1000 Monte-Carlo simulated location sets was the average distance to the water channels 334 m or lower (Monte Carlo p -value = 0.027). When analyzing all the detected cases together, we found significant spatial clustering of confirmed rabid dogs: overall, cases were detected more closely together than we would expect at random (Figure 3). However, some of these cases that were close to each other were detected several months apart. The median distance between each detected case and the next case was 2.253 km, and 86.7% of the cases were detected one km or farther from the previous case. Importantly, at least 43% of the rabid dogs in this study had bitten at least one human and 76% of the rabid dogs were turned in to authorities by their owners. These percentages likely underestimate the actual percentages of dog bites by rabid dogs and dog ownership among detected rabid dogs.

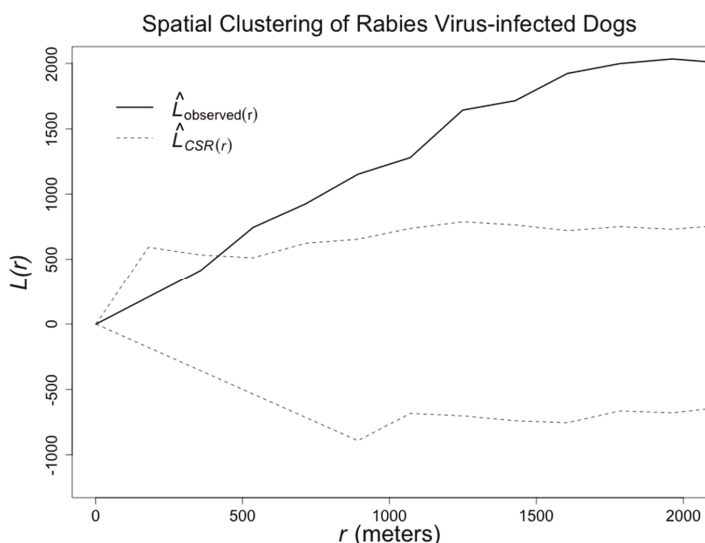


Figure 3. Strong clustering of rabid dogs evaluated with the L function. Envelopes (dashed lines) produced assuming complete spatial randomness (CSR).

4. Discussion

Rabies-virus positive dogs are spatially associated with water channels in the city of Arequipa. This association is unlikely to be related to sampling bias, as the rabies surveillance program is mostly passive: the majority of rabid dogs detected during this outbreak were reported by their owners. Free-roaming dog ecology and rabies virus transmission (e.g., habitat, movement, interactions) could be strongly influenced by water channels, as these urban structures—where trash is commonly dumped by dwellers—can produce an “ecological assembly” [23] within the city, increasing the density of dogs. Water channels may also form ecological corridors [24], increasing the connectivity of spatially-distant dog populations. It is possible that by increasing the connectivity of dog packs, the water channels facilitate the persistence of the virus by allowing it to reach new susceptible packs, similar to what has been observed at larger scales in raccoon rabies [25].

In the city of Arequipa, the high spatial heterogeneity created by the water channels poses challenges to modeling contact and networks that capture the spatial dog population structure, and analyzing models that include long-distance interactions between individuals or packs [26]. In addition, what facilitates dog movement could impede human movement: the water channels are barriers for pedestrians and could decrease access to vaccination points, thereby creating pockets of low canine vaccination coverage, which could enable the persistence of rabies virus [27].

Clearly, containment activities have not controlled the outbreak; cases have been reported regularly through June 2017 in spatially disparate areas. A first look at the spatial distribution of cases could lead to the determination that cases are generally close to each other, which is also captured by the clustering analysis. The clustering analysis simply suggests that cases are not dispersed randomly all over the city. However, the distance between each case and the next detected case suggests that subsequent cases are not occurring close—or at least not close enough—to support focalized containment activities; thus, a citywide response is needed. The initial focalized containment strategy may have diverted resources [27] and delayed population-level responses such as mass dog vaccination [27] that have proven to be successful in other settings, but are not reaching the appropriate coverage levels [5,28–30] in Arequipa [31]. It is worth highlighting that Davlin and VonVille, in a systematic review on canine rabies vaccination, concluded that understanding of the local dog population ecology is important to achieve effective vaccination coverage [32]. The unrealistic vaccination coverage estimates reported during the first year of the outbreak reflect inaccurate estimates of the dog population in the city of Arequipa. The human-to-dog ratio used to estimate the dog population was 10:1 in 2015; that ratio has been reduced to 6:1 for 2016 and 2017, approaching the true ratio, but a better knowledge of the canine population is still necessary.

In Arequipa, there are a number of plans to control the canine population that is found in the water channels. To some extent, these plans recognize the spatial heterogeneity produced by urban structures. However, if plans include the culling of free-roaming dogs, there may be negative unintended consequences [10,11,33–36]. In addition, these measures may not take into account the potential functionality of these ecological urban corridors [24] on free-roaming dog ecology. Additionally, these water channels serve a political function: they delineate boundaries between districts, which determine jurisdictions for rabies health inspectors. These areas within the water channels become a no-man's-land in the surveillance system; dead dogs are left uncollected and undiagnosed. A centralized surveillance system at the city level could better address these areas that seemingly do not belong to any district jurisdiction. The design of urban landscapes has received much attention around the world to improve health outcomes. However, most of this attention is focused on chronic disease, mental health, violence and injuries, and pollution [37]. Landscape ecology has the potential to be used in urban areas to understand zoonotic diseases and guide land management, as it has been applied for rabies in wild and rural areas [25,38–42].

One limitation of our study was the lack of locations for negative dogs—more detailed data accompanying the samples sent to the laboratory would allow for analyses with fewer assumptions. Related to the surveillance system, it is important to note that surveillance systems that are based on meeting a pre-defined quota of samples frequently receive and analyze samples of non-suspicious animals and miss the vast majority of cases [43]; therefore, the 30 rabies-positive dogs detected during the first year of this outbreak most likely represent only a fraction of the total number of cases. New surveillance systems based on active rabid-dog finding guided by triage data from dog-bite patients has the potential to increase the probability of case detection by several orders of magnitude [43]. Given that a great extent of cases were most likely missed, there is the possibility that our results of spatial clustering may be different if it were possible to account for those cases not detected by the surveillance system. It is also possible that other less ecological and more social phenomena are explaining the association between cases and water channels: dog vaccination status could be lower around the water channels due to spatial differences in educational attainment, economics, logistical constraints, among other factors. Dog ownership practices also affect dog ecology: households closest to the water channels may restrain their animals less, which would result in an increase of the free-roaming dog population in the water channel areas. Further investigations on vaccination patterns, the urban landscape, and the ecology of dogs in Arequipa could inform surveillance and control activities.

Supplementary Materials: The following are available online at www.mdpi.com/2414-6366/2/3/38/s1, S1 Abstract. Resumen en español (alternative language abstract).

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Author Contributions: R.C.N., E.Z., Y.M., and M.Z.L. conceived and designed the study; E.Z., Y.M., R.F.B., and I.C.R. acquired the data, V.A.P.S. and M.Z.L. administered and supervised the study; and R.C.N., V.A.P.S. and M.Z.L. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Heterogeneity of Rabies Vaccination Recommendations across Asia

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Abstract: Asian countries bear the greatest burden of the disease, with a majority (59%) of rabies-related deaths occurring in Asia. In order to promote best practices, we summarized national human vaccination guidelines across this region, to highlight differences and similarities and to discuss the aspects that would benefit from updates. National management guidelines for rabies were retrieved from various sources to extract information on rabies pre- and post-exposure prophylaxis (PrEP, and PEP), booster vaccination, and route of administration. Rabies guidelines recommendations for wound management and PrEP across Asia are broadly aligned to the World Health Organization (WHO) guidelines. For PEP, the 5-dose Essen, and the 4-dose Zagreb are the regimens of choice for intramuscular (IM), and the Thai Red Cross regimen for intradermal (ID), administration. Several national guidelines have yet to endorse ID vaccine administration. Most guidelines recommend rabies immunoglobulin in category III exposures. Booster recommendations are not included in all guidelines, with limited clarity on booster requirement across the spectrum of risk of rabies exposure. In conclusion, national recommendations across Asian countries differ and while some guidelines are closely aligned to the WHO recommendations, resource-saving ID administration and use of rational abbreviated schedules have yet to be endorsed.

Keywords: Asia; guidelines; prevention and control; rabies; vaccination

Highlights

- The recommended practices for rabies pre- and post-exposure prophylaxis vary widely from country to country in Asia.
- Overall, the WHO recommendations for wound management are consistent in national guidelines. The post-exposure prophylaxis (PEP) 5-dose Essen and 4-dose Zagreb intramuscular (IM) regimens are uniformly recommended.
- The value of intradermal (ID) administration in reducing costs is not considered in several country guidelines.
- In the majority of the national recommendations, concurrent administration of rabies immunoglobulin (RIG) in category III exposures is recommended; however, there are concerns regarding availability and cost.
- Booster recommendations are not included in all guidelines, with limited clarity on booster requirement across the spectrum of risk of rabies exposure.
- Limited recommendations are available for special populations including pregnant women, aged population, and immunocompromised patients.

1. Introduction

Despite being entirely preventable, human rabies is estimated to cause 59,000 global deaths annually, of which 59% occur in the Asia region [1,2]. Bites from infected dogs cause 95% of human rabies deaths, 40% of which occur in children [3,4]. If prompt vaccination is not given, rabies infection causes death in virtually all cases [3]. However, the death toll can be considerably reduced through access to post-exposure prophylaxis (PEP), consisting of wound cleaning, rabies immunoglobulin (RIG) and vaccination. Additionally, the broader use of pre-exposure (PrEP) vaccination and mass dog vaccination would further reduce deaths and help control and eventually eliminate dog-mediated human rabies [1,4,5]. Human and canine rabies control are therefore interdependent, and experience has shown that a collaboration between human and animal health sectors is required to enhance cost-effectiveness of rabies control measures, and effectively reduce rabies incidence and associated societal burden [6–8]. This approach, under the “One Health” framework, with the goal to eliminate dog-mediated rabies by 2030, is endorsed and jointly advocated by the World Health Organization (WHO), the World Organisation for Animal Health (OIE), the Food and Agriculture Organization of the United Nations (FAO), and the Global Alliance for Rabies Control (GARC) [5].

Activities aiming to control rabies disease have been initiated, and some governments of disease-endemic countries have committed to its elimination by 2030. In this process, it is important to have reliable country-level epidemiology data, active surveillance systems seeking to register any new cases, appropriate vaccine requirement forecasting, and other such tools that can track progress made towards achieving this goal [3].

However, national reporting systems and public awareness are often lacking [2,3]. People living in countries with endemic rabies disease are sometimes insufficiently aware of the risk and the need for vaccination. Furthermore, the total cost of vaccination—including, for instance, travel expenses to-and-from the vaccination center, and lost work income—is a major consideration for these individuals [2,4,9–13]. Moreover, healthcare personnel are sometimes unaware of appropriate wound management, of PEP regimens, and of the existence of PrEP [4,14].

In this context, safe and effective, yet cost-saving and/or shorter regimens are appealing. Both intramuscular (IM) and intradermal (ID) vaccine schedules are endorsed by the WHO (Table 1). ID administration requires fewer vaccine vials than IM, reducing the direct vaccination cost by 60–80% [13]. For optimal cost benefit of the ID administration, the health seeking rate should be sufficiently high to utilize the entire vial within 6–8 h [1,13]. Importantly, patients receiving IM or ID cell culture rabies vaccination should reach 0.5 IU/mL or higher titers of rabies virus neutralizing antibodies (RVNA) within 14 days from vaccination (the level considered indicative of adequate immune response to vaccination). Both vaccines administration methods demonstrate acceptable safety profiles. In this respect, in an otherwise healthy population, ID vaccination is clinically equivalent to that of IM [13]. The shorter, dose-sparing vaccination regimens are equally effective alternatives, improving compliance and allowing for more animal bite victims to successfully complete a full vaccination course [1,13,15–20]. However, these have not yet been endorsed by all countries with endemic disease in Asia region. To achieve the 2030 goal for rabies elimination, national PrEP and PEP strategies and clear public health guidelines following WHO recommendations must be implemented to increase access to vaccination with optimal efficacy of the vaccine and control the disease [1,4]. A Working Group on rabies vaccines and rabies immunoglobulins established by the WHO Strategic Advisory Group of Experts on Immunization (SAGE) is currently reviewing new evidence on country practices in the use of RIG, PrEP, and the cost-effectiveness of the interventions. The findings will be discussed and SAGE will consider recommendations on the WHO position on rabies during its October 2017 meeting [21,22].

Table 1. World Health Organization (WHO) recommendations for human rabies management.

Categories of Exposure to Suspected or Confirmed Rabid Animal and Actions Required	
Category I	<ul style="list-style-type: none"> • Touching or feeding animals • Licks on intact skin • Contact of intact skin with secretions or excretions of a rabid animal or human case <p><i>Action required:</i> not regarded as exposures, no post-exposure prophylaxis is required</p>
Category II	<ul style="list-style-type: none"> • Nibbling of uncovered skin • Minor scratches or abrasions without bleeding <p><i>Action required:</i> thorough local wound care and vaccine injection as soon as possible</p>
Category III	<ul style="list-style-type: none"> • Single or multiple transdermal bites or scratches • Contamination of mucous membrane with saliva from licks • Licks on broken skin • Exposures to bat bites or scratches <p><i>Action required:</i> thorough local wound care and administration of vaccine and RIG as soon as possible</p>
Passive Immunization: Rabies Immunoglobulin (RIG)	
Target population	<ul style="list-style-type: none"> • All people with category III exposure, bites to the head, neck, face, and genitals • Immunodeficient people with category II exposure • Not for previously vaccinated individuals
Type, dose	<ul style="list-style-type: none"> • Human rabies immunoglobulin: 20 IU/kg body weight • Equine immunoglobulin: 40 IU/kg body weight • F(ab)₂ products of equine immunoglobulin: 40 IU/kg body weight
Time and site	<ul style="list-style-type: none"> • One administration as soon as possible, and within seven days from vaccination • Into or around the wound site or sites. The remaining RIG (if any) should be administered IM at a site distant from the vaccination site
Active Immunization: Vaccines	
Cell culture vaccine (CCV) and embryonated egg-based vaccines (CCEEVs):	
Types	<ul style="list-style-type: none"> - Purified Vero cell rabies vaccine (PVRV) - Purified chick embryo cell vaccine (PCECV) - Human diploid cells vaccine (HDCV)
Potency	<ul style="list-style-type: none"> - Purified duck embryo vaccine (PDEV) - ≥ 2.5 IU per single IM
Route of administration, dose, vaccine type, and injection sites	<ul style="list-style-type: none"> • Intramuscular (IM), 1.0 mL or 0.5 mL (volume depending on the type of vaccine). <p>Sites</p> <ul style="list-style-type: none"> - Vaccine type: any CCEEVs - Adults and children ≥ 2 years: in the deltoid area of the arm - Children < 2 years: in the anterolateral area of thigh - Never in the gluteal area • Intradermal (ID), 0.1 mL, per ID site <p>Vaccine type: only PVRV or PCECV</p> <p>Sites:</p> <ul style="list-style-type: none"> - The deltoids - Lateral thighs - Suprascapular areas

Table 1. *Contd.*

Categories of Exposure to Suspected or Confirmed Rabid Animal and Actions Required	
Pre-Exposure Prophylaxis	
Indication	<ul style="list-style-type: none"> Anyone at continual, frequent, or increased risk of exposure to the rabies virus, either as a result of their residence or occupation (laboratory staff, veterinarians, animal handlers and wildlife officers, etc.) Travelers with extensive outdoor exposure in rural high-risk areas Children living or visiting rabies-affected areas
IM vaccination regimen	3 doses, one IM dose on each of days 0, 7, and 21 or 28
ID vaccination regimen	3 doses, one ID injection of 0.1 mL on each of days 0, 7, and 21 or 28*
Booster injections	Only for those working under continuous or frequent risk of exposure [†] , if rabies-virus neutralizing antibody titers is <0.5 IU/mL
Post-Exposure Prophylaxis	
Indication	<ul style="list-style-type: none"> No vaccination is required for category I Immediate vaccination is required for category II Immediate vaccination is required for category III (and administration of RIG)
Wound care	<ul style="list-style-type: none"> Immediate thorough washing and flushing, for 15 min, with soap or detergent Topical application of povidone iodine or other substances with virucidal activity A bleeding wound must be infiltrated with RIG Postpone suturing if possible; if suturing is necessary ensure that RIG has been applied locally Antibiotics and tetanus prophylaxis should be applied as appropriate for potentially contaminated wounds
IM vaccination regimen [‡]	<p>5-dose Essen regimen (1-1-1-1-1), one dose on each of days 0, 3, 7, 14, and 28</p> <p>4-dose Zagreb regimen (2-0-1-0-1 or 2-1-1-1): 2 doses on day 0 (one in each of the two deltoid or thigh sites) followed by one dose on each of days 7 and 21</p> <p>4-dose shortened Essen regimen (1-1-1-1-0) for fully immunocompetent, exposed people who received wound care + high quality RIG + WHO prequalified rabies vaccine:</p> <p>One dose on each of days 0, 3, 7, and 14</p>
ID vaccination regimen [§]	<p>For immunocompromised individuals including patients with HIV/AIDS:</p> <p>5-dose CCEEV regimen + wound care + local infiltration with human RIG. Evaluation of the rabies-virus neutralizing antibody 2-4 weeks after vaccination and administration of an additional vaccine dose if needed.</p> <p>The updated Thai Red Cross regimen (2-2-2-0-2):</p> <p>Injections of 0.1 mL at two sites (deltoid and thigh) on each of days 0, 3, 7, and 28</p>
Short Post-Exposure Prophylaxis for Previously Vaccinated Individuals	
ID vaccination regimen [§]	<p>Exposed or re-exposed individuals, or individuals with rabies-virus neutralizing antibody titers of ≥0.5 IU/mL:</p> <ul style="list-style-type: none"> One CCEEV IM dose OR one CCV ID dose on each of days 0 and 3, OR One visit four site: four ID injections at a single visit <p>No RIG should be applied</p> <p>Individuals exposed or re-exposed three months after complete vaccination:</p> <ul style="list-style-type: none"> Wound care and booster vaccination if the dog or cat is healthy, vaccinated and available for an observation period of 10 days <p>Individuals with category III re-exposure who were vaccinated with a vaccine of unproven potency, or have received an incomplete course of vaccination:</p> <ul style="list-style-type: none"> Full post-exposure vaccination course + RIG

Sources: Rabies vaccines: WHO position paper (2010) [13] and WHO Expert Consultation on Rabies (2013) [1]; Abbreviations: CCEEV, cell culture vaccine and embryonated egg-based vaccine; CCV, cell culture vaccine; HDCV, human diploid cells vaccine; ID, intradermal; IM, intramuscular; PCECV, purified chick embryo cell vaccine; PDEV, purified duck embryo vaccine; PVRY, purified Vero rabies vaccine; RIG, rabies immunoglobulin; WHO, World Health Organization; * within 6-8 h, several individuals should be vaccinated in order to utilize all the volume of the opened vials, reducing thus the overall cost; † antibody monitoring, is preferred to booster injections. Every six months for those at risk of exposure to high concentrations of live rabies virus (e.g., laboratory workers dealing with rabies virus and other lyssaviruses), and every two years for those not at continuous risk of exposure (e.g., veterinarians and animal health officers); ‡ at exceptional circumstances and when it is not possible to complete post-exposure prophylaxis with the same CCEEV, a rabies CCV fulfilling the WHO requirements should be used.

We collected current national human vaccination recommendations across Asia to summarize and highlight differences and similarities, and to identify best practices. The ultimate aim was to increase knowledge of current needs and identify gaps, in turn encouraging adoption of uniform rabies vaccination methods across all countries to ensure optimization of resource utilization.

2. Methods

We aimed to retrieve national guidelines on management of human rabies exposures from 21 Asian countries: Bangladesh, Bhutan, Brunei, Cambodia, China, Hong Kong, India, Indonesia, Japan, Lao People's Democratic Republic (PDR), Malaysia, Myanmar, Nepal, Pakistan, Philippines, Republic of Korea, Singapore, Sri Lanka, Taiwan, Thailand, and Vietnam. The research for the guidelines was performed between January and March 2017. Our investigation included scientific literature review searches, Ministry of Health web pages, other web pages (e.g., travel information pages), surveillance platforms and databases, medical association guidelines, publications for healthcare professionals and the public, and personal communications with people working on the specific field in each country. English and country-specific websites were reviewed in the local language. Due to the nature of this research, systematic review methods could not be applied. Indeed, national guidelines are not expected to be necessarily published in scientific journals, hence this work did rely mostly on information provided by Ministry of Health websites (often in local language), by national experts, etc. Findings were classified by document type, publisher, and year of publication. We aimed (a) to summarize PEP and PrEP recommendations and compare them to the WHO recommendations; (b) to explore variations between countries in human rabies prophylaxis and discuss opportunities for a harmonized approach; (c) to identify pitfalls and drawbacks in the adaptation of successful human rabies treatment and prophylaxis programs sufficiently harmonized with the WHO recommendations; (d) to highlight best practices.

3. Findings

3.1. Overall Findings

We retrieved national vaccination guidelines from 13 of the 21 countries considered: Bangladesh [23], Bhutan [24], Cambodia [25], China [26], India [27,28], Indonesia [29], Malaysia [30], Pakistan [31], Philippines [32], Sri Lanka [33], Taiwan [34], Thailand [35], and Vietnam [36]. Official documents, outlining current rabies prevention and treatment practices, were retrieved for Hong Kong [37–39], Japan [40], Lao PDR [41], and the Republic of Korea [42]. The national guidelines retrieved were usually issued by the Ministry of Health. We did not succeed to retrieve national documentation for four countries: Brunei, Myanmar, Nepal, and Singapore (Figure 1 and Table 2).

As expected, all national guidelines were considerably detailed and incorporated the WHO recommendations for PrEP and PEP vaccination. All recommended vaccines were cell culture vaccines and embryonated egg-based vaccines (CCEEVs). The schedules are summarized in Table 2. Overall characteristics are given below.

3.2. Post-Exposure Prophylaxis (PEP)

3.2.1. Wound Care

Overall, the WHO recommendations for wound management are consistent in national guidelines. The guidelines from China [26], India [27], and Pakistan [31] were more detailed than others, containing explicit recommendations for wound care with photographs. The Indian [27], Pakistani [31], Philippine [32], and Sri Lankan [33] guidelines recommend avoidance of wound suturing to allow for antibody (RIG) diffusion throughout the tissues, unless there is life-threatening bleeding. The Pakistani guidelines recommend daily dressing instead of suturing, except for very loose suturing for severe facial bites, with proper suturing 2–3 days after initial wound management [31]. Cauterization is no

longer recommended in India as it does not offer additional benefit over washing while tetanus and antibiotics should be given if required, and if sepsis prevention is needed [27]. The Philippines recommend adhesive strips as an alternative to suturing, and also include detailed recommendations on antibiotic treatment [32]. In Sri Lanka, wound dressing is recommended, but not suturing [33].

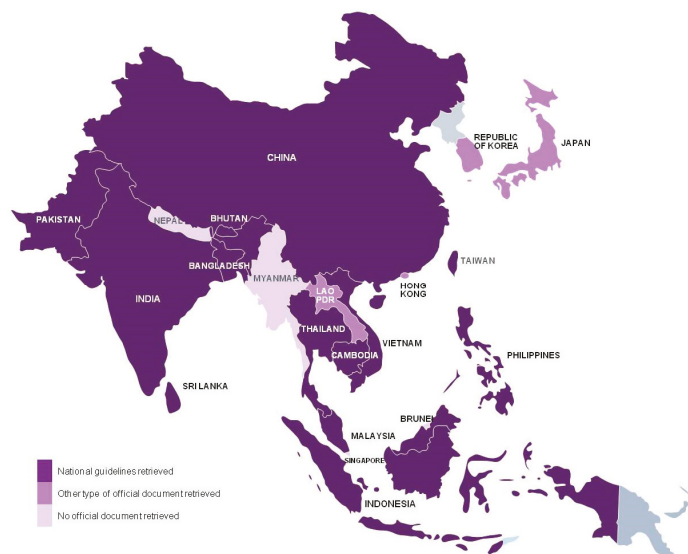


Figure 1. Countries for which national guidelines or other type of rabies-related official document on rabies human vaccination schedules were retrieved and included in the analysis.

3.2.2. RIG

Most guidelines follow WHO recommendations regarding RIG administration; however, there are some concerns related to the associated cost. Thus, for Lao PDR, the 2000 WHO report shows that RIG is rarely used due to its high cost [41], and in a number of countries [25,32,35], equine immunoglobulin (ERIG) is more commonly used than human immunoglobulin (HRIG) because it is cheaper and therefore more commonly available for free. The Philippine guidelines also contain details on the size of needles, skin tests to check whether human RIG should be preferred, special considerations for bites to the finger and toes, and they recommend HRIG for multiple bites and in symptomatic patients infected with human immunodeficiency virus (HIV) [32].

3.2.3. PEP Vaccination Schedules

- The 5-dose Essen, 4-dose Zagreb, or both schedules were the regimens of choice for IM administration. The Essen is used in Bhutan, Cambodia, China, India, Philippines, Sri Lanka, Taiwan, Thailand, Vietnam; and Zagreb in Bangladesh, Cambodia, China, Indonesia, Pakistan, Philippines, Sri Lanka (Table 2). The 4-dose shortened Essen was the regimen of choice in the Malaysian recommendations, and was also recommended in the guidelines of Philippines (Tables 2 and 3).
- We found recommendation for ID administration in 9 of the 13 retrieved national guidelines; the guidelines of China, Indonesia, Malaysia, and Taiwan do not include such recommendation (Tables 2 and 3). Also in the official documents retrieved for Japan and Lao PDR we did not find ID recommendations (Tables 2 and 3). All guidelines with ID recommendation suggested the updated Thai Red Cross regimen (Tables 2 and 3).
- The Japanese guidelines recommend only subcutaneous (SC) administration as shown in Table 2.

Table 2. Summary of available information on vaccination schedules, within the retrieved documents, by country.

Country	Document Title	Published by (Year)	Available Vaccine Types (Route)	PEP Vaccination Schedule	PREP Vaccination Schedule
Bangladesh	National Guideline for Rabies Prophylaxis and Intra-dermal Application of Cell Culture Rabies Vaccines [23]	Disease Control Unit, Ministry of Health and Family Welfare (2010)	PVRV (IM, ID) PCECV (IM, ID) ID administration is recommended as cost-effective and the technique is preferred for immunocompromised individuals	IM: 4-dose Zagreb (Essen schedule described but not favored by the government) ID: Updated Thai Red Cross Re-exposed previously vaccinated: 2 doses, one injection (IM or ID) on each of days 0 and 3, if re-exposure was <5 years after full PEP + wound washing (RIG not necessary)	IM: 3 doses, one injection on each of days 0, 7, and 21 or 28 ID: 3 doses, one injection on each of days 0, 7, and 21 or 28 Booster: 1 dose when titer <0.5 IU/mL for individuals working under risk for rabies exposure, monitoring Ab titers every 6 months
Bhutan	National Guideline for Management of Rabies [24]	Ministry of Health, Department of Public Health/Zoonotic Disease Program (2014)	HDCV (IM) PVRV (IM, ID)	IM: 5-dose Essen ID: Updated Thai Red Cross Re-exposed previously vaccinated: 2 doses (IM or ID), each on days 0 and 3, for those who have documented previous full vaccination.	IM: 3 doses, one injection on each of days 0, 7, and 21 or 28 ID: 3 doses, one injection on each of days 0, 7, and 21 or 28 Booster: 1 site ID at 1 year and every 3 years to lab staff, veterinarians, animal handlers, dog catchers, wildlife workers. Regular (exact timing not specified) monitoring of Ab titers and administration of a booster dose when titers <0.5 IU/mL.
Cambodia	Rabies Vaccine Procedure SOP-OPT-025-004 [25]	National Institute of Public Health, National Public Health Laboratory (2012)	PDEBV (IM) PCECV (IM, ID) HDCV (IM) PVRV (IM, ID)	IM: 5-dose Essen 4-dose Zagreb ID: Thai Red Cross 8-site Oxford (8-ID-4d-1) injection sites on days 0, 7, 28 and 90 Re-exposed previously vaccinated: 2 doses, one injection on each of days 0 and 3, in individuals who had receive at least 3 doses of any PEP regimen	IM: 3 doses, one injection on each of days 0, 7, 21–28 ID: 3 doses, one injection on each of days 0, 7, 21–28
China	Technical Guidelines for Human Rabies Control and Prevention [26]	Chinese Center for Disease Control and Prevention (2016)	PVRV (IM) HDCV (IM) PHRCV (IM) PCECV (IM)	Re-exposed previously vaccinated: 2 injections each on days 0 and 3, if 20–30 units have passed from previous vaccination, if <20 units have passed from previous vaccination, and individual is healthy, vaccinated and accessible for observation	IM: 3 doses, one injection on each of days 0, 7, and 21 or 28 Booster: 1 booster dose when titers <0.5 IU/mL, for individuals working under risk for rabies exposure (monitoring every 6 months for veterinarians/animal health officers)
Hong Kong	Web page: Rabies [27] Web page: Vaccine and Prophylaxis—Rabies Vaccination [39] Scientific Committee on Emerging and Zoonotic Disease—Prevention and Control of Rabies [38]	Center for Health Protection, Department of Health (2017) Travel Health Service, Department of Health (2012) Center for Health Protection (2005)	(not specified) (not specified) (not specified)	IM: 5-dose Essen 4-dose Zagreb Re-exposed previously vaccinated: 2 injections each on days 0 and 3, if 20–30 units have passed from previous vaccination, if <20 units have passed from previous vaccination, and individual is healthy, vaccinated and accessible for observation Thorough wound cleansing Vaccination if necessary	Travelers to endemic areas
India	National Guidelines on Rabies Prophylaxis [27] Indian Academy of Pediatrics (IAP) Recommended Immunization for Children Aged 0 through 18 years—India, 2016 and Updates on Immunization [28]	National Center for Disease Control (2015) Indian Pediatrics (2016)	PCECV (IM, ID) PVRV (IM, ID) HDCV (IM) PDEBV (IM) PVRV (IM) ID not in individual practice	IM: 5-dose Essen ID: Updated Thai Red Cross Re-exposed previously vaccinated: 2 doses (IM or ID), each on days 0 and 3, for those who have received full vaccination and re-exposed IM: 5 doses, one dose on each day 0, 3, 7, 14, and 30, and for optional 6th dose on day 90 Re-exposed previously vaccinated: 2 doses for those who have received full vaccination and re-exposed	IM: 3 doses, one injection on each of days 0, 7, and 21 or 28 Booster: 1 booster dose when Ab titers <0.5 IU/mL, for individuals working under risk for rabies exposure (monitoring every 6 months for the first 2 years and every 24 months thereafter) IM: 3 doses, one injection on each of days 0, 7, 21, or 28 (day 28 is preferred)
Indonesia	National Guidelines for Rabies Vaccination [29]	Center for Disease Control, Ministry of Health (2011)	PVRV (IM) PCECV (IM)	IM: 4-dose Zagreb Re-exposed previously vaccinated: 1 dose if re-exposure occurred 3–12 months from full vaccination below 3 months, full vaccination over 12 months	IM: 3 doses, one injection on each of days 0, 7, 21, or 28
Japan	Questions and answers on rabies [40]	Ministry of Health (2017)	(not specified) ID is not approved RIG is not approved	SC: 5 doses on days 0, 3, 7, 14, 30 and 90 Re-exposed previously vaccinated: 2 injections for those who have received full vaccination, on days 0 and 3	SC: week 0, 4 and month 6–12

Table 2. Contd.

Country	Document Title	Published by (Year)	Available Vaccine Types (Route)	PEP Vaccination Schedule	PREP Vaccination Schedule
Lao People's Democratic Republic	Intradermal application of rabies vaccines. Report of a WHO consultation. Bangkok, Thailand 2000 [41]	WHO, Communicable Disease Control and Control (2000)	PVRV (IM) PCECV (IM) ID not yet used due to high cost. The ID route is not used	(not specified)	(not specified)
Malaysia	Intrinsic guidelines for human rabies prevention & control [30]	Ministry of Health, Disease Control Division (2014)	PVRV (IM)	IM: 4-dose shortened Essen Re-exposed previously vaccinated: 2 doses on days 0 and 3	(not specified)
Pakistan	Country Guidelines for Prevention of Rabies. Rabies Control Programme [31]	WHO in consultation with Ministry of Health, Departments (2013)	PVRV (IM, ID) PCECV (IM, ID) PDEV (IM)	IM: 4-dose Zagreb ID: The updated Thai Red Cross Re-exposed previously vaccinated: 2 injections (IM or ID) for those who have received full vaccination, on days 0 and 3	IM: 3 doses, one injection on each of days 0, 7, 21, or 28 ID: 3 doses, one injection on each of days 0, 7, 21, or 28
Philippines	New Guidelines on the Management of Rabies Exposure [32]	Republic of Philippines, Department of Health (2014)	PVRV (IM, ID) PCECV (IM, ID) Among the first countries that adopted ID	IM: 5-dose Essen 4-dose Zagreb ID: The updated Thai Red Cross Re-exposed previously vaccinated: 2 injections (IM or ID), each on days 0 and 3 if the re-exposed individual had previously received full vaccination. Re-exposed if he/she had not receive full vaccination course	IM: 3 doses, one injection on each of days 0, 7, 21, or 28 ID: 3 doses, one injection on each of days 0, 7, 21, or 28 Booster: Routine booster for individual with occupational risk: 1 dose after one year from full vaccination and one more thereafter in case of Ab titers <0.5 IU/mL
Republic of Korea	Recommended Adult Immunizations for Foreign Travel [42]	Korea Society of Infectious Disease (2012)	(not specified)	IM: 5-dose Essen ID: Updated Thai Red Cross Modified 4-site (4-2-2-0-2); one dose at 4 sites on day 0 and one dose at 2 sites on days 3, 7, and 30	3 doses
Sri Lanka	Protocol for anti-rabies post exposure therapy [33]	Director General of Health Services (2016)	PVRV (IM, ID) PCECV (IM, ID)	Re-exposed previously vaccinated: 2 injections (IM or ID), one on each of day 0 and 3, OR 4 ID at four sites on the same visit	IM: 3 doses, one injection on each of days 0, 7, 21, or 28 ID: 3 doses, one injection on each of days 0, 7, 21, or 28 Booster: One injection on the 1st year following full vaccination, and one more every five years
Taiwan	Post-exposure guidelines [34]	Centers for Disease Control (2016)	(not specified, ID not recommended)	IM: 5-dose Essen	IM: one dose, on day 0, 7, 21 or 28 (6 doses) Booster: For individuals at high risk (e.g., laboratory workers), 2 injections, one on each day 0 and 4 when Ab titers <0.5 IU/mL. For workers at high risk, one injection on each of days 0 and 4 following full vaccination and every 3-5 years thereafter
Thailand	Thai rabies management guidelines [35]	Queen Suvabha Memorial Institute (2015)	PCEVC (IM, ID) PVRV (IM, ID) PDEV (IM)	IM: 5-dose Essen ID: Updated Thai Red Cross Re-exposed previously vaccinated: (a) 2 injections, each on day 0 and 3 if exposure occurred within 6 months from vaccination, no RIG; (b) same as previous or 4-site ID on a single visit if >6 months have elapsed from previous vaccination to exposure	IM: 3 doses, one injection on each of days 0, 7, 21, or 28 ID: 3 doses, one injection on each of days 0 and 28 Booster: For individuals at continuous or frequent risk of exposure. One booster vaccination if Ab titers <0.5 IU/mL
Vietnam	Guidelines on human rabies surveillance and prevention [36]	Ministry of Health (2014)	Cell culture vaccines (not specified)	IM: 5-dose Essen ID: Updated Thai Red Cross Re-exposed previously vaccinated: (a) 2 injections, each on days 0 and 3 if time from previous full vaccination <5 years; (b) Full vaccination if previous was not completed or >5 years have elapsed from previous full vaccination	IM: 3 doses, one injection on each of days 0, 7, 21, or 28 Booster: One injection on 1st year from previous full vaccination and every 3 year thereafter

Abbreviations: Ab, antibody; APCRI, Association for Prevention and Control of Rabies in India; HDCV, human diploid cells vaccine; IAP, Indian Academy of Pediatrics; ID, intradermal; IM, intramuscular; PCECV, primary chick embryo cell vaccine; PDEV, purified duck embryo vaccine; PEV, post-exposure prophylaxis; PHKCV, primary hamster kidney cell vaccine; PREP, pre-exposure prophylaxis; PVRV, purified Vero rabies cell vaccine; SC, subcutaneous.

Table 3. National guidelines endorsing the WHO recommended PEP vaccination regimens: Essen, Zagreb, and the updated Thai Red Cross.

Country	5-Dose Essen (1-1-1-1-1)	4-Dose Zagreb (2-0-1-0-1 or 2-1-1)	4-Dose Essen (1-1-1-1-0)	Updated Thai Red Cross (2-2-2-0-2)
Bangladesh	X	X		X
Bhutan	X			X
Cambodia	X	X		X
China	X	X		
India	X			X
Indonesia		X		
Malaysia			X	
Pakistan		X		X
Philippines	X	X	X	X
Sri Lanka	X	X		X
Taiwan	X			
Thailand	X			X
Vietnam	X			X

3.2.4. PEP Vaccination for Re-Exposed Individuals

Not all guidelines advise on the post-exposure management that should be followed when an individual who has previously received rabies vaccination is re-exposed (Table 2). The guidelines of Bangladesh [23], Bhutan [24], China [26], India [27], and Philippines [32] recommend new full vaccination when previous full exposure cannot be documented or is uncertain.

3.3. Pre-Exposure Prophylaxis (PrEP)

3.3.1. PrEP Vaccination Schedules

With the exception of Japanese guidelines, all other guidelines recommend IM or ID vaccination schedules (Table 2). IM administration is included in all of the 13 retrieved national guidelines. ID is included in only seven national guidelines, these of Bangladesh, Bhutan, Cambodia, Pakistan, Philippines, Sri Lanka, and Thailand (Table 2). The Malaysian interim guidelines do not include any reference to PrEP [30].

3.3.2. Booster after PrEP Vaccination

Eight national guidelines include booster recommendations and there are differences between countries; all relevant recommendations are described in detail in Table 2. For individuals working under a high risk (laboratory workers dealing with rabies virus and other lyssaviruses) or continuous risk (veterinarians and animal health officers) of exposure to rabies, all recommendations agree that a booster vaccination should be given when antibody titers fall below 0.5 IU/mL.

3.4. Vaccination Recommendations for Special Populations

We found PEP recommendations for special populations in the guidelines of Bangladesh [23], Bhutan [24], Cambodia [25], China [26], India [27], Malaysia [30], Pakistan [31], Philippines [32], and Sri Lanka [33]. Chinese guidelines have an additional entry for PrEP recommendations in those populations [26].

PEP recommendations

- Pregnant and lactating women: The guidelines of Bangladesh, Bhutan, Cambodia, India, Pakistan, Philippines, and Sri Lanka state that there is no contraindication for vaccination in this population. The Chinese guidelines do not directly state whether PEP should be given or not, however they

make reference to studies demonstrating that rabies vaccines are safe for pregnant women and for the fetus.

- Aged population and individuals with comorbidities: the same as above in the guidelines of Bangladesh, India, Pakistan, Philippines, and Sri Lanka.
- Immunocompromised population: full PEP and IM route is recommended in the guidelines of Bangladesh, Bhutan, India, Malaysia, Philippines, and Sri Lanka. There is no special reference to this population in the guidelines of Cambodia and Pakistan. Chinese guidelines indicate that passive immunization can be administered in patients with immunodeficiency disorder and that the antibody response should be closely monitored.
- Individuals on treatment for malaria taking chloroquine: ID is contraindicated and IM is recommended in the guidelines of Bangladesh, Cambodia, India, Malaysia, Pakistan, Philippines, and Sri Lanka.
- Other populations: Philippine's guidelines also note that IM is contraindicated for individuals with hematologic conditions, for whom the ID route should be chosen and that ID is contraindicated for individuals with chronic liver disease.

Chinese PrEP recommendations: Only in the Chinese guidelines, we found recommendations specific to PrEP. According to these, PrEP can be delayed in case of (a) pregnancy; (b) acute febrile disease or other acute disease; (c) active chronic disease; (d) use of steroids and immunosuppressive products. PrEP is not recommended for patients with immunodeficiency disease.

4. Discussion

4.1. Characteristics of Human Rabies Burden

In endemic areas rabies disease is largely underreported, and reliable occurrence data are often scarce or non-existent [2,3,43]. Patients often seek no treatment [44], and some leave hospital against medical advice believing there is no cure [45] or because treatment cannot be offered [3]. Laboratory confirmation is sought in only a limited number of cases [44,46] because of limited resources and training [46,47]. Information systems for the collection of rabies cases may not be available [46] and often local authorities do not report rabies cases to central authorities [44,46]. In many countries, the officially reported numbers of rabies cases and deaths are substantially lower than the actual numbers [1]. In Cambodia for example, the incidence of human rabies deaths was estimated to be 5.8 per 100,000, which was 15 times higher than the officially reported incidence [48].

Younger children who are unable to protect themselves are at higher risk because they may interact with dogs in a manner perceived as threatening by the animal, e.g., stepping on the tail or trying to play when the dogs are eating [3,4,49,50]. Because of their smaller stature, children are more prone to get bitten multiple times on the face, head, and neck, being thus exposed to the more severe type of bites with the shortest incubation period [51,52]. Furthermore, children do not always report minor bites or exposures by licks to their parents [52,53].

4.2. Rabies: An Unjustified Disease Burden

The development of vaccines against rabies started 100 years ago, and highly effective life-saving vaccines are currently available [19]. There is also a heightened awareness of rabies symptoms and inevitable fatal outcome without appropriate treatment [5,54,55]. Despite these advances, important knowledge gaps still exist. For example there are still those unaware that the wound must be immediately washed with soap and water [55,56], or that a laboratory test can confirm or reject suspected rabies in biting animal [55]. Furthermore, the rural population has limited access to vaccination centers as they are usually located in big cities, many animal bite victims do not seek medical care, and most infected individuals die at home [2,13]. In addition, RIG is often not made available because a large fraction of the population cannot afford it [56,57]. Poor people, living far from

vaccination centers cannot afford to travel back-and-forth for injections, even if the vaccine is provided free of charge, and they often fail to complete complicated vaccination schedules [48,56]. The problem is further accentuated by a lack of access to vaccines and RIG globally.

4.3. Rabies Vaccination Schedules Across Asia: Current Situation

PEP Recommendations

Adequate wound cleaning can reduce the number of infectious viral particles inoculated via saliva during the bite from the rabid animal [58]. Improper or incomplete wound care is one of the reasons for PEP failure [59]. Thorough washing of the wound can eliminate or substantially reduce the viral load [60]. Unfortunately, this critical intervention is often undervalued or ignored [60]. The level of detail on wound washing techniques were highly heterogeneous in the national guidelines, suggesting a local need for specific operational guidelines on wound treatment.

Although international guidelines on the provision of RIG are clear, local recommendations are often different and almost universally the use of RIG in practice is not aligned to international recommendations due to significant access barriers [61].

The 5-dose Essen and 4-dose Zagreb IM regimens are considered equivalent in countries where both are recommended, except for the Bangladeshi guidelines that clearly favor the Zagreb regimen. The Zagreb regimen, however, is not endorsed by all national guidelines.

ID PEP vaccination was endorsed in nine national guidelines; however, the value of ID administration in reducing costs was not considered regarding PEP in six national guidelines or other official documents. Clearly, there is still a need to emphasize the cost savings achieved using ID administration as opposed to the cost of the five IM regimen of similar vaccine efficacy. However, these cost savings can only occur in facilities with well-trained staff and with sufficient patients presenting with bite wounds to ensure that the maximum number of doses are extracted per vial of vaccine. With no preservative in the available vaccines, the vial should not be left open for longer than 6–8 h [1,13] and for smaller clinics this will not result in any cost saving vs. IM.

There are limited recommendations on the vaccination of patients who have a history of vaccination against rabies (PEP or PrEP). This could lead to overtreatment of patients presenting for treatment on multiple occasions. Given the risk factors for being bitten are associated with socio-economic factors, e.g., rural environments, working outdoors, it is likely that those who receive a bite are at a higher risk of subsequent bites than the general population. As such, multiple exposures are likely to occur. Clearer guidance could result in reduced use of vaccine and, where available, RIG for these patients.

Pregnancy, lactation, infancy, older age and comorbidities are not considered contraindications in the few guidelines that make reference to vaccination for special populations. Immunosuppressed individuals might have an inadequate antibody response in rabies PEP; however, specific recommendations can be found in very few guidelines.

4.4. PrEP Recommendations

Most country recommendations on patients for whom PrEP should be considered are aligned to WHO. Although recommendations are in place, uptake remains low because of complicated schedules, cost, and competing priorities especially within the context of limited PEP vaccine supply or resources for rabies control [62]. Less complicated vaccination schedules with shorter regimens and fewer doses would make PrEP simpler and reduce associated costs [62]. In addition, educational campaigns and rabies prevention and elimination programs should be conducted in areas where the infection rate is high; one such program was introduced in 2007 in the Bohol district of the Philippines, an area with the highest rabies incidence in the country [63]. The program included free routine PrEP for children aged 5–14 years and lasted four years (2007–2010) [52,62]. Up to April 2010, this program achieved high PrEP vaccination coverage (47%) of the target population [62].

Booster recommendations are not included in all guidelines and this may become an additional impediment to the optimal use of PrEP as there is little agreement on duration of protection or clinical benefit. Guidelines are more established for individuals at continual risk (lab workers, veterinarians, animal health workers) than for individuals at increased risk (children living in endemic areas, or travelers), and the intervals for serological follow-up (a test that is not consistently available everywhere) vary. It should also be noted that serological follow-up, even when recommended, is rarely practical or affordable. PrEP may be associated with cost savings because a previously vaccinated person needs shorter PEP and no RIG [62]. Children may benefit from receiving PrEP from 1 year of age and clear boosting recommendations are needed [62].

4.5. Country-Specific Information for Countries Whose National Guidelines Were Not Retrieved

In *Hong Kong* public hospitals, 10,255 individuals received PEP between 2000 and 2004, all of whom received rabies vaccine and 1% also received RIG [38]. The country's rabies control program contains disease surveillance systems, laboratory diagnostic testing, PEP, and wound management [38].

For *Lao PDR*, the SEARS initiative (South-East Asia Rabies Strategy, 2013) indicates the rabies control program was not clearly defined, human resources were short, the surveillance system was inadequate, and laboratory confirmation was unavailable [64].

Brunei and *Singapore* are considered as free-of-rabies areas [43]. Singapore has been free from rabies since the 1950s and this was achieved through specific legislation, with which several rabies prevention strategies were implemented [43].

For *Myanmar*, the Association of Southeast Asian Nations 2016 report drew attention to the lack of a national rabies control strategy, low level of awareness and surveillance, and limited funding for rabies control [43].

In *Nepal*, public hospitals provide free post-exposure vaccination since 2007 [65]. RIG is available only in Kathmandu, and even there is mainly used by tourists and expats who can afford it [65].

4.6. Future Perspective

The Sustainable Development Goals (SDG), established by the September 2015 UN General Assembly, included the target to end epidemics of Neglected Tropical Diseases (NTDs) by 2030 [66]. Coinciding with these SDGs, WHO and OIE in collaboration with the FAO and supported by the GARC, have jointly set the goal for rabies elimination by 2030 [3,5]. Lessons learned from areas of the world where rabies has been successfully eliminated show that the "One Health" agenda will be the only way to achieve the 2030 goal of elimination of disease in Asia [5]. This framework resulted in substantial decrease in dog rabies incidence in 21 countries of Latin America and the Caribbean region, and several countries have been declared free of human rabies cases [67]. This was achieved thanks to a strong political commitment to control rabies in coordination with the Pan American Health Organization (PAHO). The program promoted mass canine vaccination, epidemiological surveillance, and provision of PEP and PrEP to people at risk. PEP became broadly available and, as part of the program, rabies vaccination centers were decentralized. PAHO's Veterinary Public Health Program provided technical support for a disease notification system and coordinated actions between Ministries of Agriculture and Health and the executive councils of the WHO and OIE. Community education and involvement was part of the program to engage people in mass dog vaccination. The educational resources of the GARC were used to achieve this. Mass media campaigns on the radio, and in schools, health facilities, and similar organizations were staged to convince people to have their dogs vaccinated [67]. Similarly, in 2015, the Pan-African Rabies Control Network was launched, to integrate the One Health approach in the 33 member states, aiming to meet the 2030 target [68]. In a similar way, the Association of Southeast Asian Nations (ASEAN) developed the ASEAN Rabies Elimination Strategy in a One Health approach, integrating political, organizational, sociocultural, and technical collaboration to achieve rabies elimination by 2020 in the ASEAN member states, plus China, Japan, and Korea [43].

It is hoped that findings presented here might be useful to the authorities, health care providers, and patient organizations in the development and implementation of their initiatives for rabies elimination. This is more likely attainable through increased awareness of disease and encouraging the adoption of uniform rabies vaccination across Asia to ensure optimization of resource utilization, most often in resource-constrained environments. In that respect, the findings of the SAGE Working Group on rabies vaccines and rabies immunoglobulins will be highly significant.

To the best of our knowledge, this was the first work summarizing national rabies guidelines of the endemic Asian countries. However, we were unable to recover all national guidelines. Lack of information on vaccines' origin and shortages are further limitations. Our work could not retrieve real-life data on proportions of use of ID and IM; a future prospective study would be necessary in that respect.

5. Conclusions

National recommendations across these countries differ and while some guidelines are closely aligned to the WHO recommendations, other countries specify PEP schedules that are very demanding on resources. A lot of progress has been made with respect to rabies control programs in many countries; however, efforts should be continued through closer collaboration between human and animal health sectors to meet the 2030 goal for rabies elimination. These efforts will have the opportunity to incorporate the most current findings of the SAGE Working Group on rabies vaccines and rabies immunoglobulins.

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Abbreviations

Ab	antibody
APCRI	Association for Prevention and Control of Rabies in India
CCEEV	cell culture vaccine and embryonated egg-based vaccines
CCV	cell culture vaccine
ERIG	equine immunoglobulin
FAO	Food and Agriculture Organization
GARC	Global Alliance for Rabies Control
HDCV	human diploid cells vaccine
HIV	human immunodeficiency virus
HRIG	human immunoglobulin
IAP	Indian Academy of Pediatrics
ID	intradermal
IM	intramuscular
NTD	Neglected Tropical Diseases
OIE	World Organization for Animal Health
PAHO	Pan American Health Organization
PCECV	purified chick embryo cell vaccine

PDEV	purified duck embryo vaccine
PEP	post-exposure prophylaxis
PHKCV	primary hamster kidney cell vaccine
PrEP	pre-exposure prophylaxis
PVRV	purified Vero rabies vaccine
RIG	rabies immunoglobulin
RVNA	rabies virus neutralizing antibodies
SAGE	Strategic Advisory Group of Experts
SC	subcutaneous
SDG	Sustainable Development Goals
SEARS	South-East Asia Rabies Strategy
UN	United Nations
WHO	World Health Organization

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Article

Public Preference for Pet-Rabies Prophylaxis: Opportunities and Information Dissemination

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Abstract: Risky human behavior and high density of rabies vectors in urban environments combine to increase the risk of rabies. Pet vaccination, wildlife vector management, and public health education may be the most efficient ways to prevent urban rabies epidemics. Racial, ethnic, and socio-economic factors influence the use of low-cost rabies vaccination clinics, understanding rabies reporting requirements, and learning preferences. In collaboration with the City of Greensboro and Animal Control in Guilford County, NC, we conducted a survey of rabies prevention and transmission across socio-economic strata representing Latinos, African Americans, and Whites, and different income and education levels. Compliance with vaccination was low among Latinos; African Americans and Latinos were not aware of low-cost rabies vaccination clinics; and most respondents were willing to report rabid animals but did not know whom to call. White respondents preferred online information delivery, whereas Latinos and African Americans preferred postal mail. Communication targeting the public requires the consideration of different message decoding and interpretation based on the ethnicity, income, and educational level, and other barriers such as language. Differing message delivery methods may be required to achieve full dissemination.

Keywords: rabies; rabies prevention; public health outreach; race; ethnicity

1. Introduction

Several factors are combined to make urban environments ideal for the spread of zoonotic diseases [1]. First, human population densities are highest in urban areas. Second, population densities of wildlife species which serve as rabies vectors (e.g., raccoons, foxes, and coyotes) are often highest in urban areas because these species are highly adaptable and take advantage of anthropogenic factors (e.g., garbage, denning sites in buildings, green areas protected of larger predators) that facilitate high reproduction rates and increased survival [2]. Third, pet densities are highest in urban areas. Finally, human behaviors in urban areas, such as attempting to help approachable animals, feeding wildlife purposively or not (e.g., feeding corn to raccoons, leaving pets' food outside), increases the risk of disease transmission in urban environments. This may happen by directly relating to wildlife or by attracting a larger number of animals to a particular area.

Rabies is an important zoonosis that affects wild and domestic animals, as well as humans. The current rabies epidemic in the eastern United States is associated with a raccoon (*Procyon lotor*) variant of the virus [3]. Raccoons are widespread throughout North America and are present in high densities in urban environments [4,5]. They are well adapted to urban and suburban areas, using human housing to den, and garbage, pet food, plants in residential gardens, and urban proximate crops as a food sources [6]. These animals are hosts to a large number of pathogens (e.g., *Leptospira interrogans*,

and canine distemper, rabies, and feline panleukopenia viruses) that can infect other animal species and humans [7]. Therefore, interactions between raccoons, humans, and their companion animals have led to increasing public health concerns [6].

Pet vaccination, wildlife vector management, and public health education may be the most efficient ways to prevent a rabies epidemic in urban environments [8]. Public health officials in Greensboro, NC, indicated that compliance with rabies vaccination laws is low. For example, of the 11 cases of pets that had been in contact with a suspected rabid animal in 2012, 10 had to be euthanized or quarantined because they did not have the proper rabies vaccination [9]. Although rabies vaccination by professionals is required by law for dogs, cats, and ferrets, and is reportable in NC, rabies immunization and reporting is difficult to enforce and the number of unvaccinated animals is difficult to estimate. There has not been any other comparable reporting method in place.

Cultural norms may contribute to challenges with pet immunization efforts. Ethnic minorities—particularly Hispanic/Latinos—are increasing in the United States, and are becoming a critically important focus of wildlife management and outreach programs [10]. Engaging minorities in wildlife management and public health programs requires the creation of language-appropriate, culturally-sensitive, and relevant information to be delivered in an accepted format and through media outlets favored by the target population. Compared to Whites, Hispanic/Latinos and African Americans know less about the signs of rabies infection in animals, transmission routes, wildlife vectors, and about first response after rabies exposure [11]. Health and illness perception, risk-taking or -aversion behaviors, and lack of access to information, may increase exposure to rabies or the reporting of suspected rabid animals [11]. The challenge of reaching minorities has been documented in other public health areas, including sexually transmitted diseases (STDs) and oral health [12].

Guilford County, NC, USA was a good place to find solutions for some of these challenges. In 2006, Guilford County had 37 confirmed cases of wild animal rabies, and 20 of these cases were raccoons (others included one cat, one coyote, two bats, four foxes, and five skunks). In addition, Guilford County animal control officials were anecdotally reporting a low turnout of ethnic minorities to their pet rabies vaccination clinics. After the 2006 rabies report, Guilford County Environmental Health officers were concerned with raccoon population dynamics in urban areas and in particular in Greensboro (a major city in the county), and decided to reach out to North Carolina State University to express the need for public health research. Their concern also extended to public awareness, understanding of rabies transmission, symptoms, and early treatment, as well as low-cost options for pet vaccination. The city has a total population of 237,423, of whom Hispanic/Latinos (15,412) and African Americans (88,587) account for roughly half of the population [13]. This diverse population made the city a good case study to answer some of the concerns presented by Environmental Health officials and to gain insight about the public's knowledge, get an estimate of pet vaccination status, and understand how people would respond to an encounter with a rabid animal. Therefore, we conducted a bilingual (English/Spanish) survey in Greensboro, NC, to determine pet vaccination compliance and awareness and use of low-cost rabies vaccination clinics. Information about the understanding of the requirements for reporting suspected rabid animals (e.g., who they would report to) and differing public preferences for communication content, format, and delivery of rabies information were elicited from survey participants.

2. Materials and Methods

Four neighborhoods located within the northwest quadrant of the city of Greensboro were selected for the survey, given the high number of rabies-positive raccoon cases reported in 2006 and 2007 [14]. We conducted the survey during October and November 2009. We interviewed the adult (18 years or older) who answered the door in every third dwelling in each of the selected neighborhoods. If nobody answered or the person who answered refused to participate in the survey in the selected house, the next dwelling was considered the replacement. This systematic sampling strategy ensured we

engaged diverse audiences often left out of sample frames based on phone numbers or formal mailing addresses [15,16]. Surveys were conducted on weekdays and weekends, alternating mornings and afternoons to decrease possible bias associated with sampling weekday and time. A total of nine interviewers participated, four males and five females, who worked in pairs with at least one bilingual interviewer in each pair. All interviewers were trained by the primary author, and carried English and Spanish copies of the questionnaire. Each respondent was asked which language (English or Spanish) they preferred; when Spanish was chosen, the respondent was asked if they wanted to talk to one of the bilingual interviewers.

Survey design and administration—An English version of the survey was designed and translated into Spanish by two of the authors who are bilingual. The survey was then back-translated to English in order to check for semantic and conceptual equivalence. Information was elicited on: learning about rabies, attitudes towards and reporting on rabies, pet-vaccination status, demographic and socio-economic status i.e., previous year's income classified into categories of 0 (\leq \$14,999) to 8 (\geq \$60,000); age; education, categorised as 0 (grammar school) to 4 (graduate degree); years of residence in the area; number of household residents; and gender and ethnicity. Ethnicity included Hispanic/Latino and race options were White, Asian, Black or African American, Native American, and Hawaiian or other Pacific Islander as defined in US Census Bureau [17]. Twelve questions (12 closed-ended and 1 open-ended) pertained to pet vaccination status and knowledge and use of rabies vaccination clinics in the county, reaction to a rabid animal, and choice of outlets to receive rabies-associated public health information. This is a subset of the questionnaire; results from other portions of the survey have already been published in a previous manuscript [11].

Data analysis—Data are presented in tables with frequencies and percentages. Chi-square testing using SAS [18] was used to compare frequencies for specific questions with an alpha value of \leq 0.05.

3. Results

Socio-economic and demographic characteristics of respondents—The overall ethnic/racial distribution of the 301 respondents was: 220 White non-Hispanic/Latino (Whites 75%); 33 Hispanic/Latino (11%); 40 African American (13%). Only 23 participants required a Spanish version of the survey. Hispanic/Latinos and African American respondents had lower income levels than Whites. Income varied by ethnic/racial groups, with 65% of the Hispanic/Latino respondents reporting earnings of less than \$20,000/year while 77% of the White respondents reported earning more than \$35,000/year. Hispanic/Latino and African American respondents were younger and had lived in the area for less time compared to Whites [11]. Hispanic/Latinos had lower education levels than African American and Whites, with 39% of the Hispanic/Latino respondents having only completed grammar school. College completion was eight times higher among White respondents (65%) than African Americans (8%).

Pet ownership and care—Overall, half of the respondents indicated owning one pet; however, only 30% of the Hispanic/Latino respondents owned a pet (Chi-square p -value $<$ 0.00001 for comparison between Whites and Hispanic/Latino and African Americans combined). Of the respondents that owned pets, 85% indicated that they vaccinated for rabies in the last 12 months. Reasons for no rabies vaccination were: vaccination is only done for outdoor pets, cost of the vaccine too high, pet was too young to vaccinate, or they could not catch the animal to have it vaccinated. African Americans (60%) and Hispanic/Latinos (89%) reported no knowledge of low-cost rabies vaccination clinics offered by Guilford County, whereas a larger percent of White respondents (65%) were aware of the clinics. More Whites ($n = 93$) than Hispanic/Latinos and African Americans combined ($n = 9$) were aware of the low-cost clinics run by Guilford County Animal Control (Chi-square p -value = 0.000472). Considering the people who were aware of the rabies clinics, 69% had heard about them from the local media (e.g., radio, television, or newspaper), but only 38% of them had vaccinated their pets at the low-cost clinics (Table 1).

Table 1. Frequency of answers regarding pet vaccination status and awareness and use of rabies vaccination clinics in Greensboro, NC, 2009.

Survey Questions and Possible Answers	Responses % (n)			
	Total Responses	Hispanic/Latinos	African Americans	Whites
Do you own or live with someone who owns a cat, dog, or ferret?				
Yes	58.19 (174)	28.13 (9)	50 (20)	64.38 (141)
No	41.81 (125)	71.88 (23)	50 (20)	35.62 (78)
Have these pets been vaccinated against rabies in the last 12 months?				
Yes, all of them	84.57 (148)	66.67 (6)	95 (19)	84.51 (120)
Yes, some of them	8.57 (15)	11.11 (1)	5 (1)	8.45 (12)
No	6.86 (12)	22.22 (2)	0	7.04 (10)
Why did you not vaccinate some of the pets?				
I only vaccinate pets that live outside	6.67 (1)	0	0	9.09 (1)
I only vaccinate pets who live inside	13.33 (2)	33.33 (1)	0	9.09 (1)
Is too expensive	13.33 (2)	33.33 (1)	0	9.09 (1)
The pet is too young	13.33 (2)	0	0	18.18 (2)
The pet is too old	6.67 (1)	0	0	9.09 (1)
Given three-year vaccination	20 (3)	0	100 (1)	18.18 (2)
I have no time to take the pets to the veterinarian	6.67 (1)	0	0	9.09 (1)
Cannot catch the animal	13.33 (2)	0	0	18.18 (2)
Lack of attention to pet's vaccinations	6.67 (1)	33.33 (1)	0	0
Are you aware of the low-cost/free rabies clinics offered by Guilford County?				
Yes	60.34 (105)	11.11 (1)	40 (8)	65.96 (93)
No	39.66 (69)	88.89 (8)	60 (12)	34.04 (48)
Have you ever vaccinated your pets in the low-cost/free rabies clinics offered by Guilford County?				
Yes	38.1 (40)	50 (1)	71.43 (5)	34.41 (32)
No	61.9 (65)	50 (1)	28.57 (2)	65.59 (61)
How did you find out about the low-cost/free rabies vaccination clinics offered by Guilford County?				
Media (newspaper, radio, TV, internet, email)	68.75 (66)	100 (1)	42.86 (3)	70.59 (60)
Family or friends	23.16 (22)	0	28.57 (2)	22.62 (19)
Veterinarian	18.75 (18)	0	28.57 (2)	16.47 (14)
Shelter	5.21 (5)	0	0	5.88 (5)

When asked what they would do if they encountered a dog which they suspected had rabies, most respondents, regardless of ethnicity, indicated that they would call someone to handle the animal. Latinos indicated they would call Animal Control but did not have the number. Conversely, African Americans and Whites indicated they would call Animal Control, and did have the number (Table 2). A total of 30% of respondents indicated that they would call 911. The majority of respondents of all ethnic groups indicated that they would go to the emergency room if they were bitten by a dog. Similarly, the majority of the respondents indicated that they would report the offending dog to Animal Control (Table 2).

A total of 53% of the White respondents indicated they would like to receive future information about rabies over the internet, whereas Latinos (43%) and African Americans (51%) preferred to receive the information by postal mail. When asked what would be the best way to deliver information about future rabies clinics, most respondents (35%) chose local media outlets such as local radio and TV (Table 3).

Table 2. Frequency of answers regarding rabid animal reporting and first response after rabies exposure in Greensboro, North Carolina, 2009.

Survey Questions and Possible Answers	Responses % (n)			
	Total Responses	Hispanic/Latinos	African Americans	Whites
If you were to encounter a large dog you suspect has rabies in your neighborhood, what would you do?				
Try to capture the animal to try to help it	0.67 (2)	3.03 (1)	0	0.46 (1)
Try to scare the animal away	2.01 (6)	0	5.13 (2)	1.83 (4)
Try to kill the animal	2.34 (7)	0	2.56 (1)	2.74 (1)
I would do nothing	1 (3)	0	2.56 (1)	0.91 (1)
Call someone that can take care of it	93.65 (280)	96.97 (4)	89.74 (35)	93.61 (205)
If you have to call someone about a rabid animal, who would be the easiest for you to call?				
Family member, friend, or neighbor	0.68 (2)	0	0	0.92 (2)
Animal control (I have the number)	35.37 (104)	12.9 (4)	47.37 (18)	36.41 (79)
Animal control (I do not have the number)	31.97 (94)	61.29 (19)	31.58 (12)	27.65 (60)
Local Public Health Department (I have the number)	0.68 (2)	0	0	0.92 (2)
Local Public Health Department (I do not have the number)	0.68 (2)	0	2.63 (1)	0.46 (1)
Police/911	30.61 (90)	25.81 (8)	18.42 (7)	33.64 (73)
How would you respond to a dog biting your hand?				
Call a doctor	16.67 (50)	15.15 (5)	10 (4)	18.72 (41)
Care for the wound yourself	4 (12)	3.03 (1)	0	5.02 (11)
Go to the emergency room	60.33 (181)	69.67 (23)	77.5 (31)	55.25 (121)
Find the dog's owner and ask for vaccination records	19 (57)	12.12 (4)	12.5 (5)	21 (46)
Would you report the dog to anyone?				
Yes	96.66 (289)	93.94 (31)	100 (40)	96.33 (210)
No	3.34 (10)	6.06 (2)	0	3.67 (8)
If you have to report the dog, who would you report to?				
Family member, friend, neighbor	1.71 (5)	6.45 (2)	0	1.4 (3)
Animal Control	71.92 (210)	51.61 (16)	84.21(32)	72.56 (156)
Local Public Health Department	5.82 (17)	9.68 (3)	5.26 (2)	5.58 (12)
Police/911	20.55 (60)	32.26 (10)	10.53 (4)	20.47 (44)

Table 3. Frequency of answers regarding rabies information outlet preference in Greensboro, North Carolina, 2009.

Survey Questions and Possible Answers	Responses % (n)			
	Total Responses	Hispanic/Latinos	African Americans	Whites
If you want to learn more about rabies, what would be the BEST way to deliver that information to you?				
Internet	47.98 (119)	32.14 (9)	32.43 (12)	53.11 (94)
Mail	34.27 (85)	42.86 (12)	51.35 (19)	29.94 (53)
Television	15.32 (38)	14.29 (4)	16.22 (6)	15.25 (27)
Radio	2.42 (6)	10.71 (3)	0	1.69 (3)
How would you like to be informed about future low cost/free rabies clinics offered by Guilford County?				
Media (newspaper, radio, TV, Internet, email)	35.34 (41)	75 (6)	20 (4)	35.63 (31)
Family of friends	17.24 (20)	0	20 (4)	17.24 (15)
Veterinarian	12.93 (15)	25 (1)	5 (1)	14.94 (13)

4. Discussion

Pet vaccination rates reported by respondents was surprisingly high (85%). In surveys when the public are asked about regulatory compliance without penalty, there is a tendency to respond what it

is expected and not what actually is done. Therefore, the results may reflect a response bias—more specifically, an expectation bias. Pet owners know that they should be vaccinating their pets for rabies and report they have done it, even when they have not. The average one-year tags for vaccination in the state of North Carolina is 520 and for three-year tags is 445; these are tags issued by veterinary or animal control agencies [19]. The pet population in Greensboro is expected to be of the order of 53,000 dogs and 58,000 cats using the American Veterinary Medicine Association Pet Ownership Calculator (<https://www.avma.org/KB/Resources/Statistics/Pages/US-pet-ownership-calculator.aspx>). These numbers exceed the reported averages of rabies tags issues by veterinarians or animal control agencies.

Most Hispanic/Latino respondents indicated they were not aware of the rabies vaccination clinics offered by the county. Lack of information in Spanish is a possible explanation, in combination with a delivery system not favored by Hispanic/Latinos. The responses highlighted specific information outlets, giving public health officials a very clear direction on how to disseminate information. In NC there are a few Spanish-speaking radio stations, one television channel, and a couple of newspapers either sold at low cost or provided for free at stores selling food or products from Latin America and the Caribbean. Hispanic/Latinos frequent stores owned by other Hispanic/Latinos, and information regarding low cost/free rabies vaccination clinics and preventive information can be posted on information boards usually available at these places.

Information regarding whom to contact if an animal suspected of having rabies is encountered should be made available to the public. Although most survey respondents indicated that they would report a rabid animal to Animal Control, they were unaware of the correct number to call. Many respondents (30%) indicated they would report their concerns to 911, especially after being asked if they knew the number for Animal Control. Animal Control could provide the public with educational resources such as multilingual information leaflets, refrigerator magnets, and calendar reminders to disseminate rabies information about reporting rabies suspicious animals, telephones to contact authorities, vaccination protocols and laws, and reporting an animal bite. Furthermore, 911 emergency respondents could be provided with a protocol for responding to calls about rabies while dispatching Animal Control.

Our results suggest that outreach efforts would benefit from considering how ethnicity, income, and education impact the information outlets used by the public. Hispanic/Latinos and African Americans responded that they would like to receive information through postal mail, whereas Whites indicated that they would prefer the information be made available via the internet. These differences may reflect differential access to the internet, as only 51% of Hispanic/Latinos and 49% of African Americans have a home internet connection, compared to 66% of non-Hispanic/Latino Whites in 2011 in United States [20]. Similarly, 41% of those surveyed by Pew Research Center reported earning less than \$30,000 and having internet access at home, and only 22% of people without high school diploma had home internet access [20]. The mean income for Hispanic/Latinos in our study was \$19,000 a year, and had lower education levels than any other demographic group (39% did not have a high school diploma). Most African Americans in this study fared similarly to Hispanic/Latinos in income (less than \$30,000/year); however, they reported a higher level of education (45% reported having a high school diploma and some college education). Half of the Hispanic/Latinos and African Americans reported access to a mobile internet connection. Many state and county websites are not designed to be accessed by phone, and present limited content when accessed by mobile devices. Websites enhanced for mobile access are a possible system to use adding to the other preferred methods to receive information [20]. Cellular phones could also be used to send information to the public about rabies, rabies vaccinations, and exposure notifications [21], particularly as these messages can be delivered to target segments of the population by creating geographical boundaries for message recipients.

Risk perception and aversion is associated with culture and complicated by socio-economic and demographic status. Language is one of the many factors affecting message decoding and interpretation, but is a very important one. Knowing the population at risk is fundamental to

establishing a public health program for rabies and other zoonoses. Studies like the one presented offer a glimpse of where to start, not only in one county in NC, but other places where a public health campaign is needed. Our studies have prompted county public health officials throughout the country to request our survey instrument. Several officials have shared the need to better inform different segments of the population, and the lack of research efforts in the area of outreach and publicity of vaccination and other community-service oriented efforts.

The Centers for Disease Control and Prevention 'Simply Put' guide for creating educational materials was used as the base for the elaboration of educational materials on rabies and other zoonotic diseases [22]. The educational materials are available for public, Animal Control, and non-governmental and animal rescue organizations, as well as public institutions. A bilingual trifold was developed; this contains a cutout portion where the public can write the numbers of the local Animal Control and Public Health. The effectiveness evaluation of the educational materials is pending. Additionally, a bilingual rabies coloring book, various brochures, and leaflets were made available to the Guilford Animal Control after the study completion; radio and television appearances and short messages were published in Spanish-language local newspapers.

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Conflicts of Interest: The authors declare no conflict of interest.

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Article

Field Studies Evaluating Bait Acceptance and Handling by Dogs in Navajo Nation, USA

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Abstract: Mass parenteral vaccination remains the cornerstone of dog rabies control. Oral rabies vaccination (ORV) could increase vaccination coverage where free-roaming dogs represent a sizeable segment of the population at risk. ORV's success is dependent on the acceptance of baits that release an efficacious vaccine into the oral cavity. A new egg-flavored bait was tested alongside boiled bovine intestine and a commercially available fishmeal bait using a hand-out model on the Navajo Nation, United States, during June 2016. A PVC capsule and biodegradable sachet were tested, and had no effect on bait acceptance. The intestine baits had the highest acceptance (91.9%; 95% confidence interval (CI), 83.9–96.7%), but the fishmeal (81.1%; 95% CI, 71.5–88.6%) and the egg-flavored baits (77.4%; 95% CI, 72.4–81.8%) were also well accepted, suggesting that local bait preference studies may be warranted to enhance ORV's success in other areas where canine rabies is being managed. Based on a dyed water marker, the delivery of a placebo vaccine was best in the intestine baits (75.4%; 95% CI, 63.5–84.9%), followed by the egg-flavored (68.0%; 95% CI, 62.4–73.2%) and fishmeal (54.3%; 95% CI, 42.9–65.4%) baits. Acceptance was not influenced by the supervision or ownership, or sex, age, and body condition of the dogs. This study illustrates that a portion of a dog population may be orally vaccinated as a complement to parenteral vaccination to achieve the immune thresholds required to eliminate dog rabies.

Keywords: rabies; bait; dog; oral vaccination

1. Introduction

Dogs (*Canis lupus familiaris*) account for approximately 95% of all rabies cases reported worldwide, and they are also responsible for most human cases. Globally, the burden is not equal among countries [1]. Dog-mediated rabies has been eliminated from Europe, North America, and parts of Latin America. However, canine rabies remains a serious health problem in many countries in Africa and Asia. The most cost-effective method to control dog rabies is mass dog vaccination [2]. However, these campaigns have not always succeeded in achieving sufficient levels of herd immunity to interrupt the transmission cycle among dogs [3–5]. Poorly-supervised, free-roaming dogs play a pivotal role in transmission of rabies [6]. In some countries, a large proportion of the overall dog population is free-roaming, regardless of ownership. For example, over 50% and 66% of the owned dog population in Haiti and Bali, Indonesia, respectively, is allowed to roam freely [3,5]. During mass vaccination campaigns, these dogs are often not accessible for

parenteral vaccination, or can only be vaccinated after considerable efforts to capture the free-roaming individuals [7,8]. Hence, alternative strategies—such as offering dogs vaccine baits—to reach these inaccessible dogs have been explored [9,10]. Initially, this novel approach targeted fox rabies in Europe and Canada. Later, this effective wildlife disease management tool was adapted for other reservoir species such as raccoon dogs (*Nyctereutes procyonoides*), raccoons (*Procyon lotor*), coyotes (*Canis latrans*), golden jackals (*Canis aureus*), and domestic dogs [11,12]. One of the essential components of this concept is a bait that is well accepted by the target population. Fishmeal baits used for the oral rabies vaccination (ORV) of red foxes (*Vulpes vulpes*) have been poorly accepted by dogs in many areas [13–15]. Nevertheless, a commercial fishmeal bait was accepted in previous studies on the Navajo Nation [16]. Several baits have been developed and tested in dogs around the world, including among others Mexico [17], Tunisia [18,19], Egypt [20], Turkey [13], the Philippines [21], and Guatemala [22]. Machine-made baits are generally not as well accepted as baits made from local available food sources [13,23]. However, the preparation of large quantities of baits from local food sources is problematic. Therefore, a well-accepted bait that can be produced on a large scale in a short period of time with quality assurance is required.

Most dogs on Navajo Nation lands are not subject to movement restrictions by their owners, and have little or no access to routine veterinary care, including vaccinations [16]. To react swiftly in the case of the re-emergence of rabies in the Navajo Nation, veterinary authorities have investigated the possibility of ORV in dogs, and bait studies have been previously conducted there [16,24,25]. The present study represents a continuation of these studies. The objective of this study was to evaluate the bait handling and acceptance of a recently developed bait by free-roaming dogs in the Navajo Nation. The new egg-flavored baits tested were previously found to be readily accepted by captive beagles and wolves (*Canis lupus*) [26].

2. Materials and Methods

2.1. Study Area

The Navajo Nation is a sovereign Native American Diné first nation located in the arid to semi-arid areas of high elevation (1600–3000 m) within northeastern Arizona, southeastern Utah, and northwestern New Mexico in the U.S. The reservation is 71,000 km².

The Chinle (Chinle, Tsaile, Many Farms, Del Muerto, Lukachukai, Round Rock, and Rough Rock) and Shiprock (Shiprock, Hogback, and Cudii) Navajo Nation communities were selected for the bait trial, with the greater Chinle community located in Arizona, and the greater Shiprock community located in New Mexico (Figure 1). The study area comprised a combination of rural and semi-urban areas.

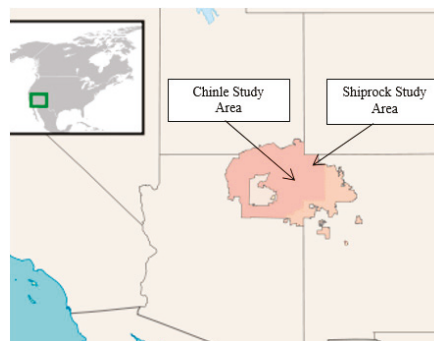


Figure 1. Location of the Navajo Nation (marked area) within the U.S., and the Chinle and Shiprock communities where the bait studies took place.

2.2. Field Trial

Three different baits and two different blister systems were tested (Table 1). All blisters contained dyed water and no active ingredients. As part of the overall field evaluation, the dye was used to visually aid field personnel to see if any of the liquid from the bait was in the mouths of the dogs as they ingested the bait. The dye was Patentblau V (Sigma Aldrich), a blue dye used in the food industry that is safe for human consumption and therefore was assumed to be safe for dogs. All three baits have recently been tested with captive beagles and European wolves [26] (Figure 2). In addition to a new bait comprised of gelatin and egg powder, a bait matrix of boiled section of cow intestine and a bait matrix identical to a commercially available bait made from vegetable fats and fishmeal used in ORV in red foxes was tested. One blister was a PVC capsule sealed by aluminum foil previously tested in Turkey and the Philippines [21,27] and the other was a sachet made from biodegradable foil and is still under final development.

Table 1. Bait and blister components making up the combinations field tested on Navajo Nation, June 2016.

	Material	Size (cm)	Weight (gr)
Bait			
Intestine	boiled local cow intestine sections	8–12 cm long	20–30
Fishmeal	vegetable fats + fishmeal	8.5 × 4.0 × 1.2	43
Egg	gelatin + egg powder	8.5 × 4.0 × 1.2	43
Blister			
Capsule	PVC + aluminum foil	6.5 × 3.0 × 0.7	3.5 mL
Sachet	Biodegradable foil	5.0 × 3.0 × 0.4	3.5 mL



Figure 2. The two blister types used (left top; left—biodegradable, right—PVC) and the experimental egg-flavored bait (right top—yellow), fishmeal bait (right-top—brown), and intestine bait with capsule (bottom).

Three person teams visited their allocated sections of the study area and systematically searched for dogs between 09:00 and 19:00 during 13–15 June 2016. If a dog was located, it was first determined if the dog had an owner and, if so, the dog was only offered a single bait with consent of the owner. Owners were given a leaflet that contained additional information and a phone number to report any potential adverse reactions. If the dog did not appear to have an owner, it was determined based on behavior if the dog was a stray/unowned (free-ranging dogs that survived and reproduced independently of human interaction or assistance) or community owned (free-ranging dogs that were fed and cared for by the community, but were not claimed by or residing with one single resident) by the ease of approachability of the dog. Community dogs were friendly and easily approached, while stray dogs were more cautious and would scare easily. All located dogs were offered a bait; stray/unowned and community dogs were approached and offered baits similar to owned dogs, with the exception that unowned dogs were approached more carefully to not scare them away. Bait type offered was randomly pre-determined. Half of all three bait types contained a hard PVC-capsule and the other half of all three bait types contained a soft biodegradable sachet. For every four experimental egg-flavored baits offered, one intestine and one fishmeal bait was included. The 4:1:1 ratio of bait flavor and the ratio of blister type were included in the randomization procedure. Since no data was available for bait uptake of the new egg-flavored population, this ratio were used to obtain as much data as possible on this bait. The intestine bait and fishmeal bait was used as a positive and negative control for comparison, respectively; it was assumed that the intestine bait would be well accepted and the fishmeal bait would be accepted poorly based on previous studies in the Philippines and Turkey [21,27]. A form was filled out during every baiting attempt to collect data on bait acceptance and handling. Bait handling describes what the dog does with the bait after acceptance, including handling time (time spent by the dog manipulating the bait; time was grouped into time intervals: <10 s, 10–30 s, 30–60 s, or >60 s) and if the vaccine container was perforated, discarded or swallowed. Based on these observations, an assessment was made by the observer if the dog would most likely have been successfully vaccinated based on the release of the contents of the blister into the oral cavity (“effectiveness”). Additional information recorded for each dog included: ownership, level of supervision, sex, age, size, and body condition. If the dog discarded the vaccine blister or if the dog did not accept the bait, the bait was recollected by the observer.

Data were initially analyzed using the statistical software package Modde 10.0, Umetrics by fitting the data in a model by multilinear regression or partial least squares (PLS). However, all but one factor examined were qualitative (blister type, day, ownership, level of supervision, sex, age, size, and body condition). The responses measured (bait acceptance and vaccinated) were also qualitative (yes, no). Since qualitative factors tend to negatively influence the statistical power, the model had an extremely poor fit and most of the model coefficients were not significant; only bait type, intestine (positive), and fish meal (negative) had a significant effect. Therefore, data were analyzed with chi-squared using the statistical software package GraphPad Prism 6.

3. Results

A total of 532 baiting attempts were recorded. Twenty-two data sheets with conflicting data were omitted from the analysis. Eighteen of the 22 omitted data sheets originated from a single team on the first day; most entries on those data sheets were not filled out or were filled out incorrectly. Also, two dogs ran off immediately when approached, and one dog was chased away by a passing car before it reached the bait offered. Hence, a total of 507 data sets was used for analysis, although it was not always possible to fill out the complete form. If no data was available for a specific parameter investigated, the data set was not included in the analysis for that parameter. Additional analysis on data collected for the various parameters of this study are included as Supplementary Tables S1–S9.

The ownership, confinement status, gender, age classification, body condition, and body size for the target dog population was documented and summarized (Figure 3). There were no significant effects on bait acceptance associated with dog age, sex, body condition, and size by contingency

analysis. The overall bait acceptance (consumption) was 80.6% (95% CI, 76.8–83.9%; Table 2); the intestine baits were refused significantly less often than the other two baits ($\chi^2 = 9.091$, $df = 2$, $p = 0.01$). The type of blister had no significant effect on bait acceptance ($\chi^2 = 1.276$, $df = 1$, $p = 0.26$). The dogs' level of confinement (restricted vs. unrestricted) or ownership status (owned vs. community, including ownerless) had no significant effect on bait acceptance (Figure 4a,b). Also, no significant effect in bait acceptance was observed for the time of day that baits were offered to the dogs ($\chi^2 = 3.765$, $df = 4$, $p = 0.44$).

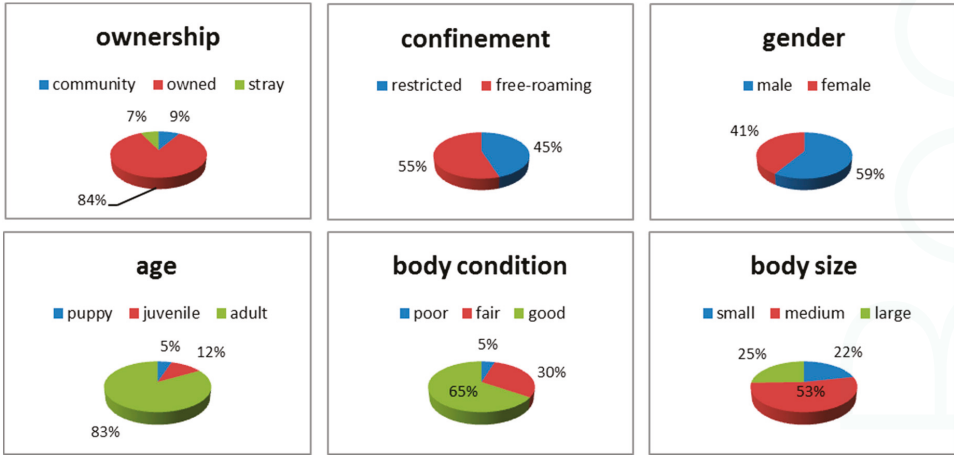


Figure 3. Characteristics of the dogs included in this study.

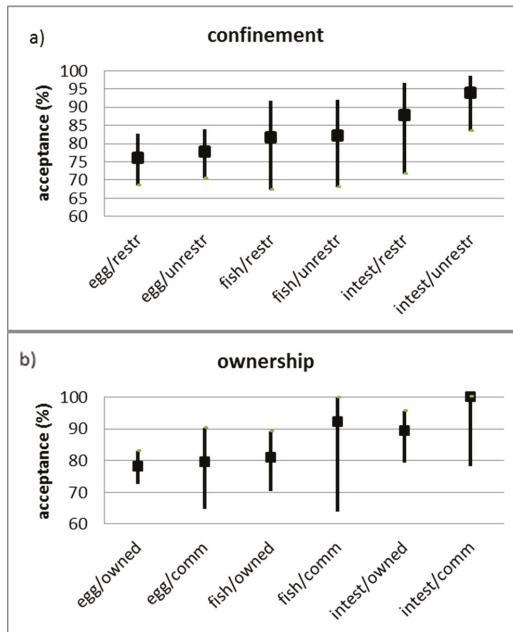


Figure 4. Bait acceptance (%) (mean percentage and 95% confidence interval) associated with (a) level of confinement (restr—restricted; unrestr—unrestricted) and (b) ownership status (comm—community).

Table 2. Summary of bait acceptance and handling, fate of blister and final assessment if vaccine was released in oral cavity of the dog (vaccinated), included 95% confidence intervals (95% CI). Except for bait acceptance all numbers are based on dogs that (partially) consumed the bait after it was accepted.

Bait	Blister	Bait Accepted			Bait Consumed Completely			Blister Swallowed			Blister Perforated			'Vaccinated'		
		n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI
egg	capsule	140	74.5	69.3–82.1	106	86.9	79.6–92.3	57	42.2	33.8–51.0	112	94.9	89.3–98.1	114	91.2	84.8–95.5
	sachet	110	81.5	73.9–87.6	90	90.0	82.4–95.1	82	80.4	71.4–87.6	90	94.7	88.1–98.3	90	88.2	80.4–93.8
fish	capsule	40	87.0	73.7–95.1	34	87.2	72.6–95.7	6	15.8	6.0–31.3	29	78.4	61.8–90.2	20	55.6	38.1–72.1
	sachet	33	75.0	59.7–86.8	28	90.3	74.2–98.0	20	66.7	47.2–82.7	22	88.0	68.8–97.5	24	85.7	67.3–96.0
intestine	capsule	39	88.6	75.4–96.2	34	97.1	75.2–97.1	27	73.0	55.9–86.2	28	96.6	82.2–99.9	29	87.9	71.8–96.6
	sachet	40	95.2	83.8–99.4	37	97.4	86.2–99.9	37	94.9	82.7–99.4	19	76.0	54.9–90.6	23	79.3	60.3–92.0
Total		402	80.6	76.8–83.9	329	90.1	86.6–93.0	229	60.3	55.0–65.1	300	91.2	87.6–94.0	300	85.0	80.8–88.5



When baits were accepted by the dogs, 90.1% (95% CI, 86.6–93.0%) of the animals consumed the whole bait. There was no significant difference between blisters ($\chi^2 = 0.8826$, $df = 1$, $p = 0.35$) and bait type ($\chi^2 = 5.213$, $df = 2$, $p = 0.07$) if the bait offered was consumed completely or partially. The intestine baits were consumed significantly faster than the other two baits ($\chi^2 = 40.90$, $df = 6$, $p < 0.0001$) (Figure 5). The baits containing a sachet were also consumed significantly more rapidly than baits with the hard PVC capsule ($\chi^2 = 10.65$, $df = 3$, $p = 0.01$). The intestine and sachet combination was consumed significantly much faster in comparison to intestine baits with the capsule; 59% (95% CI, 42.1–74.4%) of the intestine baits accepted were consumed within 10 s ($\chi^2 = 10.95$, $df = 3$, $p = 0.01$). No significant difference in consumption time between the blister types was observed for the egg baits ($\chi^2 = 1.413$, $df = 3$, $p = 0.70$) and fishmeal baits ($\chi^2 = 4.689$, $df = 3$, $p = 0.20$).

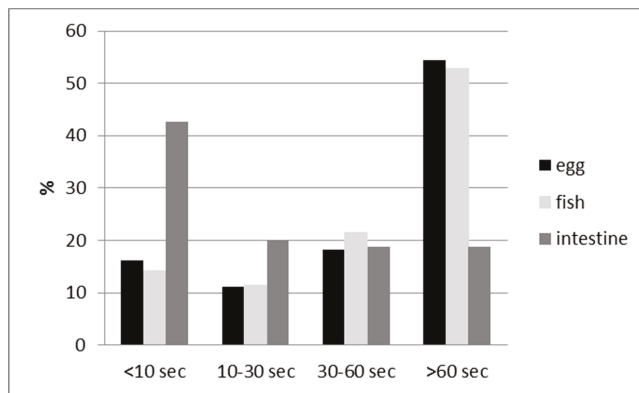


Figure 5. Bait handling time (seconds) for the 3 different baits.

There was a significant effect on the fate of the blister (swallowed or discarded) for both the bait and blister type. Almost all dogs (84.2%; 95% CI, 74.0–91.6%) that consumed an intestine bait swallowed the blister, while only 58.6% (95% CI, 52.1–61.0%) and 38.2% (95% CI, 26.7–50.8%) of the dogs that accepted the egg and fishmeal baits, respectively, swallowed the blister ($\chi^2 = 32.19$, $df = 2$, $p < 0.0001$). Irrespective of bait type, the sachets were swallowed more often than the PVC capsules; 81.3% (95% CI, 74.6–86.8%) of dogs swallowed the sachet, but only 42.9% (95% CI, 30.1–49.8%) of the capsules were swallowed ($\chi^2 = 58.05$, $df = 1$, $p < 0.0001$). However, overall, the type of blister did not have an influence on bait handling time.

The blisters in the egg baits (94.8%; 95% CI, 90.9–97.4%) were more often perforated than those in the fishmeal baits (82.3%; 95% CI, 70.5–90.8%) and intestine baits (87.0%; 95% CI, 75.1–94.6%; $\chi^2 = 10.83$, $df = 2$, $p = 0.004$). Blister type did not seem to have any influence on perforation, except for the intestine baits, in which the PVC capsules (96.6%; 95% CI, 82.2–99.9%) were significantly perforated more often than the sachets (76.0%; 95% CI, 54.9–90.6%; $\chi^2 = 5.026$, $df = 1$, $p = 0.03$).

Overall, there was no significant effect of blister type on vaccination success based on dyed water marker ($\chi^2 = 0.3145$, $df = 1$, $p = 0.5749$), but there was a highly significant effect of bait type on vaccination success (effectiveness) ($\chi^2 = 17.52$, $df = 2$, $p = 0.0002$). The fishmeal baits had a significantly lower vaccine delivery success rate than the other two baits. The content of the blister was considered successfully released in the oral cavity of 68.8% (95% CI, 55.9–79.8%) of the dogs that accepted the fishmeal baits. For the egg and intestine baits, 89.9% (95% CI, 85.2–93.5%) and 83.9% (95% CI, 72.3–92.0%) of the dogs were considered successfully ‘vaccinated’, respectively. The better accepted intestine baits had a lower vaccination success rate due to the very short handling time; dogs that consumed baits in less than 10 s were significantly more often considered ‘not vaccinated’ ($\chi^2 = 33.45$, $df = 3$, $p < 0.0001$). The low success rate of the fishmeal baits was predominantly caused by the blister

type; using the hard PVC capsule, only 55.6% (95% CI, 38.1–72.1%) of the dogs that accepted the bait were considered vaccinated, while for the sachet this percentage was 85.7% (95% CI, 67.3–96.0%; $\chi^2 = 6.668$, $df = 1$, $p = 0.01$). For the other two baits, no significant effect of the blister was detected. A total of 91.2% (95% CI, 84.8–95.5%) and 88.2% (95% CI, 80.4–93.8%) of the dogs accepting the egg baits containing the capsule or the sachet, respectively, were considered vaccinated ($\chi^2 = 0.54$, $df = 1$, $p = 0.46$).

A total of 87.9% (95% CI, 71.8–96.6%) and 79.3% (95% CI, 60.3–92.0%) of the dogs accepting the intestine baits with the capsule and sachet, respectively, were considered vaccinated ($\chi^2 = 0.84$, $df = 1$, $p = 0.36$). If we analyze bait acceptance and vaccination success in tandem, the discrepancy between acceptance and vaccination is much lower for the egg baits than for the other two baits (Figure 6). The effectiveness of the fishmeal baits was much lower than the other two bait types.

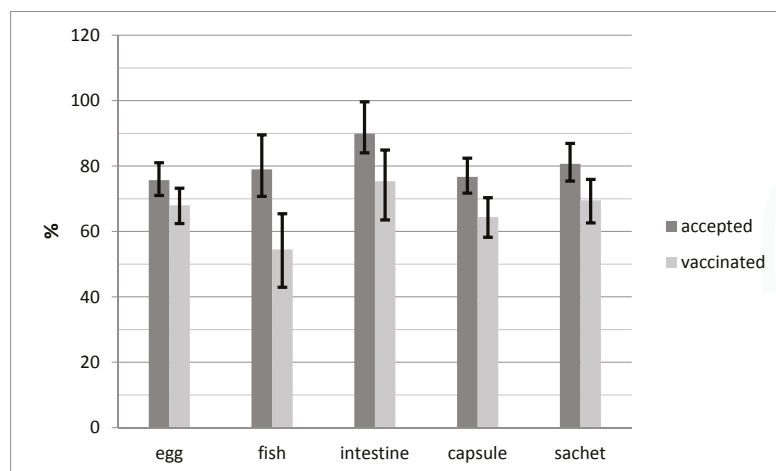


Figure 6. The difference in the proportion of dogs accepting and consuming baits and blisters and the subsequent assessment of a successful vaccination attempt (release of contents of vaccine blister in oral cavity) with associated 95% confidence intervals.

4. Discussion

An important precondition for oral vaccination is the availability of a bait that is well accepted by the target species under field conditions. Initially, efforts to develop baits for dogs were based on those previously developed for wildlife such as foxes and raccoons; a review of some of these early studies can be found in Linhart [28]. A bait that is poorly accepted by dogs has no use for oral vaccination of these animals against rabies, even if all of the other requirements have been fulfilled. Bait acceptance studies using a variety of baits have been conducted in different countries, and showed regional differences in bait preference [13,17–22]. For example, the significantly better accepted boiled intestine baits used in this study were only taken by 58.8% of the dogs in Guatemala. Poultry flavored baits were accepted more often than other bait types in Guatemala [22]. No significant difference in bait acceptance was observed between the fishmeal and egg-flavored baits in the Navajo Nation. During a previous study conducted on the Navajo Nation with confined shelter dogs, five flavors were tested using a commercial bait matrix: bacon, cheese, sweet, egg, and fish. The sweet-flavored baits were the least preferred, and among the other flavors no significant difference could be observed [25]. Interestingly, the acceptance of the egg-flavored baits (79.9%) was quite similar to that observed in the current study (77.4%), although a different ground bait substance was used. In another study at the dog shelter in the Navajo Nation, seven different flavors added to a commercial bait matrix were tested

using the two-food-preference test approach [24]. Here, a dog-food flavor had the highest preference. However, the preference for dog-food flavor may have been a result of the familiarity of the dogs with this food source in the shelter. Bergman et al. [16] tested four different commercially available bait matrices, and found that the bait acceptance of free-roaming dogs in the Navajo Nation fluctuated between 56.5% and 84.5%. The fish-flavored coated sachet had the highest bait consumption among the four baits tested. The relatively high acceptance rate of the fishmeal baits in these previous studies and our study in Navajo Nation was rather unexpected, considering that fish is an unusual food source for these animals and the poor results obtained with fish-flavored baits in free-roaming and confined dogs in other areas [13,14,26]. However, in Sri Lanka, a commercially available fish-flavored bait was well accepted (84%) by owned dogs [29]. In several studies, a low acceptance of manufactured baits by free-roaming dogs was explained by the lack of familiarity with the taste, smell, shape, and texture of the baits [13]. Free-roaming dogs are often fed on household leftovers or offal. Hence, it can be expected that baits based on these resources are more attractive [30]. Thus, baits with a high acceptance rate in one area can be refused by dogs in another area, due to different local food preferences and experiences. Although several studies indicate high acceptance rates for manufactured baits [15,23,29], generally, baits made from locally available material have a much higher acceptance rate [13,21,22]. During a field study in Turkey, 96% of all dogs accepted the locally-made bait based on minced-meat mixed with bread crumbs [13]. In the Philippines, more than 90% of the dogs accepted baits made from different local materials [21]. Locally produced baits are often not favored by field personnel due to difficulties in preparing, handling, and storing the baits [22]. However, due to the observed regional differences in bait acceptance, it may be difficult to develop a universally well-accepted bait for dogs. Furthermore, dog rabies is predominantly a problem in developing countries with limited financial resources available for rabies control. The cost of purchasing imported manufactured vaccine-loaded baits (including costs for their transportation and cold-storage) will most likely exceed the cost of the importation of vaccine-loaded blisters that could be incorporated locally in a suitable bait [21].

As shown in this study, when a dog accepts a bait, it does not automatically mean that the animal will be successfully vaccinated. The investigational phase and bait handling period upon acceptance should not last too long, because it increases the risk that external factors will lead to an interruption, and, consequently, a failed vaccination attempt. Hence, the selected bait should be immediately attractive to the dogs [13]. The handling time of the intestine bait was significantly shorter than for the other two bait types. However, the animal should not swallow the bait immediately, including the vaccine blister; the vaccine needs to be released in the oral cavity, and, therefore, the blister has to be punctured before it is swallowed or discarded. Actually, it was shown that the dogs consuming the bait within ten seconds were significantly more often considered 'not vaccinated'. The biodegradable sachet was significantly more often swallowed than the PVC capsule, but the type of blister did not influence the handling time of the bait. Dogs may separate the blister from the bait matrix, or it may drop out of the dogs' mouth before being punctured due to poor adherence with the bait matrix. In this study, it was observed that the fishmeal baits often broke into pieces when tossed to the dogs, consequently separating the vaccine blister from the bait matrix. Even when the blister is punctured, it does not guarantee a successful vaccination; sometimes large amounts of the vaccine are retained within the blister, and the released amount would not be sufficient to induce a protective immune response. Finally, during bait handling, the vaccine virus released from the punctured blister can be spilled on the ground [21]. Hence, not only does bait palatability need to be investigated and optimized, but also the influence of the shape, size, and texture of the bait matrix, as well as the blister, needs careful consideration in terms of the efficient release of the vaccine in the oral cavity. Experimental studies where the uptake of a single vaccine bait protected the dogs against a subsequent challenge infection have shown that this is feasible [27,31].

The swallowing of the PVC capsule can cause an adverse reaction (gastric intolerance) in dogs when swallowed [32], although no such adverse events were reported after the present study. The biodegradable sachet will cause less intolerance when swallowed. The swallowing of the blister

after prolonged chewing actually has an advantage, by reducing the amount of discarded blisters in the environment. At the moment, it is suggested to re-collect all of the discarded vaccine containers to reduce unintentional human contact with the vaccine virus. However, sometimes the dog does not consume the bait immediately, but walks away with it, and subsequently the vaccine blister cannot be re-collected. If a vaccine container was used that is frequently swallowed, the number of discarded blisters not re-collected would be further reduced.

This study found no significant effect of the dogs' age, sex, body condition, and size on bait acceptance. Therefore, the age distribution, gender ratio, or health of a local dog population is not expected to impact the bait acceptance or the vaccination success of a population when using the bait types assessed in this study. There was also no significant effect on bait acceptance associated with ownership status or the dogs' level of confinement. Thus, similar conclusions could be drawn on the population regardless of the ownership status and level of confinement of the dogs.

It can be concluded that both bait types, manufactured or made from local material, were well accepted by the local dog population, but the bait, the blister, and the interaction between these two clearly influenced bait handling, and consequently the outcome of the vaccination attempt. There is a discrepancy between the bait acceptance rate and the subsequent estimated vaccination rate. It seems that the difference between these two was the lowest for the newly developed egg-flavored baits. However, this bait can be further optimized, and potentially this discrepancy could be further reduced. It was shown that several dogs had problems with the shape and texture of this bait. The animals had problems picking up the bait from the ground (a condition of shape) and sometimes seemed to consider the bait more as a toy than as a food item (a condition of texture). Both aspects increased the handling time, and subsequently the risk of disturbance and discontinuation of bait consumption. With minor improvements to the bait and further research, the egg-flavored baits have potential to be effectively used as an ORV bait for dogs in the Navajo Nation.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2414-6366/2/2/17/s1>, Table S1. Bait acceptance for the 3 bait types and 2 blister types; unknowns not further included in statistical data analysis, Table S2. Amount (%) of bait consumed by dog upon acceptance for the 3 bait types and 2 blister types; unknowns not further included in statistical data analysis, Table S3. Bait handling time (seconds) upon acceptance for the 3 bait types and 2 blister types; unknowns not further included in statistical data analysis, Table S4. The number of animals that swallowed or discarded the blister after bait acceptance for the 3 bait types and 2 blister types; unknowns not further included in statistical data analysis, Table S5. Number of blisters perforated/ruptured (yes) or not (no) after bait acceptance for the 3 bait types and 2 blister types; unknowns not further included in statistical data analysis, Table S6. Number of dogs that accepted a bait and were considered vaccinated (likely) or not (not likely) in case that the blister would have contained a vaccine for the 3 bait types and 2 blister types; unknowns not further included in statistical data analysis, Table S7. Bait acceptance and the effectiveness (dog likely to be vaccinated) for the 3 bait types and 2 blister types. Only animals were included for which data on both parameters, acceptance and effectiveness (likely vaccinated), were available, Table S8. Bait handling time (seconds) and the subsequent effectiveness of the vaccination attempt (likely or not likely vaccinated) for the 3 bait types; unknowns (effectiveness) not included in data analysis, and Table S9. Bait acceptance by owned and community (including ownerless) dogs for the 3 bait types and 2 blister types; unknowns (ownership status) not included in data analysis.

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Author Contributions: The author contribution to this study is as follows: S.B., D.B., A.V. and R.C. conceived, designed and performed the field experiment; S.B. and A.V. analyzed the data; A.V. provided the placebo baits and blisters for this study (S.B., D.B., R.C. and A.V. prepared the intestine baits that were locally sourced) and S.B., D.B., R.C., A.V. and A.M. collaboratively wrote and reviewed multiple drafts of this manuscript prior to submission.

Conflicts of Interest: A.V. is a full-time employee of IDT Biologika, Germany, a company that manufactured oral rabies vaccine placebo baits for this study. He was involved in the design of the study; in the collection, analyses and interpretation of data as well as the writing of the manuscript. However, I can affirm no direct funding was received from the vaccine company for this study. R.C. is the National Rabies Management Coordinator for the USDA, APHIS, Wildlife Services program and indirectly provided discretionary Federal funding to support field work and the writing of the manuscript. The Navajo Nation provided salaries of Navajo Nation employees (including S.B.) who participated in the field work.

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Article

Modeling Raccoon (*Procyon lotor*) Habitat Connectivity to Identify Potential Corridors for Rabies Spread

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Abstract: The United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Wildlife Services National Rabies Management Program has conducted cooperative oral rabies vaccination (ORV) programs since 1997. Understanding the eco-epidemiology of raccoon (*Procyon lotor*) variant rabies (raccoon rabies) is critical to successful management. Pine (*Pinus* spp.)-dominated landscapes generally support low relative raccoon densities that may inhibit rabies spread. However, confounding landscape features, such as wetlands and human development, represent potentially elevated risk corridors for rabies spread, possibly imperiling enhanced rabies surveillance and ORV planning. Raccoon habitat suitability in pine-dominated landscapes in Massachusetts, Florida, and Alabama was modeled by the maximum entropy (Maxent) procedure using raccoon presence, and landscape and environmental data. Replicated ($n = 100/\text{state}$) bootstrapped Maxent models based on raccoon sampling locations from 2012–2014 indicated that soil type was the most influential variable in Alabama (permutation importance $PI = 38.3$), which, based on its relation to landcover type and resource distribution and abundance, was unsurprising. Precipitation ($PI = 46.9$) and temperature ($PI = 52.1$) were the most important variables in Massachusetts and Florida, but these possibly spurious results require further investigation. The Alabama Maxent probability surface map was ingested into Circuitscape for conductance visualizations of potential areas of habitat connectivity. Incorporating these and future results into raccoon rabies containment and elimination strategies could result in significant cost-savings for rabies management here and elsewhere.

Keywords: circuit theory; habitat suitability; Maxent; pine; *Pinus*; *Procyon lotor*; rabies; raccoon; risk model

1. Introduction

Rabies kills approximately 59,000 humans world-wide annually [1,2], but due to considerable control efforts, human cases are relatively rare in the developed world [1]. However, rabies control is expensive. Annual costs of living with rabies in the U.S. have been estimated at \$300 million [3] (\$646 million in 2017 USD [4]). Oral rabies vaccination (ORV) has proven effective for achieving wildlife rabies control [5] with noteworthy successes from a number of locations [6–12]. Raccoon (*Procyon lotor*) variant rabies (raccoon rabies) is currently present along the entire eastern seaboard, from Florida west to Alabama, north to the Canadian frontier, and west to Ohio. Consequently, it is one of the most important terrestrial variants currently circulating in North America in terms of incidence rates and proximity of raccoons to humans. Elimination of raccoon rabies is currently a high priority in the U.S. The United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Wildlife Services (WS) and cooperators have conducted ORV aimed at preventing the spread of raccoon rabies to the west (Phase I) since 1997. Phase II planning is underway and will focus on eliminating raccoon rabies from enzootic areas in the eastern U.S.

Considerable effort has been made to understand and model the spread, perpetuation, control, and economics of rabies. Deterministic and stochastic models for the spread of rabies in wildlife and for control scenarios with varying vaccination levels and barrier widths were evaluated, and the latter outperformed the former at rabies persistence and elimination simulations [13]. In Ontario, a time-series analysis was employed to try to understand regional differences in the dynamics of Arctic fox (*Alopex lagopus*) rabies in red foxes (*Vulpes vulpes*) [14], which ultimately led to the delineation of different rabies units with independent management strategies. Modeling has also demonstrated geographic clustering of raccoon rabies cases in New York, and allowed for consideration of possible causes for these and related temporal patterns [15]. Additionally, modeled likely consequences of global climate change include a potential primary reservoir shift for Arctic fox variant rabies from Arctic to red foxes in Alaska [16], and range expansion for common vampire bats (*Desmodus rotundus*) [17]. Economic models of rabies and its control have been created for diverse scenarios, with the monetary burden from raccoon rabies estimated at \$1.1 billion without ORV intervention over a 22-year horizon from 2012 to 2033 [18].

The concept of connectivity, or the ease of movement between landscape features, is important to understand in the contexts of both conservation and eco-epidemiology [19]. Connectivity modeling procedures are designed to identify critical movement areas for species of concern to achieve any of a number of management goals [20,21]. A relatively new approach to connectivity modeling is the adoption of circuit theory, in which the principles of electrical circuits are applied in ecology for creating more robust representations of and effects from known sources of resistance and multiple available pathways [22]. Through the incorporation of random walk theory [23], and Ohm's Law-like resistances (wherein current = voltage/resistance, or $I = V/R$) in the form of resistance (or conductance) maps generated through habitat suitability index (HSI) modeling, circuit theory modeling can suggest pathways for the greatest likelihood of movement of members of a species between nodes or regions [22].

In the absence of complete information on habitat or site occupancy, assessments of distribution and habitat or site preference become problematic. The Maxent approach [20,24] allows for the use of occurrence data without absence information, along with environmental data, to estimate distribution likelihood for a given landscape. The Maxent procedure output includes a receiver operating characteristic (ROC), with the area under curve (AUC) provided as a measure of performance of the model in terms of assessing habitat suitability. Used in tandem with Circuitscape [25], a visualization of likely areas of concern for managers is created and in the case of rabies, can delineate priority areas for surveillance and control.

Comparisons between Maxent and other available procedures suggest at least equal performance and often superiority for Maxent in many cases. For example, Poor et al. [26] compared Maxent to the expert-based analytic hierarchy process (AHP) for developing habitat suitability models (HSM).

They deemed the overall performance of the two HSM procedures to be similarly satisfactory, except that they considered Maxent's cell-based habitat analyses inflexible compared with the expert-based approach in which temporal and spatial analysis units can be manipulated. However, they felt that Maxent performed somewhat better at ultimately producing more corridors that contained pronghorn antelope (*Antilocapra americana*) locations when the surfaces produced were utilized in connectivity models. A consideration worth noting is that in cases of relatively small sample sizes, information criteria procedures such as Akaike's information criteria for small sample sizes (AICc) [27] or Bayesian information criteria (BIC) [28] provide somewhat better results than does the Maxent default AUC measure of model performance [29]. In another example, traditional movement models for golden-headed lion tamarins (*Leontopithecus chrysomelas*) in Brazil used Circuitscape [25] with an HSI resistance map to increase the authenticity of the modeled conservation situation in terms of tamarin movements between patches [30]. Circuitscape was also compared to least cost method (LCM) procedures for modeling connectivity using surfaces created through both the Maxent and AHP processes. While the corridors created using Maxent input generally contained more pronghorn antelope locations, the LCM proved superior in terms of generating pronghorn-containing corridors [26]. However, the ease with which Circuitscape's seamless integration of Maxent outputs provides useful visualizations of landscape connectivity help it retain its appeal.

The WS ORV program along the Appalachian Ridge and the New England/New York-Canada frontier is designed to take advantage of research findings indicating relatively low raccoon densities at higher elevations (>610 m), as established by raccoon density indexing (RDI) [31], and which are presumed to result in reduced raccoon contact rates. Also of importance, the spread of raccoon rabies appears to be negatively affected by rivers [32–34] and certain types of forested habitats [33,34].

Much of the area targeted for Phase II is comprised of pine-dominated (*Pinus* spp.) forests. For example, pitch pine (*P. rigida*) occurs in coastal areas from New England south through New Jersey; Virginia pine (*P. virginiana*) occurs from the mid-Atlantic coast inland to the Appalachian Ridge-south; longleaf pine (*P. palustris*) is found in coastal areas from southern Virginia to Texas, and loblolly pine (*P. taeda*) is found in large commercial plantings in the southeast [35]. Relatively low RDIs have been developed for pitch pine and pitch pine-scrub oak (*Quercus* spp.) forest types of southeastern Massachusetts and New Jersey [36]. Similarly, RDIs developed within loblolly and longleaf pine-dominated landscapes were lower relative to adjacent types [34,37].

We modeled raccoon habitat suitability in pine-dominated landscapes in Massachusetts, Florida, and Alabama by the maximum entropy (Maxent) procedure [20] using raccoon presence and landscape and environmental data to optimize ORV operations in pine-dominated landscapes of the eastern United States. As is the case for many generalist mesocarnivores, absence data for raccoons are difficult to acquire. Consequently, the presence-only Maxent procedure was employed given the relatively large number of locations available to us as byproducts of rabies management activities. Environmental surfaces generated by Maxent were ingested into Circuitscape for conductivity analysis [21,22,25,26,38], which then illustrated potential risk pathways for the spread of raccoon rabies. These results provide us an opportunity to assess the potential utility of circuit theory modeling for providing insight into critical areas for consideration when developing rabies control strategies.

2. Methods

Raccoon sampling location data are collected as part of routine rabies virus and post-ORV rabies serological monitoring which occurs in diverse habitats, including pine-dominated sites. Post-ORV live-trapping was conducted for the purpose of program assessment randomly throughout treated areas using Tomahawk Model 608 live-traps (Tomahawk Live Trap LLC, Hazelhurst, WI, USA). Raccoon handling was as described in Slate et al. [39]. Data from unique raccoons ($n = 2986$) sampled in Massachusetts, Florida, and Alabama during 5 January 2012–26 June 2014 were analyzed in Maxent. This sampling timeframe was selected to overlap with contemporary land use status as recorded within the National Land Cover Database 2011 (NLCD 2011) [40]. Trap location selection

was based on opportunity (safe locations—for trappers, raccoons, and the public), where observed microhabitats suggested that potential undiscovered (by the public) raccoon capture was likely. While this does not reflect true random sampling, we made every effort to remove the influence of assumptions about density to achieve the greatest degree of randomness we could expect given the study limitations. Consequently, 1869 raccoons met study selection criteria (captured/collected by WS since 1 January 2012; not captured within 30 days prior; not captured as part of density indexing; and with a geo-location available), and their locations were imported into the GIS (Table 1).

Additional selection within the study areas in three states and GIS layer coverages resulted in 1770 raccoon sample locations available for Maxent model training and Circuitscape risk analysis.

Table 1. Raccoon risk model sample data for pine-dominated landscapes of Massachusetts, Florida, and Alabama: 2012–2014. ¹

State	Dates Sampled	Sample Size	Age Ratio ^{2,3} (n)	Sex Ratio (M:F; n)
Massachusetts	25 January 2012–26 June 2014	171	4.3:1 (32)	1.7:1 (168)
Florida	19 January 2012–30 May 2014	431	15.4:1 (278)	1.7:1 (427)
Alabama	5 January 2012–20 December 2013	1267	NA	1.2:1 (1251)
Total		1869	12.5:1 (310)	1.3:1 (1846)

¹ Selection criteria for study inclusion: captured/collected by USDA, APHIS, Wildlife Services personnel since 1 January 2012; no capture within 30 days prior; not captured as part of a density study; geo-location available. Not all samples selected were ultimately included in each analysis. ² Age ratios are adult: juvenile; juvenile status ≤1 year as aged by the *cementum annuli* procedure, Matson’s Laboratory, Milltown, MT [41]. ³ Age data not available for Alabama.

2.1. Landscape Data

Land class and use types were represented by 30 m NLCD 2011 data [40] for the areas of interest. Although not the only available coverage for assessing forest cover, land use, and other surface feature types, NLCD 2011 is the standard used by WS for flight planning. Consequently, modeling results based on this product can be more easily translated into management actions. Study area characteristic analysis indicate that evergreen forest (NLCD 2011 Class 42) ranks 3/15 classes available for the Massachusetts study area, 9/13 for the Florida study area, and 4/15 in Alabama. NLCD 2011 Class representation among raccoon sample locations used in initial analyses indicated that 8.8% of raccoon sample locations in Massachusetts fell into the evergreen class, while only 5.3% and 6.2% were in that class in Florida and Alabama, respectively (Table 2).

The Digital General Soil Map of the U.S. (STATSGO2) represents an inventory of soils mapped at 1:250,000 scale [42] and at the level of soil taxonomic order that provides appropriate resolution data for consideration at raccoon home range scales. Study area characteristic analysis in terms taxonomic soil orders (soils) indicates that entisols (dunes, floodplains) rank highest among soil orders for the Massachusetts study area (>98%), while in the Florida and Alabama study areas, soils from the alfisols (common in semiarid-moist regions) dominated at >67% and almost 42%, respectively. In terms of soils representation among the sample points used in initial analyses, >99% (*n* = 171) of raccoon sample locations in southeastern Massachusetts fell into the entisols, while in Florida the majority (almost 88%) fell into the alfisols. A greater diversity of soils was represented among the Alabama raccoon sample locations (alfisols at 34%, entisols at 37%, inceptisols (characterized by varied productivity; layer formation developing) at 19%, and ultisols at 9%).

Table 2. NLCD 2011 ¹ class representation in raccoon risk model study area and sample point data (5 January 2012–26 June 2014) from within pine-dominated landscapes of Massachusetts, Florida, and Alabama.

NLCD 2011 Class ¹	Massachusetts (n = 171)		Florida (n = 431)		Alabama (n = 1168)	
	Percent Study Area	Percent Sample Points	Percent Study Area	Percent Sample Points	Percent Study Area	Percent Sample Points
11-open water	2.1	NA	1.5	NA	0.9	2.1
21-developed, open space	19.6	19.3	19.0	15.8	10.3	23.7
22-developed, low intensity	17.7	40.9	13.3	7.7	2.3	4.5
23-developed, medium intensity	8.7	6.4	6.1	1.6	0.8	0.9
24-developed, high intensity	1.2	0.6	1.4	0.9	0.2	0.3
31-barren land (rock/sand/clay)	2.0	NA	1.0	NA	0.4	0.7
41-deciduous forest	11.0	4.1	NA	NA	17.0	24.7
42-evergreen forest	11.7	8.8	3.7	5.3	12.8	6.2
43 mixed forest	8.5	1.2	NA	NA	20.4	5.2
52-shrub/scrub	2.7	NA	7.3	6.5	15.0	6.2
71-grassland/herbaceous	2.9	1.2	7.7	1.9	5.3	4.8
81-pasture/hay	1.1	NA	5.6	1.6	6.2	13.2
82-cultivated crops	0.7	NA	0.9	NA	2.4	3.2
90-woody wetlands	5.6	8.2	19.9	54.1	5.1	4.3
95-emergent herbaceous wetlands	4.6	9.4	12.6	4.6	0.6	0

¹ Source: U.S. Geological Survey, 2014 [40].

2.2. Environmental Data

Euclidean distance layers for distance to National Hydrography Dataset (1:24,000) lake, pond, swamp, marshland, reservoir, and estuary (nhd24kwb; water bodies); and stream, river, canal, ditch and coastline (nhd24kst; streams) data [43] were also incorporated. Environmental inputs were 1 km Worldclim Bio1 (Annual Mean Temperature; temperature) and Bio12 (Annual Mean Precipitation; precipitation) data [44]. Worldclim elevation (elevation) data [44] derived from the Shuttle Radar Topography Mission (SRTM) [45] were also used for assessing potential effects from elevation to RDI (Table 3).

2.3. Human Environment Data

To refine our assessment of potential effects from human development that may subsidize raccoon populations through the provision of garbage, garden and farm crops beyond what is demonstrated in the development classes within the NLCD 2011 [40], 2010 census data in the form of population and housing layers were incorporated [46]. Euclidean distance to primary and secondary road layers [46] for our study areas were also included, given the potential role of roads as travel corridors or barriers to movement (Table 3).

2.4. Data Preparation and Processing

Circuitscape requires ASCII raster-formatted data for analysis. The Circuitscape Exporter add-in tool for ArcMap [47] was used to export ArcGIS vector and raster data into ASCII rasters of identical cell sizes, extents, and spatial references for use in Maxent, and for eventual ingestion into Circuitscape. Separate global Maxent model (settings: 100 replicate bootstrap analysis, 25% random-seeded test percentage, logistic output format, duplicate locations allowed, response curves created, jackknife measure of variable importance, and 5000 maximum iterations) runs were made for each state using raccoon sample locations from 2012-present and 10 ‘environmental layers’ (Table 3).

Table 3. Raccoon risk modeling: environmental variables, sources, and choice justification for pine-dominated landscapes of Massachusetts, Florida, and Alabama.

Variable	Data Type	Source	Selection Justification
National LandcoverDataset (NLCD 2011)	Landscape/categorical	30 m GeoTIFF images-http://landcover.usgs.gov/	Used by NRMP for ORV planning
Euclidean Distance to (streams)	Landscape/continuous	National Hydrography Dataset 2014 http://nhd.usgs.gov/	Raccoon foraging frequently focused in riparian areas [48]
Euclidean Distance to water (water bodies)	Landscape/continuous	National Hydrography Dataset 2014 http://nhd.usgs.gov/	Raccoon foraging frequently focused in riparian areas [48]
USDA soil taxonomic order (soils)	Landscape/categorical	STATSGO22 http://www.nrcs.usda.gov/	Standing water, and invertebrate availability [48]
Elevation (elevation)	Landscape/continuous	Shuttle Radar Topography Mission http://www2.jpl.nasa.gov/srtm/	Likely correlated with water/NLCD
Annual Mean Precipitation (precipitation)	Environmental/continuous	WorldClim, Global Climate Data http://www.worldclim.org/	Standing water, land cover/use types
Annual Mean Temperature (temperature)	Environmental/continuous	WorldClim, Global Climate Data http://www.worldclim.org/	Foraging behavior and reproduction timing
Human Housing Density (housing)	Human environment/continuous	2010 Census Population/Housing Unit Counts-Blocks: Tiger/line files www.census.gov	Human subsidies to raccoons - garbage, garden crops [48]
Human Population Density (human population)	Human environment/continuous	2010 Census Population/Housing Unit Counts-Blocks: Tiger/line files www.census.gov	Human subsidies to raccoons - garbage, garden crops [48]
Euclidean Distance to Roads (roads)	Human environment/continuous	U.S. Census Bureau http://www.census.gov/geo/maps-data/data/tiger.html	Travel corridors

Maxent created ROC plots of true versus false positive locations in the model, and an assessment of the AUC created by plotting sensitivity (1-omission rate) against 1-specificity (the fractional predicted area), which is a threshold-independent measure of accuracy and ranges from between 0.5 (the random prediction) to the maximum achievable value of 1.0 [49]. Percent contribution and permutation importance values provided measures of variable performance, with the former being useful for assessing the roles of uncorrelated variables, and the latter for an assessment based on the AUC metric of the dependence of the model on a given variable. The jackknife results provide a visual interpretation of variable importance [50]. Resulting environmental surfaces were ingested into Circuitscape for conductivity analysis [25,38].

In Circuitscape, the input resistance data (environmental data) were set to represent conductances rather than resistances, and the focal nodes (samples) consisted of 500 random points created in ArcMap for the global models. To expedite analyses, the all-to-one modeling mode was selected. Resulting Circuitscape conductance maps provide no metrics, but despite this are extremely useful for visualizing areas of concern for the prevention of the spread, or the perpetuation, of rabies in our areas of interest.

In addition, for each environmental layer class (environmental = temperature and precipitation; landscape = NLCD 2011, elevation, stream, water body, and soils; and human-based = human population, housing, and roads) separate models were constructed to further develop an understanding of the importance of these variables as classes of landscape factors. A 5-replicate bootstrap Maxent analysis (all other settings as above) was conducted for each of these models. Circuitscape images were not created for these analyses.

3. Results

Raccoon sample location distribution ($n = 171$) was relatively even across areas of interest in Barnstable and Plymouth Counties, Massachusetts. The averaged 100-replicate bootstrapped Maxent model ROC AUC (model sensitivity relative to 1-specificity) for southeastern Massachusetts was relatively high at 0.958. Higher levels of raccoon sample location probability are seen along the more heavily populated (by humans) and mixed-forest type dominated coasts. Precipitation was the most influential variable in the Massachusetts Maxent model (44% contribution; 46.9 permutation

importance), with a higher proportion of raccoon sample locations occurring where precipitation levels were higher, primarily along the outer portion of Cape Cod. The next most influential variable was NLCD 2011 (15.3%; 7) which had more raccoon sample locations (40.9%) occurring on the developed low intensity (NLCD 2011 Class 22) than any other land class available, while soils was the third most influential (11.7%; 3.8). All other variables fell below 10% model contributions, although permutation importance was higher for several of these than for the second and third highest in terms of percent contributions (Table 4).

Table 4. Raccoon ($n = 171$) risk modeling sample data for pine-dominated landscapes of Massachusetts during 5 January 2012–26 June 2014. All values are means.

Variable	Percent Contribution	Permutation Importance
Annual Mean Precipitation	44	46.9
National Landcover Dataset 2011	15.3	7
USDA Soil Taxonomic Order	11.7	3.8
Euclidean Distance to Roads	9.5	9.1
Human Population Density	5.8	11.4
Human Housing Density	4.5	7
Annual Mean Temperature	3.6	4.8
Euclidean Distance to Streams	2.4	2.6
SRTM Elevation	2.3	5.9
Euclidean Distance to Water bodies	1	1.4

The Maxent model for raccoon sample ($n = 431$) distribution in Florida performed slightly better than the model for Massachusetts, with an AUC >0.975. The distribution of raccoon sample locations and the resulting Maxent model representation for Florida demonstrate a potential difference between several urbanized areas in terms of predicted raccoon habitat suitability. Temperature played the most important role in raccoon sample collection location in Florida (30.1%, 52.1), with the majority of raccoons at locations with slightly higher annual mean temperatures. Precipitation played the next most important role (21.5%, 10.5). Soils was the third most important (20.5%, 19.4) variable. All other variables made <10 percent contributions and scored below the topmost variables in terms of permutation importance as well (Table 5).

Of the three states sampled, the Maxent model for raccoon sample ($n = 1168$) location distribution in Alabama performed the least well, with an AUC of 0.932. Here, soil was the most influential variable (39.4%, 38.3), followed by temperature (18.3%, 22.7) and elevation (12.1%, 15.9) (Table 6).

Raccoon sample locations and predicted raccoon habitat suitability for Alabama were fairly consistent. However, areas with relatively high probability predictions without raccoon samples having come from them were evident as well (Figure 1a). The Circuitscape output based on Maxent results for Alabama reveals areas of considerable risk for the movement of raccoon rabies (Figure 1b).

Table 5. Raccoon ($n = 431$) risk modeling sample data for pine-dominated landscapes of Florida during 5 January 2012–26 June 2014. All values are means.

Variable	Percent Contribution	Permutation Importance
Annual Mean Temperature	30.1	52.1
Annual Mean Precipitation	21.5	10.5
USDA Soil Taxonomic Order	20.5	19.4
Euclidean Distance to Roads	7.4	2.6
SRTM Elevation Data	7.1	6.6
Euclidean Distance to Water bodies	5.1	3
National Landcover Dataset 2011	4.6	3.1
Human Housing Density	2.9	1.8
Human Population Density	0.6	0.4
Euclidean Distance to Streams	0.1	0.5

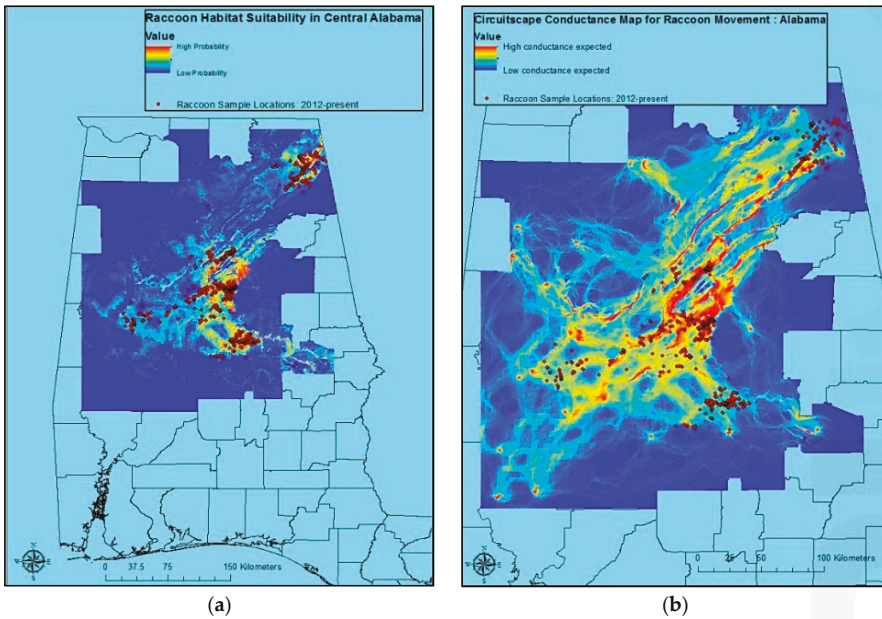


Figure 1. Maxent raccoon location probability map based on raccoon sample ((a); $n = 1168$; 5 January 2012–26 June 2014) locations, annual mean precipitation, annual mean temperature, elevation, land cover/land use, human population density, housing density, Euclidean distance to roads, USDA Soil Taxonomic Order, Euclidean distance to streams/rivers, and Euclidean distance to water bodies for central Alabama; and Circuitscape conductance map for raccoon rabies risk in Alabama (b).

Table 6. Raccoon ($n = 1168$) risk modeling sample data for pine-dominated landscapes of Alabama during 5 January 2012–26 June 2014. All values are means.

Variable	Percent Contribution	Permutation Importance
USDA Soil Taxonomic Order	39.4	38.3
Annual Mean Temperature	18.3	22.7
SRTM Elevation Data	12.1	15.9
Euclidean Distance to Roads	11	7.1
National Landcover Dataset 2011	8.3	2.4
Annual Mean Precipitation	6.1	11
Euclidean Distance to Water bodies	3.1	0.8
Human Population Density	1	1.2
Human Housing Density	0.6	0.4
Euclidean Distance to Streams	0.1	0.2

Raccoon sample locations and predicted raccoon habitat suitability were also relatively consistent in Massachusetts and Florida (Figure 2a,b). As in Alabama, Circuitscape predicts raccoon movement risk (Figure 2c,d). However, given concerns over model results relative to program understanding of raccoon ecology, these are potentially less useful for management decision-making than the model for Alabama (Figure 1b).

Evaluated as groupings of like variables, the landscape suite performed slightly better than the environmental and human-based ones in terms of average AUC, with relatively high mean AUC values in both Florida and Alabama. Only Florida had a mean AUC that exceeded 0.90 for the environmental suite of variables, while none in the human-based category did (Table 7).

Table 7. Evaluation of environmental layer classes for their influence on raccoon risk model results for pine-dominated landscapes of Massachusetts, Florida, and Alabama during 5 January 2012–26 June 2014: 5 bootstrap replicated runs. All AUC values are means.

State	Model Performance	Environmental Model ¹	Landscape Model ²	Human-based Model ³
MA	Mean AUC	0.886	0.872	0.861
	95% CI \pm	0.0023	0.0024	0.0031
	n	166	153	171
FL	Mean AUC	0.956	0.963	0.824
	95% CI \pm	0.0002	0.0004	0.0008
	n	411	396	431
AL	Mean AUC	0.858	0.916	0.749
	95% CI \pm	0.0003	0.0001	0.0002
	n	1168	1168	1168

¹ annual mean temperature and precipitation at the 1 km resolution; ² NLCD 2011, elevation, Euclidean distance to streams, Euclidean distance to water bodies, and soils; ³ human population, human housing, and Euclidean distance to roads.

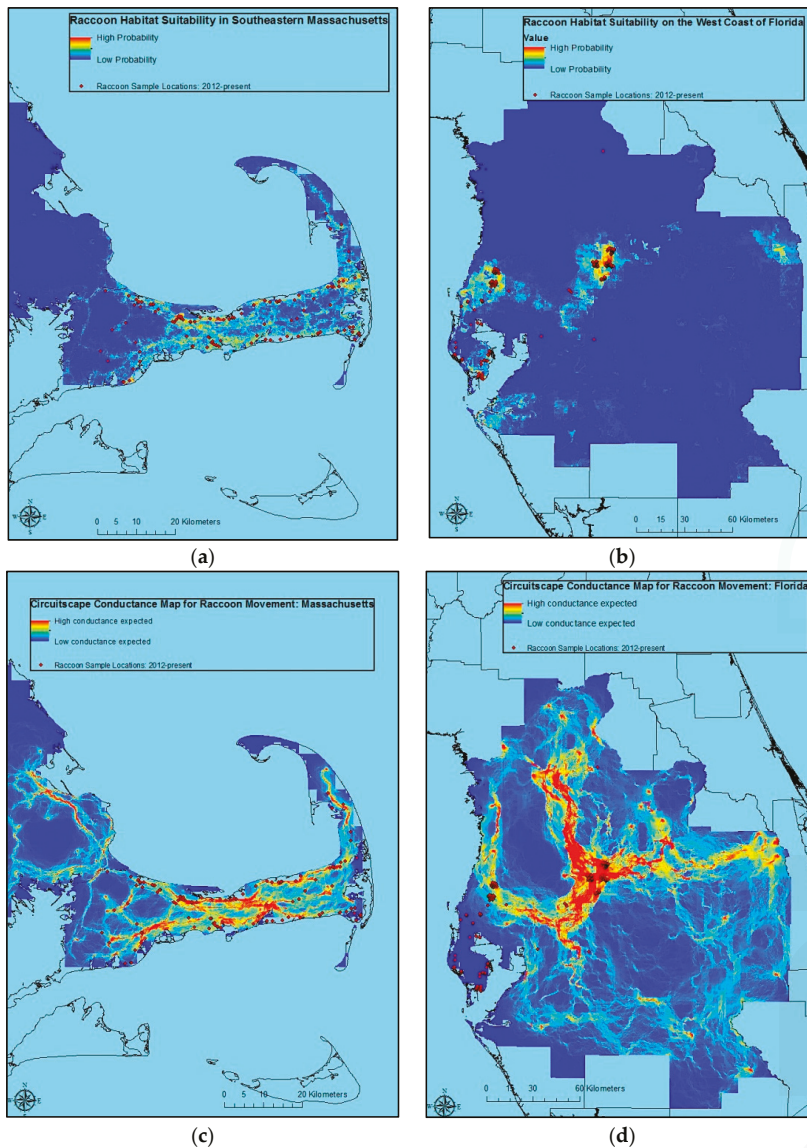


Figure 2. Maxent raccoon location probability map based on samples from Massachusetts (a; $n = 171$) and Florida (b; $n = 431$) collected during 5 January 2012–26 June 2014 plus annual mean precipitation, annual mean temperature, elevation, land cover/land use, human population density, housing density, Euclidean distance to roads, USDA Soil Taxonomic Order, Euclidean distance to streams/ rivers, and Euclidean distance to water bodies; and related Circuitscape conductance maps for raccoon rabies risk in Massachusetts (c) and Florida (d).

4. Discussion

The Maxent procedure provided unexpected results suggesting relatively strong environmental influence on raccoon distribution probability for both Massachusetts (precipitation) and Florida

(temperature), while the contribution of soil in each was less important than would have been expected, given its relationship to landcover. In Alabama, the high percent contribution of soils data was less surprising given the influence of soils on not only the distribution of forest types, but also on water retention and very likely the distribution of invertebrate prey items. The strong influence of environmental variables suggests further investigation is warranted for Massachusetts and Florida. The three additional Maxent models run to explore the relative influence of groupings of variables in the absence of other variable types revealed a somewhat higher level of influence for the landscape class in terms of their average AUC, which is more in line with our expectations given what is known about raccoon ecology.

No variable or variable class ultimately emerged as the best all-around predictor in the settings modeled. However, the most influential variables in each state had strong effects in comparison to second tier variables. For example, in Massachusetts, precipitation with a percent contribution of 44 was also extremely influential when evaluated by itself, with the highest level of permutation importance (46.9) (Table 4). For Florida and Alabama, differences between percent contributions of the best performing variables were less dramatic.

Also in Massachusetts, the NLCD 2011 variable (15.3%; 7) was expected to be highly influential. However, it performed only slightly better than soils (11.7%; 3.8), whereas soils greatly outperformed NLCD 2011 in Florida and Alabama. Results from the soils indicated that almost all (>99%) raccoon samples came from within the entisols in Massachusetts, which characterize disturbed areas and which may be explained by relatively high levels of development and attendant soil disruption. In Florida, the alfisols dominated with almost 88% of samples originating there. Not surprisingly, these soils are somewhat characteristic of humid locations. In contrast, raccoon sample locations in Alabama were distributed among a number of soil types, with alfisols and entisols being almost equally represented among raccoon samples there at 34.4% and 36.6%, respectively. One exception was the inceptisols, which while they represented >25% of the Alabama study area, accounted for only 19.1% of raccoon sample locations.

Although neither human population nor housing ever represented >5.8% contribution toward influencing of raccoon sample locations, planning for ORV bait distribution within residential and recreational areas has always been a priority given our knowledge of raccoon use of household wastes, garden crops, and invertebrates found in lawns, etc. However, based on our results, it appears that bypassing relatively small residential areas when conducting large scale ORV operations may have little impact on overall program success. Further confirmation of this from directed density and movement assessments could lead to greater understanding and considerable cost-savings given the complications of baiting in areas such as these. Roads was never among the top-performing variables, with a high of only 11 percent influence in Alabama. This suggests raccoons may not be dependent on them as travel corridors, nor deterred by them as potential obstacles. A potential bias from road kill- and roadside trapping-based sampling among surveillance and trapped raccoons, may have confounded this finding, however.

The consistently poor performance of streams, water bodies, human population and housing variables was somewhat surprising given the frequently documented attractiveness of these features to raccoons [48,51]. While it seems unlikely, the apparent scarcity of resources available to raccoons in pine-dominated landscapes may also extend to riparian areas contained within them, possibly explaining the phenomenon. It is also possible that raccoon population densities are low enough in some of the pine-dominated landscapes sampled that even these localized resources are not abundant enough to attract and sustain them.

The relatively high level of influence from temperature in all three states is somewhat surprising as well (Tables 4–6). Although these findings suggest further consideration of the potential role of temperature in predicting raccoon sample locations, it might also be important to consider revising this analysis to incorporate higher-resolution temperature and precipitation data to better determine if there is a real measurable influence.

This modeling effort presented a number of challenges. For example, the raccoon samples utilized were byproducts of other research and management efforts. Much of the work done in these areas, and in particular in Massachusetts, was undertaken as emergency response to raccoon rabies epizootic front movements, barrier breaches, and perceived threats based on surveillance. In addition, samples were frequently road kills, and from residential areas where concerned citizens reported them. Finally, our primary knowledge of raccoon densities in these areas comes from 10-day, 500 trap night (with some allowance for initial results-based modifications), 3-km² density estimation procedures designed to provide enough information for responsive ORV and conducted on the fly and with limited resources [31]. More refined findings may also be required for full understanding of the role of commercial pine plantations in the south. For instance, seral stage was an important predictor for determining raccoon usage of commercial loblolly pine plantation habitat, and raccoon home ranges overlapped more frequently in commercial pine forests than in mixed forest types in one study in Alabama [34]. Consequently, further analysis incorporating remotely-sensed imagery capable of elucidating seral stage in near real time may be necessary for full understanding on commercial pine plantation-dominated landscapes. In addition, since edge habitats have emerged as important predictors of relative raccoon density in pine-dominated forest types [36], incorporating this landscape feature into future Maxent assessments may prove useful.

Applying the Maxent and Circuitscape procedures to problems such as we addressed here was useful. However, the application of these findings to future management efforts will occur only after a process that includes other inputs. Our examination of variable permutation importance aids in discerning variable input on model performance. However, for similar future analyses, we feel additional evaluation in the form of correlation analysis will provide an even clearer understanding of the potential problem of collinearity. Nonetheless, these Circuitscape outputs make clear to us areas for strong consideration for sampling in rabies surveillance efforts, and for concentrating treatment by ORV, or in limited cases, by trap-vaccinate-release (TVR). Although many of the higher risk corridors suggested jibe with what we know about raccoon rabies eco-epidemiology in these regions, we are aware of the limitations of modeling for management, regardless of the procedures utilized. The NRMP has traditionally modified management strategies based on modeling only after careful consideration of outputs in light of what is known from actual work on the ground, and then only where over-arching programmatic risks can be minimized.

5. Summary

Relatively new modeling tools such as the maximum entropy procedure (Maxent) [20,25] hold promise for use in the field of eco-epidemiology by helping managers increase their understanding of the systems in which they work to control important diseases such as rabies. Although Circuitscape conductance maps provide no metrics, they are extremely useful for visualizing areas of concern for the prevention of the spread or the perpetuation of rabies in areas of interest. As such, they actually represent the input of greatest interest to discussions of management options and strategy formulation. If ORV or trap-vaccinate-release operations are to be implemented, these will help direct those efforts geographically in much the same way that forest fire managers strategize to achieve control. In addition, potentially wasteful efforts in low conductance areas may be avoided, adding cost-savings.

No variable or variable class emerged as the best all-around predictor for the three locations under consideration. However, the Maxent procedure for Alabama provided us with useful results suggesting optimal raccoon habitat (Table 6, Figure 1a), as well as conductance surfaces for use in visualizing risk corridors potentially useful for planning ORV and contingency actions in response to ORV zone breaches in Circuitscape (Figure 1b). These results are consistent with anecdotal information from a number of years and sources on areas of optimal raccoon habitat, and highest raccoon rabies spread in that state. The resulting NLCD 2011 data were somewhat less informative in modeling raccoon habitat suitability than soils, except in Massachusetts where NLCD 2011 performed slightly better. However, the ease with which NLCD 2011-based research and planning are translated to

field decisions make it unlikely that a switch to soil data in decision-making, research, etc. would occur. Further exploration of the potential ties between these types of information, and the possible development of algorithms for incorporating soils information into field decisions may be worth exploring, however.

Functionally equivalent forest types dominated by other members of *Pinus* occur along coastal portions of the Atlantic and Gulf of Mexico coasts as well, so the apparent pine effect noted here may be widespread within Phase II ORV treatment areas. Pine-dominated forests comprise significant proportions of the forest types of the eastern U.S. For example, 69 million acres of the southeastern U.S. is coniferous forest, with the majority of the area occupied by loblolly and longleaf pine [52], and pitch pine forests occur from as far north as southern Maine to northern Georgia, with concentrations found on the Atlantic coastal plain [35]. Considerable savings may accrue to ORV efforts dedicated to controlling raccoon rabies in pine-dominated regions by application of these and future findings. This effort may also suggest procedures of potential utility for controlling rabies elsewhere with different reservoirs species, and may provide important inputs for future economic modeling of rabies control.

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Article

Incorporating Direct Rapid Immunohistochemical Testing into Large-Scale Wildlife Rabies Surveillance

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Abstract: Following an incursion of the mid-Atlantic raccoon variant of the rabies virus into southern Ontario, Canada, in late 2015, the direct rapid immunohistochemical test for rabies (dRIT) was employed on a large scale to establish the outbreak perimeter and to diagnose specific cases to inform rabies control management actions. In a 17-month period, 5800 wildlife carcasses were tested using the dRIT, of which 307 were identified as rabid. When compared with the gold standard fluorescent antibody test (FAT), the dRIT was found to have a sensitivity of 100% and a specificity of 98.2%. Positive and negative test agreement was shown to be 98.3% and 99.1%, respectively, with an overall test agreement of 98.8%. The average cost to test a sample was \$3.13 CAD for materials, and hands-on technical time to complete the test is estimated at 0.55 h. The dRIT procedure was found to be accurate, fast, inexpensive, easy to learn and perform, and an excellent tool for monitoring the progression of a wildlife rabies incursion.

Keywords: dRIT; Ontario; rabies; surveillance

1. Introduction

In December 2015 a raccoon (*Procyon lotor*) from Hamilton, Ontario, Canada, was diagnosed with rabies caused by the mid-Atlantic raccoon virus variant, the first case of rabies caused by this virus variant to occur in the province in 10 years. To assess the extent of this rabies outbreak and to direct rabies management actions, large-scale testing of raccoons and striped skunks (*Mephitis mephitis*) collected within a 50-km radius of diagnosed cases was undertaken. Animals were primarily collected by partnering agencies, such as municipal animal control agencies, humane societies and roads departments. Collection was focussed on sick-acting animals, animals found dead, or roadkill, and carcasses were kept frozen until they could be collected by Ministry of Natural Resources and Forestry (MNRF) staff. Frozen carcasses were collected weekly from partners and brought back to the MNRF laboratory for weekly testing using the direct rapid immunohistochemical test (dRIT). Samples diagnosed as rabies positive by dRIT were sent to the Canadian Food Inspection Agency (CFIA) for confirmation by fluorescent antibody test (FAT). Animals that had any previous contact with humans were not tested as part of this procedure. The dRIT test was developed by the Rabies and Poxvirus Section of the US Centers for Disease Control and Prevention (CDC), and was first evaluated in the field in Tanzania with very promising results [1]. The test was developed to provide a diagnostic methodology for quickly and inexpensively testing for rabies virus without the need for specialized equipment or facilities, making rabies testing more widely available worldwide, and has since been shown in many studies to be an effective tool for rabies surveillance [2]. The dRIT procedure was initiated in the rabies surveillance programs of the provinces of Québec [3] and Ontario in 2010. Until the most recent outbreak, the MNRF typically tested 10–40 animals annually, with four staff

having been trained in dRIT procedures by CDC dRIT experts. Since raccoon rabies detection in December 2015, the dRIT surveillance program in Ontario has increased exponentially, and is currently the largest such surveillance program in Canada, testing anywhere from 23–258 (mean = 82.2) animals per week.

2. Materials and Methods

Test procedures: Brain tissue was collected by inserting a modified 3 mL or 1 mL syringe through the foramen magnum of animals and removing 1–2 mL of brain matter for touch impressions to slides for subsequent dRIT. dRIT was performed according to the CDC standard operating procedure as described previously [4], using a cocktail of two anti-nucleoprotein, biotinylated monoclonal antibodies (502 and 802) sourced from The Wistar Institute (Philadelphia, PA, USA). FAT was performed as described previously [5], using the same brain tissue samples collected for the dRIT. A fluorescein isothiocyanate-conjugated polyclonal antibody (goat serum, raised against purified ERA (Evelyn-Rokitnicki-Abelseth) rabies virus ribonucleoprotein), prepared in-house at the CFIA Centre of Expertise for Rabies (Ottawa, Canada), was used in the FAT.

Test comparison: Determination of dRIT sensitivity, specificity, and test agreement with FAT were estimated using an online calculator (EpiTools; <http://epitools.ausvet.com.au>).

3. Results

3.1. Surveillance Samples

In the 17-month period from December 2015 to April 2017, approximately 5800 mammalian wildlife carcasses were collected from southern Ontario and tested for rabies using the dRIT methodology (Figure 1). Species tested comprised primarily raccoons (80%) and striped skunks (17%). Other species tested included bat, feral cat (*Felis* spp.), coyote (*Canis latrans*), fisher (*Martes pennant*), red fox (*Vulpes vulpes*), groundhog (*Marmota monax*), muskrat (*Ondatra zibithicus*), opossum (*Didelphis virginiana*), cottontail rabbit (*Sylvilagus floridanus*), squirrel (*Sciurus* spp.), and weasel (*Mustela* spp.).

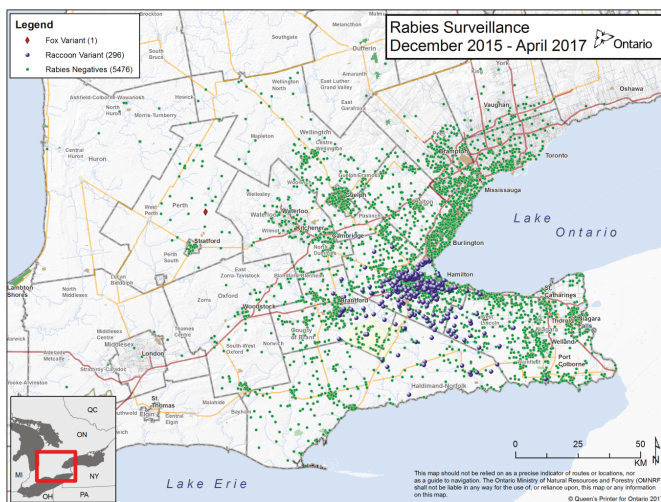


Figure 1. Ontario rabies surveillance map showing the negative (green) and positive (red and blue symbols) samples tested by dRIT.

3.2. dRIT Performance in Comparison with FAT

All dRIT-positive samples were submitted to CFIA for confirmation using FAT. Of the 5800 wildlife samples tested by MNRF, 215 raccoons, 91 skunks and 1 red fox were diagnosed as being positive for rabies in the dRIT. When the same samples were tested by FAT, 205 of the raccoons, all the skunks and the fox were positive. Five hundred and fifty dRIT-negative samples were also tested in FAT. The samples were selected from random geographic locations from a time period between February and June 2016, and comprised 490 raccoons and 60 skunks. All of the dRIT-negative samples were also negative in the FAT. The dRIT had a sensitivity of 100% and specificity of 98.2% when compared with FAT (Table 1). Overall agreement between dRIT and FAT was high (98.8%, $\kappa = 0.9744$), with negative test agreement being slightly higher than positive test agreement (99.1% vs. 98.3%). On occasion, samples processed by dRIT were found to be inconclusive, typically due to poor sample conditions. Inconclusive samples were tested a second time to make a final dRIT diagnosis. In order to verify results of inconclusive samples where a second dRIT was conducted and was determined to be negative, 40 such samples were also evaluated by FAT. Of the 40 samples, 3 were untestable by FAT due to advanced sample decomposition; the remaining 37 samples had 100% diagnostic agreement between dRIT and FAT as being negative.

Table 1. Test comparison between fluorescent antibody test (FAT) and direct rapid immunohistochemical test (dRIT) for rabies.

	FAT Positive	FAT Negative	Total
dRIT positive	297	10	307
dRIT negative	0	550	550
Total	297	560	
	Value	95% CI	
dRIT Sensitivity	100.0%	98.77%–100.00%	
dRIT Specificity	98.21%	97.74%–99.14%	
Kappa	0.9744	0.9587–0.9902	
Negative agreement	0.9910	-	
Positive agreement	0.9834	-	
Overall agreement	0.9883	-	

3.3. Cost

Through the collection and processing of approximately 5800 samples using dRIT procedures in the MNRF laboratory, an average cost of \$3.13 CAD per sample for chemicals, reagents and laboratory consumables was estimated. In contrast, the materials cost for the FAT as performed at the CFIA is approximately \$7.75 CAD per sample. Laboratory technical labour was comparable for the two tests, estimated at 0.55 h of hands-on time (i.e., not counting incubation periods), and thus labour costs are estimated to be similar, contingent upon local rates of remuneration. These estimates of cost do not include collection and transportation of samples, laboratory setup and maintenance, specimen disposal or administrative overhead, and were based on processing between 70 and 150 samples per week on average.

4. Discussion

The MNRF has responded to the most recent incursion of raccoon rabies by initiating an intensive surveillance program and testing over 5800 samples using the dRIT procedure. Since December 2015, 297 (96.4%) of the 308 cases of raccoon variant rabies diagnosed in Ontario have been diagnosed using MNRF dRIT surveillance, enabling the MNRF and partners to accurately and rapidly direct rabies control actions. Although dRIT and FAT diagnostic agreement was very high at 98.8%, it likely would have been closer to 100% were it not for a few early learning experiences with diagnostic procedures

and processes. All 10 of the cases where samples were diagnosed positive by dRIT but negative by FAT were tested within the first three months of the outbreak. Additionally, during the same time period, samples that were inconclusive after the first dRIT test were sent directly to CFIA lab for additional testing by FAT. After receiving notification of these false positives, as well as dRIT-inconclusives that tested FAT-negative, two changes were implemented with regard to staff training and diagnostic processes. First, an expert in dRIT procedures was brought in to provide first-hand training to all staff performing the dRIT, to ensure that all test procedures were consistently done in accordance with the standardized protocol. Second, a new process, in which any sample with an inconclusive dRIT result was tested a second time before making a final dRIT diagnosis, was implemented. After implementing these two changes three months into the outbreak in February 2016, and in addition to staff gaining considerable experience with the dRIT test, agreement with FAT was 100% in the following 14 months and 4500 subsequent samples tested (of which 244 were positive). These data speak to the importance of thorough training and proficiency evaluation when implementing the dRIT, or any other diagnostic test for rabies. Based on these results, the dRIT has been found to be an excellent tool for monitoring the progression of a rabies incursion and will continue to be used for enhanced wildlife rabies surveillance within the province of Ontario. These data, along with those from many previous studies, also support the use of dRIT for improved rabies surveillance in regions where FAT cannot be practicably implemented.

Acknowledgments: The authors would like to thank the organizations in Ontario that have submitted animal carcasses for rabies testing, the MNRF technicians who performed carcass collections, rabies case mapping and dRIT evaluations and the CFIA diagnostic staff who performed FAT evaluations on these samples.

Author Contributions: Christine Fehlner-Gardiner, Kevin Middel and Tore Buchanan conceived and designed the study and analyzed the data; Natalie Pulham coordinated the dRIT testing and sample exchange between laboratories; all four authors wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Progress towards Bait Station Integration into Oral Rabies Vaccination Programs in the United States: Field Trials in Massachusetts and Florida

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Abstract: Bait stations for distribution of oral rabies vaccine baits are designed for rabies management in highly-developed areas where traditional distribution of oral rabies vaccine baits may be difficult. As part of national efforts to contain and eliminate the raccoon (*Procyon lotor*) variant of the rabies virus (raccoon rabies) in the eastern United States, the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services program, distributed vaccine baits by bait stations experimentally and operationally in Massachusetts during 2006–present, and in Florida during 2009–2015. In Massachusetts, a rabies virus-neutralizing antibody (RVNA) response of 42.1% for raccoons captured in areas baited with high density bait stations during 2011–2015 was achieved, compared with 46.2% in areas baited by hand, suggesting the continuation of this as a strategy for the oral rabies vaccination (ORV) program there, and for similar locations. Non-target competition for vaccine baits is problematic, regardless of distribution method. In Massachusetts, bait station visitation rates for targeted raccoons and non-target opossums (*Didelphis virginiana*) were similar (1.18:1) during 2006–2009 ($p > 0.05$). Bait station modifications for reducing non-target uptake were tested, and in Massachusetts, reduced non-target bait access was achieved with two design alternatives ($p < 0.001$). However, no difference was noted between the control and these two alternative designs in Florida. Due to ongoing trials of new vaccines and baits, the bait station performance of an adenovirus rabies glycoprotein recombinant vaccine bait, ONRAB[®] bait (Artemis Technologies, Guelph, ON, Canada) and a vaccinia-rabies glycoprotein recombinant vaccine bait, RABORAL V-RG[®] bait (Merial Limited, Athens, GA, USA), was compared. While uptake of the ONRAB bait was greater in Massachusetts ($p < 0.001$) in this limited trial, both types performed equally well in Florida. Since bait station tampering or theft as well as potential human bait contacts has been problematic, performance of camouflaged versus unpainted white bait stations was analyzed in terms of internal temperatures and maintaining a stable bait storage environment. In Massachusetts, camouflaged bait station interiors did not reach higher average temperatures than plain white bait

stations in partially- or fully-shaded locations, while in Florida, camouflaged bait stations were significantly warmer in light exposure categories ($p < 0.05$). As ORV operations expand into more heavily-urbanized areas, bait stations will be increasingly important for vaccine bait distribution, and continued refinements in the strategy will be key to that success.

Keywords: bait stations; oral rabies vaccination; raccoon; rabies; vaccine; non-target

1. Introduction

Oral rabies vaccination (ORV) is an effective and socially acceptable strategy for wildlife rabies management [1]. Various ORV strategies have been employed successfully, including for the control of fox rabies in Western Europe [2,3] and Canada [4–6]. ORV has been used in Texas to eliminate canine rabies in coyotes (*Canis latrans*) [7,8], and to eliminate and prevent rabies in gray foxes (*Urocyon cinereoargenteus*) [9].

The United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services (WS), National Rabies Management Program (NRMP), is conducting cooperative ORV operations to prevent the westward spread of the raccoon (*Procyon lotor*) variant of the rabies virus (raccoon rabies) into the mid-western states and eastern Canada (Phase I), and has begun work towards its eventual elimination from the eastern United States (Phase II) [1]. Much of the eastern United States is highly developed and features high human densities. Traditional aerial ORV strategies are complicated in these areas by high off-times of vaccine bait distribution equipment due to treatment area development, and concern for potential human and pet contact with vaccine baits. Bait stations for distribution of oral rabies vaccine baits provide an opportunity to conduct ORV plans at minimized risk levels in settings such as these.

In 2001, WS began full-time collaboration on the Cape Cod Oral Rabies Vaccination Program (CCORV) with Tufts University and other cooperators as part of national wildlife rabies control efforts (Figure 1).

The primary objective of the CCORV was to use ORV in tandem with the physical barrier of the Cape Cod Canal to prevent the spread of raccoon rabies onto peninsular Cape Cod. In 2003, after an increase in raccoon rabies cases within the CCORV zone, vaccine bait distribution efforts were modified to reduce the risk of raccoon rabies spreading onto peninsular Cape Cod. Despite this, raccoon rabies was detected for the first time there in March 2004. A WS trap-vaccinate-release campaign plus expanded ORV efforts did not prevent the further spread of raccoon rabies. Rabies surveillance on Cape Cod became a high priority [10,11], with the aim of delineating the epizootic front to begin ORV and define priority areas for trap-vaccinate-release efforts to prevent further spread. However, in 2006, raccoon rabies was detected at the farthest tip of Cape Cod, in Provincetown. From 2006 through the spring of 2010, twice-yearly ORV treatments from Yarmouth to Provincetown were conducted using fishmeal polymer (FMP) block baits and coated sachet baits containing RABORAL V-RG[®] vaccine (a vaccinia rabies glycoprotein recombinant oral vaccine; Merial Limited, Athens, GA, USA) distributed by hand from vehicles and on foot to reduce the enzootic area.

Cornell University began investigating the use of bait stations for the distribution of vaccine baits in New York in 2003 [12], based on a design for the control of voles (*Microtus* spp.) in orchards [13]. Experimental bait stations based partially on the Cornell design were constructed from polyvinyl chloride (PVC), filled with RABORAL V-RG FMP baits, and deployed over a 3 km² study area in South Yarmouth (2006–2008) at a very high density (8/km²; $n = 24$) to assess their utility there. In addition to investigations into the use of bait stations by WS in Massachusetts in 2006, bait station trials also began in Florida in 2009 [14].

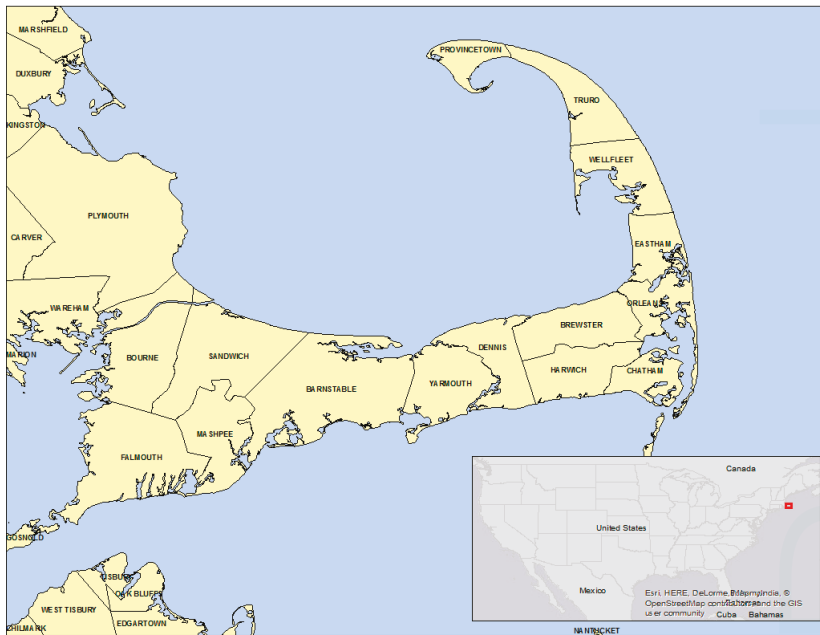


Figure 1. Cape Cod, Massachusetts, USA.

Due to partial funding loss reducing the CCORV program’s ability to distribute vaccine baits during 2006–2009, operational bait station use began in key locations of high human and pet densities and likely travel corridors in areas in which low raccoon densities has been indexed [15] in an attempt to treat the same area with fewer baits (Figure 2).

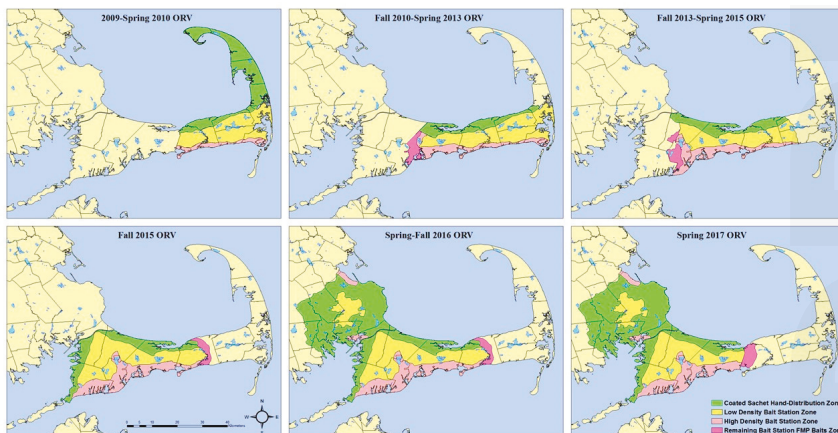


Figure 2. Cape Cod oral rabies vaccination baiting zones, spring 2009–spring 2017.

Five years of operational bait station use is reported as part of raccoon rabies elimination efforts on Cape Cod and compared to traditional hand-distribution of vaccine baits. In addition, since raccoon and non-target Virginia opossum (*Didelphis virginiana*) visitation to Cape Cod bait stations during 2006–2009 was statistically equivalent (1.18:1; $p > 0.05$), two bait station modifications designed to

reduce opossum bait uptake were evaluated in Massachusetts and Florida. The relative performance of two vaccine bait types was also assessed to be sure these were equally useful in bait stations in Massachusetts and Florida. Lastly, the interior temperature conditions of camouflaged and unpainted white bait stations were evaluated in Massachusetts and Florida to be sure the former provided both the desired effect of preventing discovery and tampering by humans, but also to maintain adequate interior temperatures for maintaining bait and vaccine integrity.

Locations for the non-target bait uptake reduction, alternate bait trials, and interior temperature conditions in both states were selected based on habitat type. South Yarmouth, Massachusetts was selected as a representative developed coastal study location due to its mix of residential, commercial, resort, and conservation lands. The Polk County, Florida study site was selected due to its mix of wooded areas, wetlands, and urbanized or moderately-developed areas. Achieving rabies control in communities like these will play an essential role in the elimination of raccoon rabies in the U.S.

2. Materials and Methods

2.1. Bait Stations on Cape Cod

From 2011 through 2015, bait stations (Figure 3) were constructed and deployed operationally on Cape Cod, Massachusetts at two densities (high density—1.8 bait stations/km²; low density—0.1 bait stations/km²) based on habitat type (mixed forest, woody wetlands, or medium intensity developed, featuring relatively high raccoon densities, and pine-dominated landscapes, featuring lower raccoon densities) (Figure 2). Habitat type was determined using the National Land Cover Database (NLCD 2001). Bait stations were affixed to trees or fence posts using heavy duty cable ties, with the openings 0.3–0.5 m above the ground. RABORAL V-RG baits ($n = 60–63$) were inserted in each bait station and monitored over a three-week period in the spring (April–May) and fall (October–November (2011–2014); September–October (2015)). Bait stations were visited weekly to ensure bait stations were functioning properly and to document bait uptake. Bait location relative to bait station, bait coating, and sachet condition were recorded at each bait station. In addition to bait station use, traditional hand-distribution of baits by vehicle and foot occurred where possible with treatment type overlap avoided.

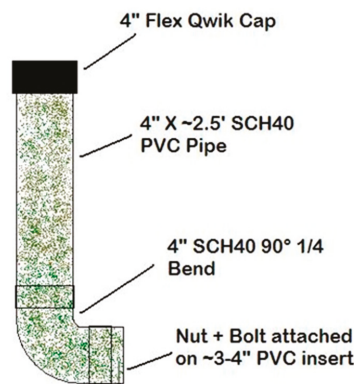


Figure 3. A USDA WS oral rabies vaccination standard bait station.

Serological surveillance occurred 4–8 weeks after each bait distribution was completed to assess rabies virus neutralizing antibody (RVNA) response in areas baited during the most recent ORV campaign. Raccoons were live captured using Tomahawk Live Trap™ (Hazelhurst, WI, USA) model 108 raccoon traps baited with Hard-Core® Raccoon Lure-coated marshmallows and a sardine in both bait zone types. Traps were checked every 24 h, in accordance with state law. Blood samples were

taken for rabies antibody prevalence and first premolar teeth were extracted for age and tetracycline biomarker analysis. Serum samples from spring 2011–spring 2015 were sent to the Centers for Disease Control and Prevention (CDC) for analysis using the rapid fluorescent focus inhibition test (RFFIT) with a positive sero-conversion cut-off of ≥ 0.05 IU/mL [16]. Fisher’s exact tests were used to determine if there was a difference between seropositive raccoons within treatment zones (hand-distribution vs. bait station). Raccoons that had been previously captured and hand-vaccinated against raccoon rabies as well as juvenile raccoons that would not have been exposed to a vaccine bait were omitted from all analysis. Tetracycline biomarker is present in all RABORAL V-RG baits; however, results were not available for analysis at the time of publication. All target species were marked with ear tags that were labeled with a unique identifier to identify recaptured animals. In order to meet programmatic goals, post-ORV sampling within high-density bait station and hand-distribution zones took precedence over sampling from within low-density bait station areas.

Baits are marked with a phone number stamped on the bait matrix and on the plastic vaccine sachets to facilitate the reporting of human or pet bait contacts. Bait contact reports between bait distribution types (i.e., bait station vs. hand-distribution) were compared by Fisher’s exact tests.

2.2. Non-Target Bait Uptake Reduction

During 2011, WS initiated efforts to reduce non-target bait uptake from standard WS bait stations (Figure 3). ‘Qwik Cap Starburst’ (QCS; 4” Fernco™ (Davison, MI, USA) Qwik Cap over the opening with a star-shape cutout), and ‘Sanitary T Station’ (STS; 4” to 2” reducer fitting to the end) bait stations were developed. Four-inch mechanical wing nut test plugs were installed in the bottom of each STS bait station to allow for easy removal of leftover baits at the end of the trials (Figure 4).



Figure 4. Candidate bait station modifications reducing non-target bait uptake in Barnstable County, Massachusetts and in Polk County, Florida, in 2011.

In April 2011, two candidate bait stations types and control bait stations were deployed over 3-km² study areas in Barnstable County, Massachusetts and in Polk County, Florida. Eight bait stations of each type (control, QCS, and STS) were deployed concurrently for ten nights in each state, located >0.4 km apart. Other bait station location considerations included the relative suitability of raccoon and opossum habitat, and the ability to conceal bait stations from the public. Bait station locations and types deployed were quasi-randomized (i.e., random selection from among available sites) because, in a suburban landscape, extra care had to be taken to avoid theft of the bait station and of the baits contained within. Bait stations were initially deployed with ten RABORAL V-RG baits. Bait counts were maintained in each bait station throughout the study by daily replacements of missing baits, with the exception of weekends. Automatic digital trail cameras (Massachusetts: Reconyx™ RC60 (Holmen, WI, USA), Reconyx™ Silent Image, and HCO™ (Norcross, GA, USA); Florida: Moultrie™ (Alabaster, AL, USA) GameSpy D55IR and Moultrie™ I40) were used to monitor bait stations throughout the study for target and non-target visits and response to the bait stations. Bait status was recorded on a daily basis for each station. Individual animal visits were tallied, using activity breaks of

15 min as a proxy for individual identification, unless identification based on unique appearances was possible. Animal response was recorded as 'successful' when animals appeared to acquire a bait from a bait station, and 'unsuccessful' when they appeared to have been deterred. Fisher's exact tests were used to assess whether visits to each bait station type differed between raccoons and opossums.

2.3. Alternate Baits for Bait Stations

ONRAB[®] baits (adenovirus rabies glycoprotein recombinant oral vaccine in Ultralite bait matrix; Artemis Technologies, Guelph, ON, Canada) have been used in Canada and the U.S. [17–19]. ONRAB bait dispersal performance in bait stations was assessed relative to the more commonly used RABORAL V-RG bait. Locations for sixteen camouflaged bait stations were established in Massachusetts and Florida (8/bait type) in suitable raccoon habitats (e.g., mixed forest, wooded wetlands, or medium-intensity developed woody, wetlands) within a 3-km² area based on the National Land Cover Database (NLCD 2001). Bait stations were placed at least 0.4 km apart. Locations and bait types were selected quasi-randomly as above. To mimic an actual ORV operation, 60 vaccine baits were loaded in each bait station; eight bait stations received traditional RABORAL V-RG baits, while the other eight received placebo ONRAB baits. Automatic digital trail cameras (Massachusetts: Reconyx[™] RC60; Florida: Moultrie[™] I40) were used to document wildlife activities at each bait station. Bait stations were checked daily, with the exception of weekends, from 6–16 March 2012 in Florida and 10–20 April 2012 in Massachusetts, and the number of baits remaining in each bait station was recorded. Bait condition (for those found on the ground) was recorded as: *bait matrix gone*, *bait matrix partially gone*, or *fully intact*; and *sachet punctured*, or *sachet unpunctured*. Trail camera images were analyzed daily and the number of interactions between any animal and bait or bait station was tallied. Number of target (e.g., raccoons) and non-target species (e.g., opossums, cats (*Felis catus*), dogs (*Canis lupus familiaris*)) were tallied, as well as individual actions when possible. Activity breaks of 15 min were used to separate visits from individual animals, unless unique identification of animals allowed otherwise. Fisher's exact tests were used to assess bait consumption by each bait type.

2.4. Decreasing Human Interference with Bait Stations

Bait stations are generally painted with camouflage paint as a way to reduce human disturbance and theft. To determine if painting bait stations significantly increases internal temperature (which may imperil vaccine viability and bait integrity), WS constructed sixteen standard bait stations (Figure 2). Eight bait stations were used in both Massachusetts and Florida. Four bait stations from each state were painted with camouflage paint and the other four bait stations remained unpainted (white).

Paired bait stations were located in full sun, partial shade, and full shade. Locations of full sun were exposed to direct sunlight throughout the entire day. Areas of partial shade had direct sun exposure for approximately one-half of a day, or had intermittent exposure. Locations in full shade were not exposed to direct sunlight at any point. Two white bait stations and two camouflaged bait stations were deployed in locations of full sun. One bait station of each color was also deployed in the partial sun and full shade locations.

Each bait station was equipped with a maximum/minimum thermometer (Taylor[™] (Las Cruces, NM, USA) 5460 Indoor/Outdoor Maximum/Minimum Thermometer) and monitored on a daily basis over 10 days in Massachusetts (16–19, 23–26, and 30–31 August 2011) and 12 days in Florida (16–19, 21–26, and 28–29 August 2011). Thermometers were suspended by string at the lowest upright part of each bait station. Maximum temperatures for each bait station were recorded daily, as well as the weather conditions and any problems encountered with the thermometers. Thermometers were also deployed outside of the bait stations to document external temperatures. T-tests were used to determine differences between internal temperatures in painted and unpainted bait stations in each light exposure type.

3. Results

3.1. Bait Stations on Cape Cod

From Spring 2011–Spring 2015, adult raccoons from within the high-density bait station zone (1.8 bait stations/km², 113 baits/km²) that had not been captured and hand-vaccinated in prior years showed seroconversion (≥ 0.05 IU/mL) rates of 42.1% ($n = 126$). Traditional hand-distribution of RABORAL V-RG coated sachet baits on Cape Cod (138 baits/km²) during the same timeframe resulted in vaccination rates of 46.2% ($n = 182$) (Table 1).

Raccoon seroconversion within high density bait station zones in Massachusetts in 2012 and 2014 resulted in higher RVNA response, however, these results were not significant (both $p = 1.00$). Seroconversion in 2011, 2013, and 2015 showed reduced seropositivity for bait stations (2011 and 2013 $p = 1.00$; 2015 $p = 0.29$) but were also not significant. Raccoons recaptured that had been previously hand-vaccinated were omitted from analysis, as well as juvenile animals that would not have encountered a bait during prior ORV operations.

Table 1. Post-ORV raccoon serology rates on Cape Cod, MA—2011–2015.

Year	Percent Seropositive by Hand Distribution (n ; 95% CI) (138 Baits/Km ²)	Percent Seropositive by High Density Bait Station (n ; 95% CI) (1.8 Bait Stations/Km ² , 113 Baits/Km ²)
2011	43.9 (41; 0.30, 0.59)	38.5 (13; 0.18, 0.64)
2012	38.5 (13; 0.18, 0.64)	41.1 (17; 0.22, 0.64)
2013	36.1 (36; 0.22, 0.52)	31.6 (19; 0.15, 0.54)
2014	38.5 (39; 0.25, 0.54)	40.5 (37; 0.26, 0.57)
2015	62.3 (53; 0.49, 0.74)	50.0 (40; 0.35, 0.65)
Total (2011–2015)	46.2 (182; 0.39, 0.53)	42.1 (126; 0.34, 0.51)

In Massachusetts, areas with bait stations had significantly less bait contact calls compared to hand-baited areas from 2010–2016 ($p < 0.001$). During 2010–2016, vaccine bait distribution campaigns in Massachusetts resulted in a total of 153 bait contact calls within hand-baited areas compared with 24 from bait station-baited areas. In hand-baited areas, at least 441 baits were found by people or their pets (0.13%). However, within areas baited by bait stations, only 38 baits were involved with a pet or human bait contact (0.02%).

3.2. Non-Target Bait Uptake Reduction

During April 2011 in Massachusetts and Florida, raccoons accounted for 74.8% of the apparent successful visits to control bait stations, while 25.2% were by opossums ($n = 107$). A similar relationship was observed for QCS bait stations, where 82.6% of apparent successful visits to bait stations were by raccoons and 17.4% were by opossums ($n = 138$). However, the ratio of raccoons photographed at STS bait stations was higher than the control and QCS bait stations (90.9%) and lower for opossums (9.1%; $n = 44$).

In Massachusetts, visitation and apparent bait retrieval by raccoons and opossums significantly differed by opening type ($p < 0.001$). Control bait stations visited in Massachusetts had a 1.8:1 raccoon to opossum ratio, while QCS and STS bait stations had raccoon to opossum visitation ratios of 55:1 and 14:1, respectively. However, apparent access by raccoons and opossums in Florida did not differ ($p = 0.131$).

3.3. Alternate Baits for Bait Stations

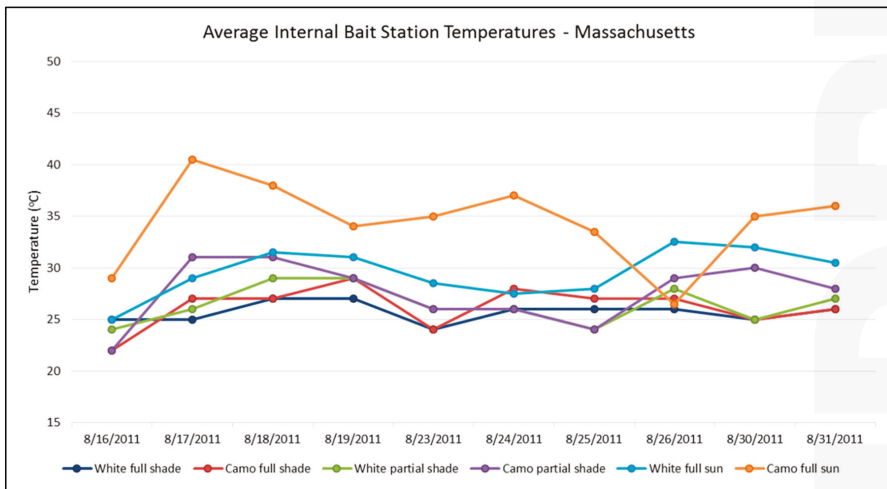
In Massachusetts, all bait stations containing ONRAB baits were emptied and all baits were consumed and/or punctured. Only one bait station containing RABORAL V-RG baits was completely

emptied of baits by the end of the study. Of the 240 RABORAL V-RG baits originally deployed, 122 remained on day 10. In Florida, all baits were taken from the bait stations by the end of the study; however, one bait station containing ONRAB baits in Florida had baits through day 9, but was emptied prior to the day 10 check. In Massachusetts, all intact baits that had been pulled out of the bait station to the ground were redeployed in each bait station to maximize documenting bait-uptake and bait station access. Baits on the ground in Florida were left in front of the bait station to document if they would be consumed after the bait station was emptied. Of the baits left on the ground at the bait stations in Florida, all were consumed by day 6. The relation between the removal of ONRAB and RABORAL V-RG baits was statistically significant ($p < 0.001$) in Massachusetts, but not in Florida ($p = 1.00$).

3.4. Decreasing Human Interference with Bait Stations

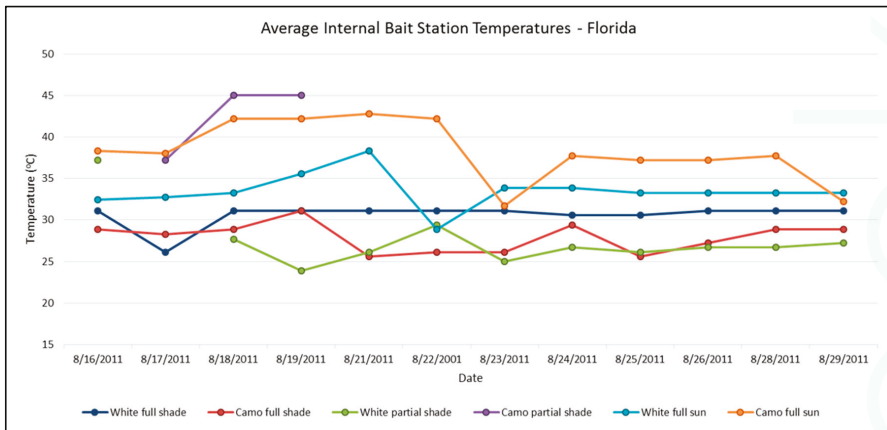
In Massachusetts, as might be expected, bait stations deployed in areas of full sun had a higher average temperature than those in shady locations. Camouflaged bait stations were also warmer than white bait stations, especially in full sun locations ($p = 0.009$), averaging 35.5 °C compared to 29.6 °C. Both camouflaged and white bait stations in full shade locations had a similar average high temperature of 26.2 °C and 25.7 °C, respectively ($p = 0.32$). Similarly, camouflaged and white bait stations also had similar average high temperatures in partial shade areas (27.6 °C and 26.4 °C, respectively) ($p = 0.13$). Several camouflaged bait stations in full sun showed high temperatures of over 40 °C (Figure 5a).

In Florida, camouflaged bait stations in partial shade had higher average temperatures (42.8 °C) than any of the other bait stations. In full sun, camouflaged bait stations were warmer (38.6 °C), but they also had a slightly cooler average low temperature than white bait stations (24.4 °C and 25.3 °C, respectively). In full shade, white bait stations were warmer than camouflaged bait stations (30.6 °C and 27.9 °C, respectively) while both remaining about the same temperature at night (24.0 °C and 24.2 °C, respectively). Average interior temperatures for camouflaged and white bait stations in Florida were statistically different in full shade, partial shade, and full sun ($p = 0.0003, 0.017, \& 0.001$, respectively). In each location, average internal temperatures within white bait stations were significantly cooler than camouflage bait stations (Figure 5b).



(a)

Figure 5. Cont.



(b)

Figure 5. (a) Average internal bait station temperatures in Massachusetts. (b) Average internal bait station temperatures in Florida.

4. Discussion

4.1. Bait Stations on Cape Cod

When WS lost cooperative program funding for the CCORV in 2009, the use of bait stations was a logical next step, given that they could be efficiently deployed in more developed areas, and to intercept raccoons moving through apparently poorer raccoon habitats where broadcast baiting is likely wasteful [15]. As the ORV zone shifted west due to decreased raccoon rabies cases in treatment areas, baiting was increasingly conducted in more highly developed areas, increasing the need to switch to bait stations to reduce potential bait contacts. The use of bait stations on Cape Cod resulted in similar RVNA responses to those areas treated by hand-distribution of baits. This suggests that bait stations hold promise for ORV in the more highly-developed areas to be encountered as the NRMP approaches elimination of raccoon rabies along the eastern seaboard [20].

Bait contact by people or pets is a concern and therefore, reduction in bait contacts is desired. Additionally, every discovered bait potentially represents a lost dose for an unvaccinated raccoon. As evidenced by the reduced number of bait contact-related phone calls and reports, the use of bait stations in developed areas has resulted in fewer contact events. The use of bait stations will continue to play an important role in reducing these bait contacts, especially in heavily developed areas.

4.2. Non-Target Bait Uptake Reduction

During a previous assessment of bait station visitation by non-target species on Cape Cod, raccoon visits to bait stations occurred at a similar frequency to non-target opossums (1.18:1; $p > 0.05$). Based on the low total number of apparent successful raccoon and opossum visits to STS bait stations compared to visits to control and QCS bait stations, it appears that this bait station prohibits easy access to baits by both species. Consequently, STS bait stations are not suitable for distribution of vaccine baits. Results of the bait station opening study did not differ between tested modifications in Florida. However, in Massachusetts, raccoon to opossum successful visitation ratios improved at QCS and STS bait stations, although the number of raccoon visits to STS bait stations decreased. Consequently, the strategy of modifying bait station openings seems to hold promise for reducing the waste represented by non-target bait consumption, and further trials will likely be conducted with better photographic equipment to more clearly document bait uptake. The control bait station design remains the best operational design until further trials are conducted.

A number of unquantifiable factors could account for differences between Massachusetts and Florida results, such as raccoon and opossum population density differences, weather, and food item competition in the surrounding landscape [21]. An obvious limitation in this assessment was the lack of complete certainty that a visit resulted in actual bait access or uptake. Future use of increasingly available video options to capture more definitive evidence that animals visiting bait stations are actually obtaining baits may help clarify this. In addition to camera capture data, serologic data would be important when comparing bait uptake from different bait station opening modifications, which would require increased geographic areas and effort.

Skunks found within the Massachusetts and Florida study locations (striped skunks, *Mephitis mephitis* (MA and FL) and spotted skunks, *Spilogale putorius* (FL)) were not documented visiting any bait stations during this study, but their roles as potential bait competitors need further attention based on their relative densities in many locations suitable for bait station use.

4.3. Alternate Baits for Bait Stations

In Massachusetts, ONRAB baits performed better in bait stations than traditional RABORAL V-RG baits based on successful removal. Both baits performed equally as well in Florida. Given the mixed results for bait removal by type between the two locations, further testing is necessary to determine whether real performance differences exist. In addition, since bait stations often dispense baits over a two- to three-week period, future bait station bait performance assessments should occur under conditions where vaccine titers can be assessed. Despite ONRAB baits preference by raccoons on Cape Cod, RABORAL V-RG baits continue to be used, and will be until further examination of ONRAB baits in bait stations is conducted.

Competition for baits, primarily in areas with high non-target populations, limits consumption by target raccoons. In Massachusetts, raccoons were photographed at bait stations containing ONRAB baits more frequently than at bait stations containing RABORAL V-RG baits, while bait stations containing RABORAL-VRG baits were more frequented by opossums. It did not appear that raccoons or opossums showed any preference towards either bait type in Florida. Further study may be necessary to examine bait-type preference on a location-by-location basis, as preference may vary by geographic region. Additional bait station modification may be necessary to prevent non-target uptake in both locations.

4.4. Decreasing Human Interference with Bait Stations

Human discovery of bait stations is problematic due to tampering, theft and increased potential for human vaccine contact. However, before bait stations could be uniformly camouflaged, it was important to assess whether these modifications could affect bait and vaccine quality. The most obvious candidate agent for reduced quality was assumed to be internal temperature. As a result of this study, it is recommended that bait stations be deployed in areas of full shade, as internal temperatures in those bait stations were cooler, regardless of color. Operationally, bait stations are rarely placed in areas of full sun due to visibility by the public and to prevent the excessive heating of vaccine baits. Where it became absolutely necessary in some instances to place bait stations in areas of full sun, white bait stations would be recommended rather than camouflage bait stations as a result of this study. Differing results between Massachusetts and Florida may be accounted for by higher ambient temperatures in Florida. However, given the need for vaccine and bait stability, these trials might bear repeating under more controlled circumstances. Additionally, bait and vaccine thermostability trials need to be conducted to examine efficacy of the vaccine at varying times throughout the typical three-week timeframe in which bait stations may contain baits.

5. Conclusions

Integrating bait stations into rabies control efforts on Cape Cod has led to similar sero-conversion rates and wildlife rabies management performance compared to traditional hand-baiting alone.

Additionally, the use of bait stations has reduced bait contact reports, maximizing the number of baits available to raccoons and other target species. Alternate bait station openings may eventually play a role in limiting non-target bait uptake. Furthermore, it was discovered that ONRAB vaccine baits were removed at a faster rate by target and non-target species when compared to RABORAL V-RG. This study also showed that bait stations should be placed in areas with limited sun exposure when possible. Although serology results are an important component to the evaluation of ORV success, the ultimate factors are the absence of raccoon rabies cases in the currently-treated zone, and those areas from where ORV has been shifted away. On Cape Cod from 2014–2016, 542 animals were tested for rabies, of which six were confirmed positive for raccoon rabies. All six animals (two raccoons and four skunks) were from west of the Cape Cod Canal. This integrated approach, subject to continued trials to achieve better bait station performance, may play an important role in rabies control and prevention in other areas of the U.S., especially as rabies management turns its focus from preventing raccoon rabies spread to elimination from the eastern U.S. However, some important questions regarding optimal bait station design and effectiveness remain.

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Conflicts of Interest: R.B.C. is the current National Rabies Management Coordinator for the USDA, APHIS, Wildlife Services, National Rabies Management Program, and D.S. is the former National Rabies Management Coordinator and both indirectly provided discretionary Federal funding to support field work.

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Article

Evaluation of Bait Station Density for Oral Rabies Vaccination of Raccoons in Urban and Rural Habitats in Florida

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Abstract: Efforts to eliminate the raccoon variant of the rabies virus (raccoon rabies) in the eastern United States by USDA, APHIS, Wildlife Services and cooperators have included the distribution of oral rabies vaccine baits from polyvinyl chloride (PVC) bait stations in west-central Florida from 2009 to 2015. Achieving sufficient vaccine bait uptake among urban raccoons is problematic, given limitations on aerial and vehicle-based bait distribution for safety and other reasons. One or three bait stations/km² were deployed across four 9-km² sites within rural and urban sites in Pasco and Pinellas Counties, Florida. Based on tetracycline biomarker analysis, bait uptake was only significantly different among the urban (Pinellas County) high and low bait station densities in 2012 ($p = 0.0133$). Significant differences in RVNA were found between the two bait station densities for both urban 2011 and 2012 samples ($p = 0.0054$ and $p = 0.0031$). Landscape differences in terms of urban structure and human population density may modify raccoon travel routes and behavior enough for these differences to emerge in highly urbanized Pinellas County, but not in rural Pasco County. The results suggest that, in urban settings, bait stations deployed at densities of $>1/\text{km}^2$ are likely to achieve higher seroprevalence as an index of population immunity critical to successful raccoon rabies control.

Keywords: bait stations; raccoons; rabies; vaccine; oral rabies vaccination

1. Introduction

Globally, rabies kills approximately 59,000 humans annually, and impacts on human and animal health result in a significant economic burden [1]. In the United States, the cost of living with the virus ranges from \$245–510 million annually [2]. Oral rabies vaccination (ORV) is an effective and socially-acceptable approach to wildlife rabies control [3]. ORV has been used to control fox rabies in western Europe [4,5] and in Canada [6–8]. In the U.S., ORV is currently aimed at the elimination and

prevention of new epizootics of canine rabies in coyotes (*Canis latrans*) [9,10], the elimination of rabies in gray fox (*Urocyon cinereoargenteus*) in Texas [11] and the containment and elimination of the raccoon (*Procyon lotor*) variant of the rabies virus (raccoon rabies) in the eastern U.S. [3].

While there are many variants of the rabies virus, and many vector species, raccoon rabies is primarily perpetuated within the raccoon. Raccoons often occur at extremely high population densities along the rural-urban interface, and are ecological generalists [12]. Raccoon rabies has spread rapidly in the abundant raccoon populations of eastern North America; however, the virus has not moved west of the Appalachian Mountain Range. Using this range as a natural barrier, USDA, APHIS, Wildlife Services (WS), National Rabies Management Program (NRMP) has implemented a large-scale ORV program to prevent the westward spread of raccoon rabies [3]. WS NRMP is conducting cooperative ORV operations to continue preventing the spread of raccoon rabies into the mid-western U.S. and eastern Canada (Phase I), and has begun work towards its elimination from the eastern U.S. (Phase II) [3], much of which is highly urbanized.

Bait stations for distribution of oral rabies vaccine baits have become an increasingly important bait delivery method in urban areas where aerial and vehicle-based (or hand) vaccine bait delivery is hampered by high human and pet densities, and in rural areas where raccoon densities are low, but target species may be concentrated in smaller localized populations, reducing the need to widely broadcast vaccine baits. Bait station use began in New York in 2003, and in key locations in Massachusetts in 2006 [13,14], with important questions regarding optimal design and effectiveness left unanswered. Although bait station design and deployment has been evaluated, including modification to reduce non-target uptake, especially by opossums (*Didelphis virginiana*), future design improvements and optimized strategies for their use require additional study [13,15,16]. Opossums are a non-target species due to their low incidence of rabies. They are attracted to vaccine baits and are able to remove baits from the bait stations with little difficulty. Direct competition with raccoons for the baits can confound rabies management efforts [17,18].

To better understand the best management strategies for using bait stations to control raccoon rabies in central Florida during 2011 and 2012, presence of tetracycline (TTCC) biomarker and rabies virus neutralizing antibodies (RVNA) as indices of bait station performance [3] were compared between two bait station densities in rural and urban settings using fishmeal polymer (FMP) baits containing RABORAL V-RG® (Merial, Athens, GA, USA) vaccine. It was predicted that placing 3 bait stations/km² would result in significantly higher RVNA and TTCC percentages than having 1 bait station/km² among the urban study sites, and that there would be no significant difference between 3 bait stations/km² and 1 bait station/km² among the rural study sites.

2. Materials and Methods

Rural study sites were selected within the Starkey Wilderness Preserve in Pasco County, Florida, which is owned and managed by the Southwest Florida Water Management District (SWFWMD), and urban sites were selected within St. Petersburg in Pinellas County, Florida (Figure 1). The rural study sites were dominated by oak (*Quercus* spp.) and pine (*Pinus* spp.) woodlands, with few to no houses in the area. In this study, there were 87 houses within the northeast corner of the study site and bait stations were set at least 0.04 km from the property lines. The rural study sites were interspersed with dirt trails maintained by SWFWMD. An understory of scrub and shrub species was throughout the rural study sites. The urban sites were located within St. Petersburg, Florida, which had a population of approximately 245,300 at the time of the studies, with a population density of approximately 3970 people/mi² (or 1533 people/km²) [19]. The landscape was dominated by residential and commercial properties. The study sites will be referenced as rural (Pasco County) or urban (Pinellas County) high bait station density (HBSD)—those sites with 3 bait stations/km², and rural or urban low bait station density (LBSD)—those sites with 1 bait station/km².

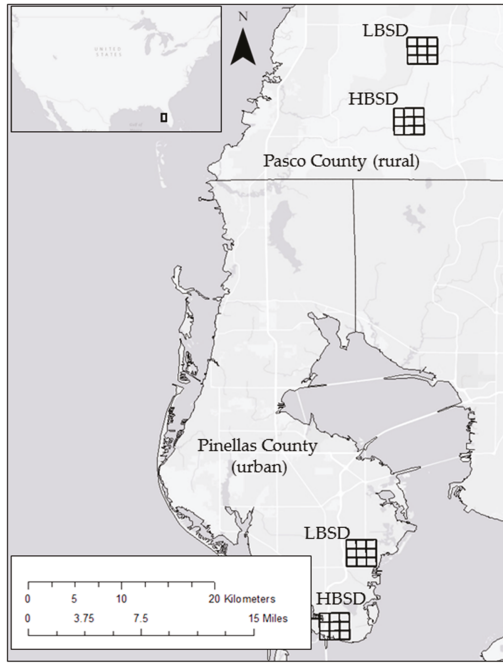


Figure 1. Location of 2011 and 2012 bait station sites within rural (Pasco County) and urban (Pinellas County) locations. The LBSD sites in each county had one bait station/km², while the HBSD sites in each county had three bait stations/km².

Bait stations were constructed of 2.5-foot sections of 4-inch diameter polyvinyl chloride (PVC) schedule 40 pipe, painted in camouflaged colors to reduce the likelihood of human tampering. Open PVC-tops were covered with 4-inch flexible Qwik® (United States Plastic Corp, Lima, OH, USA) caps to prevent rain and bait access for animals from the top. PVC elbows (90 degree angle) were attached to the 2.5-foot PVC section bottoms, and a 3–4 inch PVC pipe extended from the elbow with a nut and bolt to prevent baits from falling out of the bait station (Figure 2). The bolt acts as a stop to prevent the baits from sliding out and the nut holds the bolt in place. This design was based on the bait station design by Boulanger et al. [13], and then modified to accommodate more baits at one time.

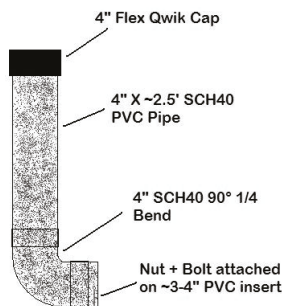


Figure 2. Bait station specifications used in Pasco and Pinellas Counties, Florida, during 2011–2012.

Bait stations were deployed over 10 consecutive nights during 9–20 May 2011 and 21 February–5 March 2012. Due to the number of bait stations to be deployed, not all bait stations were set on the same day. Each bait station area was active for 10 days, though the total number of study days was >10 days. Study sites were selected within 5 km of previous WS raccoon density study or bait station study sites to provide working knowledge of the raccoon populations in the study areas. The raccoon density in Pasco County was estimated at approximately 10 raccoons/km² during a density study conducted in 2011; however, there were no density studies conducted in Pinellas County. Target bait densities on all sites were 75/km², the standard base rate for distributing baits based on current raccoon densities [3]. The bait densities were kept constant across the study sites to ensure study sites could be compared to one another. Two 9-km² study sites were selected (within one habitat type (i.e., woodland-dominated or urban/residential-dominated) to the greatest degree possible) at least 5 km apart in each of the two counties (urban LBSD and HBSD, and rural LBSD and HBSD). Thirty-six bait stations were deployed in each county, and 1350 vaccine baits containing TTCC hydrochloride as a biomarker were deployed on Day 0 in each county. The FMP baits containing RABORAL V-RG[®] vaccine were 1.25 inch × 1.25 inch × 0.5 inch brown square blocks made of fishmeal. Inside the bait was a sachet sealed in the block with wax. The pink liquid inside the sachet was the vaccine. The amount of vaccine was intended to be a single dose.

The LBSD site in each county was equipped with one bait station/km², containing 75 baits each (9 bait stations/site). The HBSD site in each county was equipped with three bait stations/km² containing 25 baits each (27 bait stations/site). Even distribution of the bait stations within the rural sites was possible due to the rural nature (woodlands with scrub/shrub understory) of the site, and single ownership; only vegetation and a lack of trails influenced bait station distribution. These sites were dominated by saw palmettos (*Serenoa repens*), oaks, and pines. Thorny vines, like greenbrier (*Smilax bona-nox*), made human movement difficult. Access to trails in 2012 that were available for use in 2011 was reduced by storm damage. The distribution of bait stations within the urban HBSD site was clustered in several 1-km² sections based on landowner permission, vegetative cover and the need to hide bait stations from the view of the public to reduce tampering.

Bait stations were visited three to five times during each study period to monitor activity, equipment and site conditions. In the urban sites, four infrared automated cameras were positioned in LBSD, and six in HBSD, while in the rural sites, five were set in LBSD, and six in HBSD. All cameras were Moultrie[®] (EBSCO Industries, Inc., Birmingham, AL, USA), and both Gamespy D55IR and I40 Digital Game Camera models were used. The D55IR was a 5.0-megapixel camera and accepted SD cards of up to 16 GB. The I40 was a 4.0-megapixel camera and accepted SD cards of up to 4 GB. Photos were set at high image quality, with 1-minute activation intervals and with the multi-shot function turned on to capture 3 photos for each activation on both cameras. No flash was used; only the infrared flash was used at night. Sensor, aperture, and focal lengths were adjusted automatically as needed; these were not changed from the original setting as there was no means to adjust them. Cameras were set 12–24 inches from the ground, a minimum of 3 feet from the bait station, and aimed toward bait station openings to determine the species (raccoon vs. non-target) taking bait. Direction of the cameras was not accounted for, as most of the bait stations were set within clumps of vegetation so direct sunlight was not a factor. Each camera was given a unique ID number, which was printed on the photos to enable proper location of the photos. While setting the bait stations and cameras, the bait station number was recorded along with the corresponding camera ID. Any removal of vegetation in the rural sites that may have interfered with the cameras capturing photos was kept to a minimum so as not to make changes to the habitat that could deter animal visitations. In the urban sites, no vegetation changes were made, since the bait stations were set primarily on private property, and damage the landowners' plants was not desired. Camera event counters were reset during each site visit, and the time between photographs was minimized. During each bait station visit, the following information was recorded: date of visit, bait station ID, camera type, number of photos on camera (since last visit), number of images by species, and bait condition. The photos were viewed on a

laptop computer. Each new event was determined by a 15-minute interval between photos showing individual animals. If the animal could be accurately identified by its markings as the same animal in the previous event photo (15 min prior), then this was considered a new event but not a new individual. If a bait station was emptied prior to the end of the of the 10-day study period, it was removed along with the camera, if one was associated with the bait station, to reduce tampering and damage to the bait station.

Trapping began 24 days after bait stations were removed to allow sufficient time for TTCC biomarker deposition and RVNA development, and to approximate the time between standard ORV bait distribution and post-ORV sampling. Trapping occurred within 0.5 km of each study site to optimize capture rates, and ≥ 30 unique raccoons/study site were targeted to facilitate TTCC biomarker and serological analyses. Trapping was completed 84 days post-station removal. Raccoons were marked with a metal #4 ear tag (National Band & Tag Co., Newport, KY, USA) stamped with a unique identifying number so as to identify each individual raccoon captured. Given that all past and present captured raccoons are marked in the same fashion, any animals recaptured from previous studies could be easily identified and removed from testing if treated in a manner (i.e., given vaccination by injection) that would affect this study's results. Standard biological samples were collected, including blood sera to determine vaccine-induced immunity, and first premolar (PM1) teeth for biomarker evaluation. Although biomarking frequently occurs in fewer animals than actually demonstrate vaccine-induced serological responses due to extraction of a first premolar from live trapped and released raccoons, it remains useful when considered with other vaccination assessment tools [20,21]. The teeth were labeled and prepared for shipment to Matson's Laboratory, LLC (Manhattan, MT, USA) where the tetracycline biomarker analysis was performed. Methods used for this test were performed as stated in Algeo et al. [20] and Linhart and Kenelly [22]. Rabies virus neutralizing antibody tests were conducted at the Centers for Diseases Control and Prevention (CDC) in Atlanta, GA, using the rapid fluorescent focus inhibition test (RFFIT). Methods for this test were performed as stated in Smith et al. [23] and CDC [24]. A cut-off of both ≥ 0.05 and ≥ 0.1 IU/mL were used to indicate a positive RVNA response. It was desired to determine if there was a detectable difference with using the lower 0.05 IU/mL versus the higher 0.1 IU/mL.

Fisher's exact tests were used to compare RVNA rates within and between treatments and sites. GraphPad QuickCalcs (GraphPad Software, Inc., La Jolla, CA, USA) statistical software was used for analyses [25], with $\alpha = 0.05$.

3. Results

The photographs captured by each camera were examined and the number of individual animals photographed was documented. One camera was removed from the rural HBSD counts in both 2011 and 2012 for lack of photographs showing any individual animals. In 2011, total camera days were 44 (rural LBSD, 5 cameras), 42 (rural HBSD, 5 cameras), 40 (urban LBSD, 4 cameras) and 22 (urban HBSD, 6 cameras). Total camera days in 2012 were 34 (rural LBSD, 5 cameras), 24 (rural HBSD, 5 cameras), 40 (urban LBSD, 4 cameras) and 31 (urban HBSD, 6 cameras). Photographs were analyzed for individual identifiable animals by markings. If an animal was not identified as the same with certainty, then it was counted as a new individual. Raccoons were photographed more frequently in five of the eight sampling periods than were opossums, the primary non-target species in the area. However, during 2012 in the rural LBSD site, and during 2011 and 2012 in the urban LBSD site, more opossums were photographed (Figure 3). Only in the rural LBSD site did raccoons predominate amongst the photographed individuals in 2011, and opossums predominated in 2012.

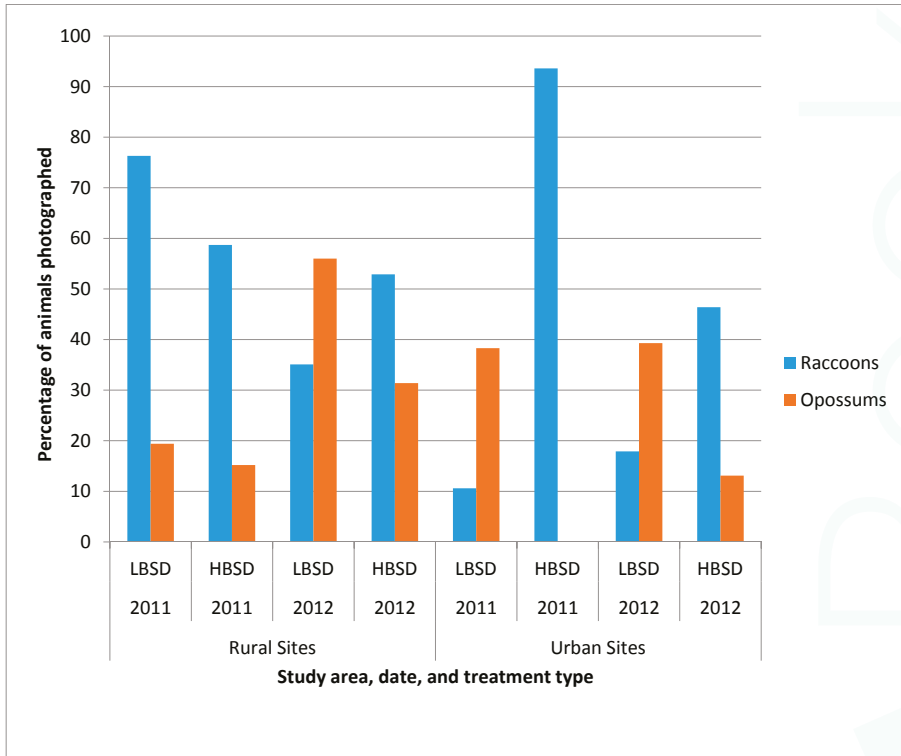


Figure 3. Comparison of raccoon and non-target opossum ratios in automatic camera images at LBSD and HBSD sites in rural and urban environments in Pasco and Pinellas Counties, Florida, during 2011–2012.

A total of 244 raccoons was trapped and sampled during 2011 and 2012; seven of these were removed from the results due to previous vaccination by injection during 2011. RVNA rates ranged from 6.3% (urban LBSD 2012) to 53.8% (rural HBSD 2012) (Table 1). The HBSD sites resulted in more elevated RVNA rates in 2012 (53.8% and 51.6%) than did the LBSD sites (44.4% and 6.3%). The 2012 rural and urban HBSD sites also had more elevated RVNA rates (53.8% and 51.6%) than both rural and urban HBSD in 2011 (35.1% and 45.2%). Tetracycline biomarker was present in more teeth collected from both rural and urban HBSD sites in 2012 (30.4% and 33.3%) than in both 2012 rural and urban LBSD sites (26.9% and 0.0%) (Table 1).

Table 1. Bait station study area designs and results for 2011 and 2012 in rural and urban sites in Florida.

	Rural				Urban			
	2011		2012		2011		2012	
Site (n bait stations; baits/station)	LBSD (9; 75)	HBSD (27; 25)	LBSD (9; 75)	HBSD (27; 25)	LBSD (9; 75)	HBSD (27; 25)	LBSD (9; 75)	HBSD (27; 25)
Baits removed from bait stations (percent of total baits)	513 (76.0%)	620 (91.9%)	675 (100%)	650 (96.3%)	179 (26.5%)	501 (74.2%)	265 (39.3%)	639 (94.7%)
Raccoons trapped	37	37	27	26	32	31	16	31
Percent raccoons RVNA ^a (n positive raccoons)	21.6 (8)	35.1 (13)	44.4 (12)	53.8 (14)	12.5 (4)	45.2 (14)	6.3 (1)	51.6 (16)
Percent raccoons RVNA ^b (n positive raccoons)	21.6 (8)	35.1 (13)	44.4 (12)	53.8 (14)	9.4 (3)	38.7 (12)	6.3 (1)	51.6 (16)
Percent biomarker present (n 1st premolar teeth biomarker present)	14.7 (34; 5)	28.1 (32; 9)	26.9 (26; 7)	30.4 (23; 7)	19.4 (31; 6)	19.2 (26; 5)	0.0 (16; 0)	33.3 (24; 8)

^a cut-off of ≥ 0.05 IU/mL was used to indicate a positive RVNA; ^b cut-off of ≥ 0.1 IU/mL was used to indicate a positive RVNA.



Bait removal from bait stations varied between sites and between years. The nine urban LBSD bait stations started each year with a total of 675 baits, and had only 179 baits removed (26.5%) by the end of the 10-day study period in 2011 and 265 baits removed (39.3%) in 2012 (Table 1). A greater percentage of baits were taken from bait stations in 2012 than 2011. Within the urban sites, a larger number of baits were taken from the bait stations in the HBSD site regardless of year. However, within the rural areas, one bait station in the HBSD site did not have any baits removed in 2012 while all the baits within the LBSD site were removed from the bait stations (Table 1).

RVNA rates were significantly higher ($p = 0.0054$ and 0.0031 , respectively) in urban HBSD sites in 2011 and 2012 using an RVNA cutoff of ≥ 0.05 IU/mL, indicating a relationship with increased bait station density in urban areas. However, the rural sites did not differ (Table 2A). RVNA rates were significantly higher ($p = 0.0081$ and 0.0031 , respectively) in urban HBSD sites in 2011 and 2012 using an RVNA cutoff of ≥ 0.1 IU/mL, indicating a relationship with increased bait station density in urban areas. However, the rural sites did not differ (Table 2B).

Table 2. Comparison of raccoon RVNA (at 75 baits/km²) with deployment of LBSD versus HBSD in rural and urban environments in Florida, 2011–2012.

County/Year	Percent RVNA (n)	Fisher's Exact Test
A. Showing results using RVNA cutoff of ≥ 0.05 IU/mL		
Rural 2011 (LBSD vs. HBSD)	21.6 (37) vs. 35.1 (37)	$p = 0.3024$
Rural 2012 (LBSD vs. HBSD)	44.4 (27) vs. 53.8 (26)	$p = 0.5867$
Urban 2011 (LBSD vs. HBSD)	12.5 (32) vs. 45.2 (31)	$p = 0.0054$
Urban 2012 (LBSD vs. HBSD)	6.3 (16) vs. 51.6 (31)	$p = 0.0031$
B. Showing results using RVNA cutoff of ≥ 0.1 IU/mL		
Rural 2011 (LBSD vs. HBSD)	21.6 (37) vs. 35.1 (37)	$p = 0.3024$
Rural 2012 (LBSD vs. HBSD)	44.4 (27) vs. 53.8 (26)	$p = 0.5867$
Urban 2011 (LBSD vs. HBSD)	9.4 (32) vs. 38.7 (31)	$p = 0.0081$
Urban 2012 (LBSD vs. HBSD)	6.3 (16) vs. 51.6 (31)	$p = 0.0031$

Tetracycline biomarker was higher in the urban HBSD site in 2012 ($p = 0.0133$; Table 3). No significant differences were found between the rural sites or the 2011 urban sites.

Table 3. Comparison of raccoon tetracycline deposition (at 75 baits/km²) with deployment of LBSD versus HBSD in rural and urban environments in Florida, 2011–2012.

County/Year	Percent Tetracycline-Positive (n)	Fisher's Exact Test Result
Rural 2011 (LBSD vs. HBSD)	14.7 (34) vs. 28.1 (32)	$p = 0.2344$
Rural 2012 (LBSD vs. HBSD)	26.9 (26) vs. 30.4 (23)	$p = 1.000$
Urban 2011 (LBSD vs. HBSD)	19.4 (31) vs. 19.2 (26)	$p = 1.000$
Urban 2012 (LBSD vs. HBSD)	0 (16) vs. 33.3 (24)	$p = 0.0133$

4. Discussion

Achieving sufficient vaccine bait uptake among urban raccoons is critical. Limitations on aerial and vehicle-based (hand) bait distribution for safety and other reasons necessitate finding other bait distribution means and optimized strategies for achieving RVNA rabies management goals. Bait stations represent one potential tool for specific settings that may achieve management goals while reducing non-target bait loss and pet and human bait contact, mitigating many concerns from managers, cooperating agencies, and the public. Even though the bait stations were studied in May 2011 and February 2012, it was anticipated that these differences in times of year did not have any impact on the results. Though warmer temperatures may impact raccoon movements during the day, when looking at the total baits removed per year, it does not appear that time of year had any effect on the results of this study. It was believed that raccoon movement was sufficient to ensure that

many raccoons came in contact with the baits in both years. The warmer temperatures during the 2011 trapping period may have been thought to negatively impact the capture rate, but capturing raccoons within the urban LBSD site in 2012 proved to be more difficult ($n = 16$; Table 1).

Bait removal from bait stations resulted in higher RVNA percentages in the urban HBSD sites, irrespective of RVNA cutoff level. The lack of uptake in the urban LBSD site (<40% of baits removed in both 2011 and 2012 out of 675 baits) may be due to a perceived relatively low localized raccoon density at the time of this study, which can be evidenced from the relatively low percentage of raccoon photos in this area in 2011 and 2012 (Figure 3). A lack of travel corridors due to roads through the area, fenced and relatively barren yards, few park and recreational areas, and people and pet interference may also have had negative impacts on raccoon movements through this site. In contrast, the urban HBSD site (74.2% and 94.7% of baits removed in 2011 and 2012, respectively, out of 675 baits) contained multiple parks, ideal tree cover, food resources, and habitats for raccoons, as well as a golf course and conservation areas with multiple fresh water ponds. Many of the house lots in this site contained several large trees as potential denning sites.

Domestic dogs (*Canis familiaris*) and cats (*Felis catus*) were both captured in photos only at the urban bait stations; however, none were documented taking a bait from the bait stations. Therefore they were not reported in the results. Since these animals did not take any baits and were not observed eating a bait that was on the ground, neither dogs nor cats were considered a non-target species for the bait.

Greater bait removal from the bait stations in 2012 than in 2011 may be due to identical bait station locations in both years, possibly resulting in bait stations being more easily found in the second year. Several authors [26–30] have documented learned behavior in raccoons—from traversing a maze after being shown the end, to pulling a lever for a reward after watching someone pull the same lever, to gaining access to garbage cans after lid modifications have been made. Female raccoons with young were documented in the 2011 photos. It is possible that those young returned the next year after learning the bait stations provided bait. A single bait station in the rural HBSD site had no baits taken, possibly due to the presence of acrobat ants (*Crematogaster ashmeadi*) that were observed covering the baits. These native fire ants potentially reduced the bait scent attractant to wildlife. Fire ants have been observed by multiple wildlife personnel throughout the southeastern U.S. covering bait in traps, as well as vaccine baits on the ground, thereby preventing raccoons and other animals access to the bait.

Two RVNA cutoff levels of ≥ 0.05 IU/mL (used by CDC [24]) and ≥ 0.1 IU/mL (suggested by Canadian and European counterparts) were examined. No comparable difference in the results was found when using the higher cutoff (Table 2). For this study, no justifiable reason was found to conclude that using ≥ 0.05 IU/mL as the cutoff was limiting or accounted for animals with false-elevated RVNA results. There remains much debate about the levels of rabies antibodies that confer resistance to rabies virus infection. No single cutoff level of RVNA is recognized as being invariably protective [31]. Repeated observations that small fractions of animals presenting detectable levels of antibody prior to challenge have shown the animals can still succumb to rabies infection, and conversely that some seronegative animals survive challenge [32,33]. While these discrepancies exist, Blanton et al. [33] did observe that no raccoon succumbed to rabies challenge after vaccination with RABORAL® V-RG, even with an RVNA level of 0.06 IU/mL at the time of challenge. This result indicates to us that a cutoff of ≥ 0.05 IU/mL for this study was sufficient.

Lower RVNA response in the urban LBSD site may be related to a lower population of raccoons (Figure 3) or a preponderance of private properties surrounded by fences and smaller lots than in the urban HBSD site. To set the bait stations on private property in the LBSD site, the bait stations were placed inside the fences, as requested by property owners. This placement may have reduced the opportunities for raccoons to find the baits. Bozek et al. [34] found raccoons in urban areas had smaller home ranges than those in rural areas. Raccoons in urban habitats have access to anthropogenic food sources and can thereby reduce their foraging distances and patterns. These human food sources may

also explain why baits were left in the urban LBSD bait stations. By providing increased bait station density within yards without fences, raccoons likely had easier access to the bait stations in the urban HBSD site, resulting in more baits taken and a significantly higher RVNA response.

Tetracycline biomarker results and RVNA rates were not compared in this study for a few reasons. First, canine teeth and mandibular bone are superior tissues for tetracycline biomarking [20,22], but first premolar teeth were collected for this study as a less intrusive procedure, so that raccoons could be released after full recovery from sedation. Canine tooth sampling would have required euthanasia and eliminated the opportunity to obtain valuable biologic information in future field trials through recaptures. First premolar teeth continue to be the most acceptable, least intrusive sample to collect from live-trapped raccoons. Second, while not noted earlier in this study, unpunctured sachet packets were found at every bait station. The FMP coating was missing, presumably eaten by a raccoon or opossum. This would result in a positive biomarker in the tooth, but no positive RVNA response. Third, background sources could have contributed to tetracycline biomarking. The most likely background tetracycline sources may have included consumption of medicated feeds sometimes used for cattle production and nonspecific fluorescence that may be found naturally [35]. While all study sites had lower biomarker percentages than elevated RVNA percentages, the 2011 urban LBSD site had a higher percentage of biomarkers present than percentage of elevated RVNA. It is unsure why this could be, unless tetracycline is present in the environment or the raccoons were avoiding the vaccine sachet and strictly eating the FMP coating. The sites with higher percentages of elevated RVNA than percentage of tetracycline biomarker may suggest a natural response to rabies in the area, poor tetracycline uptake in the first premolar tooth samples, or, as could be the case in 2012, trapping 'missed' animals from 2011 that ingested the vaccine. Additionally, the easier locating of bait stations by the animals, as evidenced by the increased number of baits removed from the bait stations in 2012, could have resulted in the higher percentages of elevated RVNA (Table 1).

The findings from this study support a higher bait station distribution density, to provide greater access to raccoons in urban settings to achieve higher RVNA to meet raccoon rabies management goals. However, additional well-designed studies are required to better understand optimized bait station density and distribution to achieve raccoon rabies elimination in the urban environments that form the mosaic of landscapes on which raccoon rabies occurs.

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Author Contributions: Dennis Slate and Tim Algeo conceived and designed the experiment; Betsy Haley performed the experiment; Tim Algeo and Ashlee Martin analyzed the data; Richard Chipman, Robert Edwin Hartin and Anthony Duffiney contributed materials; Betsy Haley, Tim Algeo, Richard Chipman, Ashlee Martin, Dennis Slate, Kathleen Nelson, and Brian Bjorklund collaboratively wrote and/or reviewed multiple drafts and substantially contributed to this manuscript.

Conflicts of Interest: Richard Chipman is the current National Rabies Management Coordinator for the USDA, APHIS, Wildlife Services program and Dennis Slate is the former National Rabies Management Coordinator and both indirectly provided discretionary Federal funding to support field work and the writing of the manuscript.

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Article

Cost and Relative Value of Road Kill Surveys for Enhanced Rabies Surveillance in Raccoon Rabies Management

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Abstract: Oral rabies vaccination (ORV) requires knowledge of the spatial-temporal distribution of rabies virus variants targeted for control. Rabies-exposure based public health surveillance alone may not provide a sound basis for ORV decisions. The value and cost of road kill surveys was evaluated for the late spring–early fall 2005–2007 as a part of enhanced rabies surveillance in northern New York, where raccoon rabies is enzootic and ORV has occurred since the late 1990s. Structured surveys were conducted to collect raccoons and other meso-carnivores for rabies testing at the New York State Rabies Laboratory. Of the 209 meso-carnivore heads collected and submitted for testing, 175 were testable by direct fluorescent antibody; none was rabid. Rabies was also not reported through public health surveillance in survey zones during 2005–2007. Overall, survey costs were \$37,118 (2016 USD). Salaries and benefits accounted for 61% of costs, followed by fuel (22%), vehicle depreciation (14%), and sample shipping (3%). Mean daily distance driven was 303 ± 37 km and 381 ± 28 km for total road kills and raccoons, respectively. Costs/road kill collected and submitted was \$176/all species and \$224/raccoon. This study provides costs for planning road kill surveys and underscores the need to continually improve enhanced rabies surveillance approaches to support ORV decision making.

Keywords: raccoon; skunk; rabies; enhanced rabies surveillance; road kill; oral rabies vaccination (ORV); meso-carnivores

1. Introduction

Rabies surveillance is characterized as being active or passive [1]. Much of the rabies surveillance in the U.S. is based on exposure events brought to the attention of public health officials [2].

While effective in protecting the public from rabies, passive surveillance is inadequate to delineate the spatial-temporal distribution of rabies in meso-carnivore reservoirs such as the raccoon (*Procyon lotor*) in near real time [3].

Enhanced rabies surveillance is characterized as a form of surveillance with special emphasis on specific areas, species, and rabies management goals [4]. Since 2005, enhanced rabies surveillance has been extensively used in proximity to oral rabies vaccination (ORV) zones in the USA to increase sampling scope and intensity to delineate rabies distribution among wildlife rabies reservoir species for improved management.

Enhanced rabies surveillance serves as a complement to exposure-based public health surveillance, and collectively this information provides a more comprehensive spatial-temporal view of rabies for strategic ORV decisions to achieve rabies management goals in the U.S. Enhanced rabies surveillance targets a range of sample sources within or near (≤ 80 km) ORV zones, without a known human or domestic animal exposure history.

Sampling scope includes:

1. Animals exhibiting unusual behaviors suggestive of rabies brought to the attention of U.S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Wildlife Services (WS), or collected by program cooperators; these typically have the highest rabies prevalence among animals not involved in human or domestic animal exposure events;
2. Animals found dead (other than road kills);
3. Road kills from formal or opportunistic surveys;
4. Animals WS trapped and euthanized or shot in specified raccoon rabies risk areas, often where recent cases warrant sampling;
5. Animals captured by Nuisance Wildlife Control Operators (NWCOS) or others near ORV zones [4,5].

Road kill surveys have been conducted for a variety of wildlife management purposes, such as indexing population trends and abundance [6–9]; wildlife disease monitoring [10]; and characterization of population genomics [11]. We assessed the relative value of structured road kill surveys as a complement to public health surveillance to determine the presence or absence of raccoon rabies variant in raccoons and other meso-carnivore species into which spillover of this rabies virus variant occurs [12] (striped skunk (*Mephitis mephitis*), red fox (*Vulpes vulpes*), gray fox (*Urocyon cinereoargenteus*), coyote (*Canis latrans*), and fisher (*Martes pennanti*)) in northern New York. ORV had been conducted since 2001 (USDA, APHIS, Wildlife Services 2001) throughout much of the area where road kill surveys were conducted. These surveys were evaluated in the context of the cost of samples acquired and their contribution to rabies surveillance in the areas surveyed.

2. Materials and Methods

From 2005 to 2007, formal road kill surveys were initiated within or near ORV zones in northern New York (Figures 1–3). Differences in routes among years were necessary in response to staffing availability. The same two personnel conducted nearly all the road kill surveys from 2005 to 2007. All personnel who participated in the road kill surveys received the recommended rabies pre-exposure vaccine series prior to conducting surveys.

Personnel were trained so that activities were uniformly conducted in a coordinated fashion, although there was not a strict protocol for speed driven by highway type or road-scanning breadth, as these may vary for safety, road shoulder width, and roadside mowing activities. Surveys were conducted in full-sized pickup trucks, which provided an improved road/road shoulder view over lower-framed vehicles.

Survey routes were driven from 6:00 am until approximately noon. State, County, and Municipal highway officials were contacted to inform them of the road kill survey times. Species subject to collection included: raccoon, skunk, foxes (red and gray), coyote, and fisher.

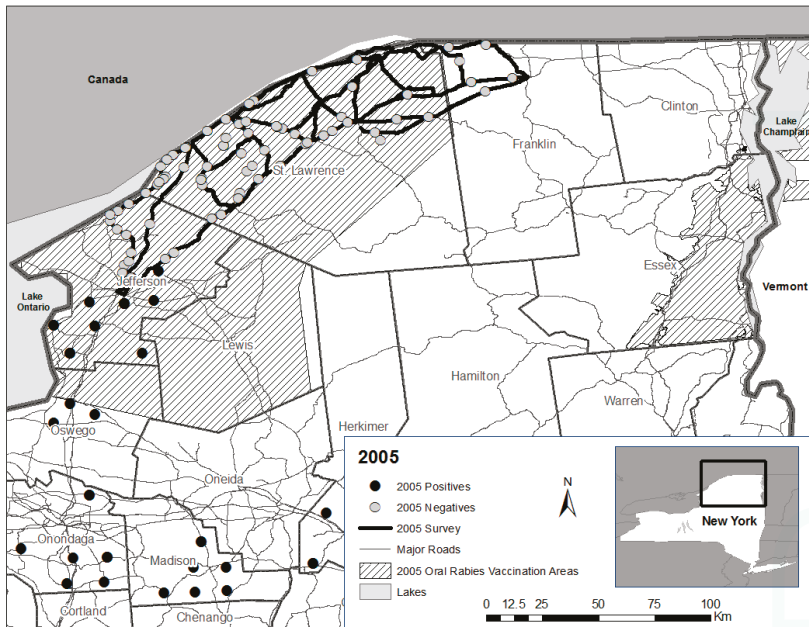


Figure 1. Rabies negative road kills collected during surveys (27 June–21 September) in Jefferson, St. Lawrence, and Franklin counties in 2005 relative to the oral rabies vaccination zone and public health positive cases.

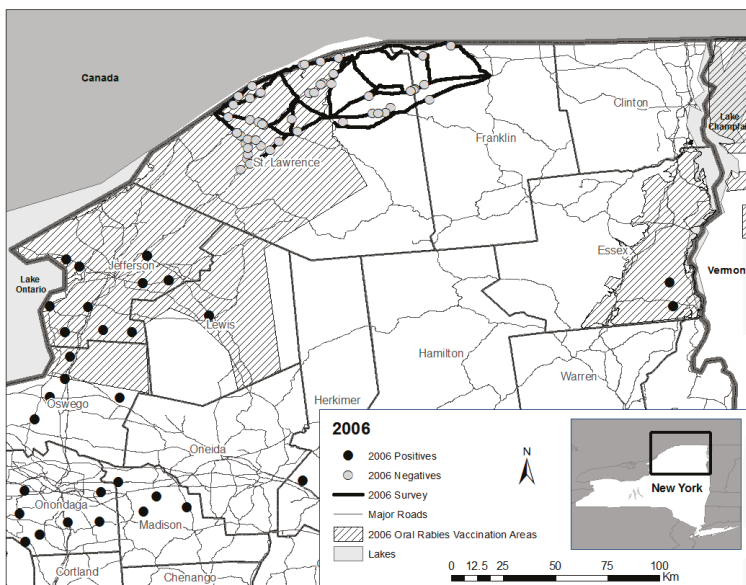


Figure 2. Rabies negative road kills collected during surveys (5 June–17 August) in St. Lawrence and Franklin counties in 2006 relative to the oral rabies vaccination zone and public health positive cases.

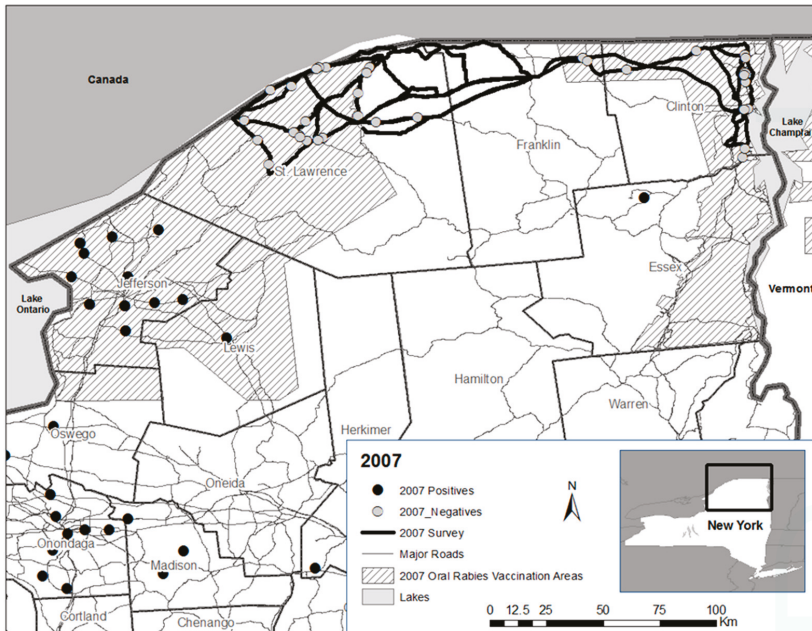


Figure 3. Rabies negative road kills collected during surveys (25 June–27 July) in St. Lawrence, Franklin, and Clinton counties in 2007 relative to the oral rabies vaccination zone and public health positive cases.

In 2005, specified routes were surveyed four days/week for 10 weeks from 27 June to 21 September (Figure 1). For staffing efficiency, the road kill survey area was divided into approximately equal east and west zones. The western zone was bordered on the west by U.S. Interstate 81 from Watertown (Jefferson County) to the St. Lawrence River, and State Route 812 between Ogdensburg and the intersection of State Route 11 near DeKalb on the east. The eastern zone included major roads (State Routes 37 and 11B) from this point East to Malone, which encompassed portions of St. Lawrence and Franklin Counties where ORV had not occurred (Figure 4). Both areas were further divided so that half of each area was sampled two days/week with minor exceptions, by two personnel (Tuesday and Thursday; and Wednesday and Friday).

In 2006, the portion of the road kill survey zone in Jefferson and southwestern St. Lawrence Counties was discontinued, reducing the zone that was sampled in 2005 by about half. The new 2006 zone was then divided approximately in half. However, unlike in 2005, each half was not further sub-divided and the entire zone was surveyed four days/week by two personnel with minor exceptions from 5 June to 17 August (Figure 2).

In 2007, the 2006 road kill survey zone was extended to include portions of eastern Clinton County, with State Routes 190 and 11 surveyed for road kills traveling to and from Franklin into Clinton County, and U.S. Interstate 87 and State Routes 9 and 22 making up much of the road matrix surveyed in the northern Lake Champlain Valley (Figure 3). Due to staffing demands, road kill surveys were conducted four days/week by two personnel during the period 25 June–27 July—a five, rather than a 10-week survey.

During sample collection, personnel wore appropriate personal protective equipment including nitrile gloves, hard hats, and safety vests for compliance with permits issued by the New York State Department of Transportation. When road kill carcasses were observed, personnel quickly and safely parked alongside the roadway to retrieve the carcass. Once personnel moved to a safe location,

each carcass was inspected to determine if a viable sample could be collected and submitted for testing. For carcasses deemed viable, GPS coordinates were recorded for the road kill location, unique numbered ear tags were attached, and the whole head was removed at the base of the neck using heavy duty brush loppers. Samples were frozen until shipping.

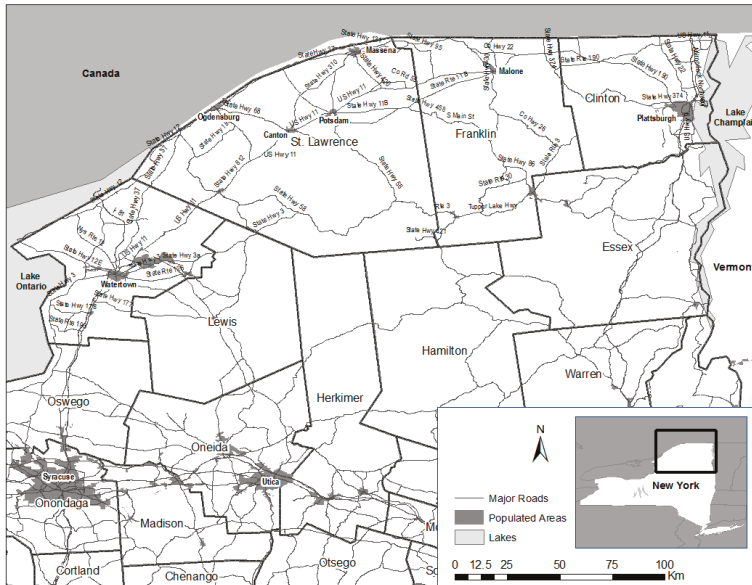


Figure 4. Reference map for counties, villages and cities, and routes that occurred within road kill survey areas, New York.

Samples were prepared and packaged for shipment to the New York State Department of Health (NYSDOH) Rabies Laboratory at Wadsworth Center, New York on Monday of each week. Intact head samples were wrapped in newspaper and double-bagged (resealable plastic bags) separately with specimen labels containing the sample ear tag numbers. Multiple intact head specimens were included in each shipment in double-walled, insulated containers, in accordance with the UN-3373 Biological Specimen Category regulations. The following data were entered into the WS National Rabies Management Program (NRMP) database: date and GPS coordinates of sample collection; species; sex; relative age (juvenile or adult); and reproductive status.

Samples were categorized for data analysis as *detected*, *submitted*, and *tested*. Samples *detected* represented species of interest found on survey routes that often included animals deemed not suitable to be submitted for testing. *Submitted* samples included those that field personnel collected and determined to be candidates for rabies testing. NYSDOH Rabies Laboratory personnel made the final determination if submitted samples were suitable to be *tested* for rabies. The direct fluorescent antibody (dFA) test was used to determine the rabies status of road kills [13]. In 2005, road kills detected but considered unsuitable for rabies testing by WS staff were not recorded. All three categories were recorded in 2006 and 2007.

Direct or estimated costs for four major components of road kill surveys included staff salary and benefits [14], fuel costs [15], vehicle depreciation, and sample shipment costs adjusted to 2016 U.S. dollars.

Statistical Analysis System (SAS) 9.3 (SAS Institute, Cary, NC, USA) statistical software was used for analyses [16].

3. Results

Six meso-carnivore species were observed during road kill surveys in the St. Lawrence River Valley (Jefferson and St. Lawrence Counties), in northern Franklin County, and the northern Lake Champlain Valley (Clinton County) along the U.S.-Canada border from 2005 to 2007. Raccoons were most frequently detected ($n = 320$), followed by striped skunks ($n = 136$), and red foxes ($n = 13$) (Table 1). These were the only species that were submitted for rabies testing in all three years. Two gray foxes were detected and submitted during 2006 that were deemed not suitable for testing by the NYSDOH. A single fisher was submitted and tested negative in 2007, but was excluded from statistical analysis because of low sample size. None of the 140 raccoons, 26 skunks, 8 red foxes, and 1 fisher ($n = 175$) tested over the survey period was rabid by dFA.

Table 1. Summary of road kill samples detected, submitted, and tested for rabies from road kill surveys conducted in northern New York during late spring-early fall 2005–2007.

Year ^{1,2}	2005 ³			2006			2007		
	Detect	Submit	Test	Detect	Submit	Test	Detect	Submit	Test
Raccoon		62	62	196	59	50	124	45	28
Striped Skunk		12	12	90	8	7	46	12	7
Red Fox		6	6	8	1	1	5	1	1
Gray Fox		0	0	2	2	0	0	0	0
Coyote		0	0	1	0	0	1	0	0
Fisher		0	0	0	0	0	1	1	1
Total		80	80	297	70	58	177	59	37

¹ Some of the road kill areas varied among years. ² Survey dates were: 27 June 2005–21 September 2005; 5 June 2006–17 August 2006; 25 June 2007–27 July 2007. ³ Road kill samples detected that were unsuitable to be submitted to the rabies laboratory were not recorded in 2005 only; all samples submitted in 2005 were tested for rabies by direct fluorescent antibody (dFA).

In 2006 and 2007, 297 and 177 total road kills were detected, and 70 (24%) and 59 (33%) were deemed suitable to be submitted, with 58 (83%) and 37 (63%) tested, respectively. Raccoons accounted for most of the detected road kills in 2006 and 2007 at 196 (66%) and 124 (70%), respectively; 59 (30%) and 45 (36%) of detected raccoons were submitted for testing, with 50 (85%) and 28 (62%) of submitted raccoons tested, respectively. In 2005, road kills detected and deemed not suitable for testing were not recorded, but all 80 samples submitted were tested for rabies.

The raccoon was the only species that occurred in all weekly samples from 2005 to 2007 (Figure 5a–c); the striped skunk occurred in an average of 73% of weekly samples during 2005–2007. Skunk representation among road kills was not different ($p = 0.03$) among the survey years relative to raccoons and averaged 19% of road kill raccoon submitted for testing annually.

On average, 19% of survey days resulted in collection of no road kills to be submitted for testing, ranging from 14% ($n = 3/22$) in 2007 to 22% ($n = 9/41$) in 2005 and 2006. No road-killed raccoons were collected for submission an average of 27% of survey days, ranging from 23% ($n = 5/22$) in 2007 to 32% ($n = 13/41$) in 2005. No road-killed skunks were collected for submission an average of 74% survey days, ranging from 68% ($n = 15/22$) in 2007 to 81% ($n = 33/41$) in 2006 (Figure 6a–c).

On average, 35% of survey days resulted in collection of one road kill that was submitted for testing, ranging from 32% in 2005 and 2006 to 41% in 2007. One road-killed raccoon was collected and submitted for testing an average of 36% of survey days, ranging from 34% ($n = 14/41$) in both 2005 and 2006 to 41% ($n = 9/22$) in 2007. One road-killed skunk was collected and submitted an average 22% of survey days, ranging from 15% ($n = 6/41$) in 2006 to 27% ($n = 6/22$) in 2007. Seven total road kills were collected for submission on single days in 2005 and 2007, which also included either red foxes or a fisher. The highest number of raccoons collected/day to be submitted for testing was six, which occurred on two days in 2005 (Figure 6a).

The mean daily km driven/raccoon collected for submission from 2005 to 2007 was 381 ± 28 (SD) for raccoons, 2099 ± 845 (SD) for skunks, and 303 ± 37 (SD) for total road kills. On the average, there was greater than a 5-fold increase in driving distance to collect a skunk to be submitted for rabies testing in comparison to a raccoon. A mean of 1.5 (range 0–6) raccoons was collected/day in all years; road-killed skunks collected/day averaged 0.4, 0.2, and 0.6 (range 0–2) in 2005, 2006, and 2007, respectively.

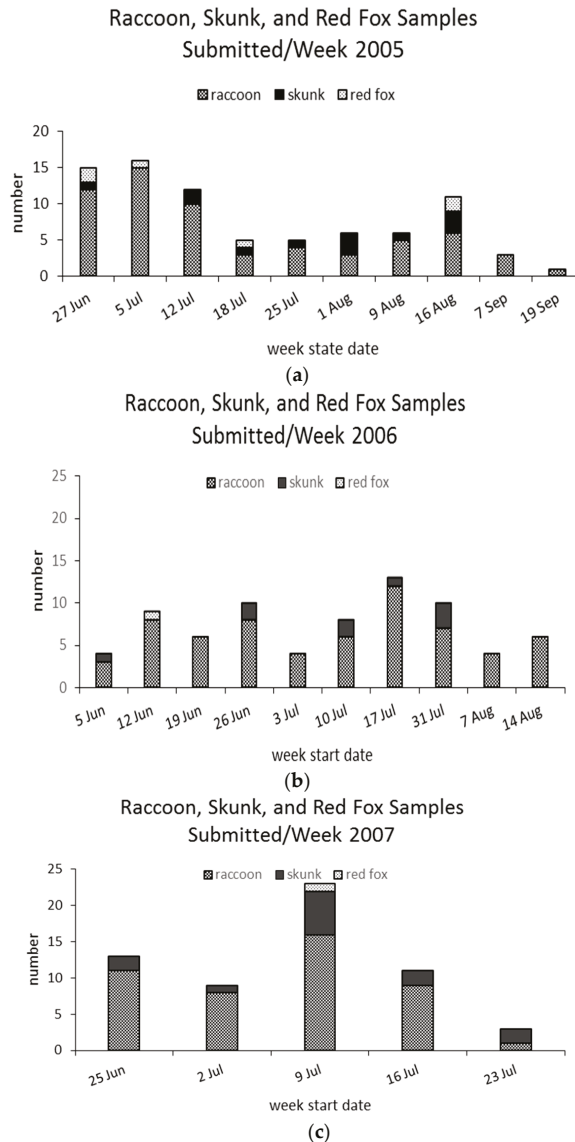


Figure 5. Number of raccoons, striped skunks, and red foxes submitted weekly for potential rabies diagnostics to the New York State Rabies Laboratory at Wadsworth Center, Albany, New York from road kill surveys in (a) 2005, (b) 2006 and (c) 2007.

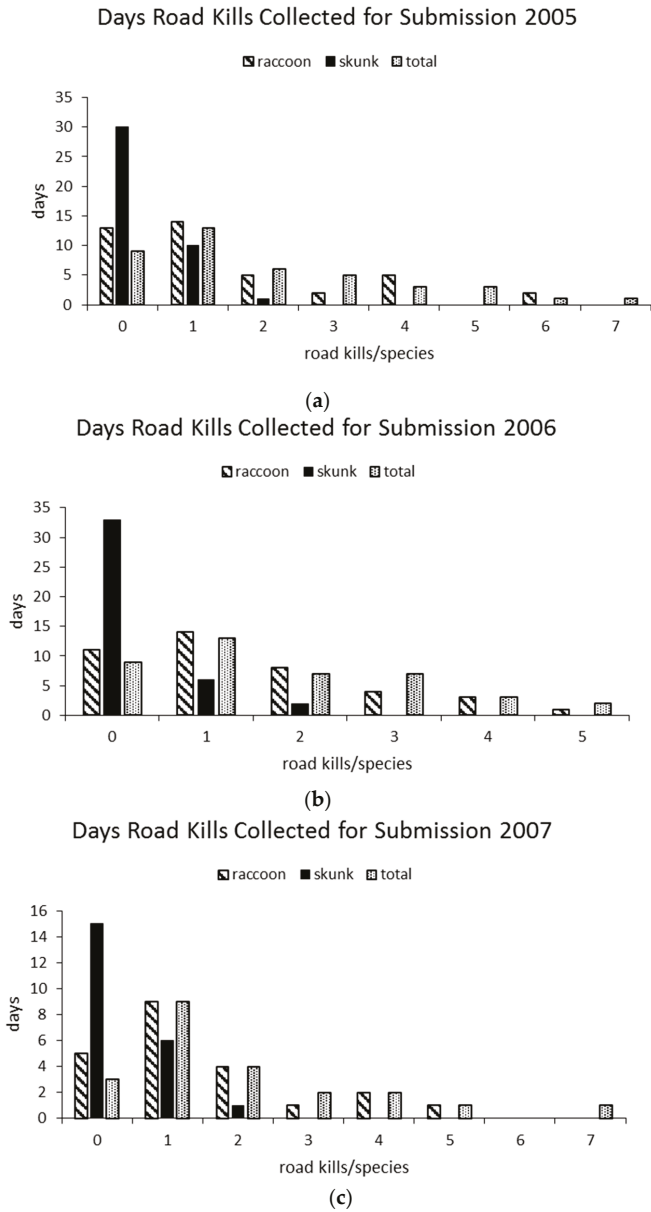


Figure 6. Frequency of days where raccoon, skunk, and total road kills collected for submission ranged from 0 to 7 (total may include red or gray fox, coyote, or fisher) in (a) 2005, (b) 2006 and (c) 2007.

Simple linear regression revealed a significant ($p < 0.02$) positive relationship between weekly km driven and road kills collected in 2005 only. Weekly km driven accounted for about half of the variation ($r^2 = 0.52$) in the dependent response weekly road kills that were collected and submitted for testing.

Weekly surveys resulted in increasing samples of juvenile raccoons during July, when young often begin traveling more extensively in family groups at north temperate latitudes [17] (Figure 7a–c). There was no difference ($p = 0.82$) in sex and relative age among road-killed raccoons within and across survey years. While there were more adult females and juveniles collected and tested in all survey years, there was not a significant association ($p = 0.48$) when juveniles were pooled with adult females in comparison to adult males.

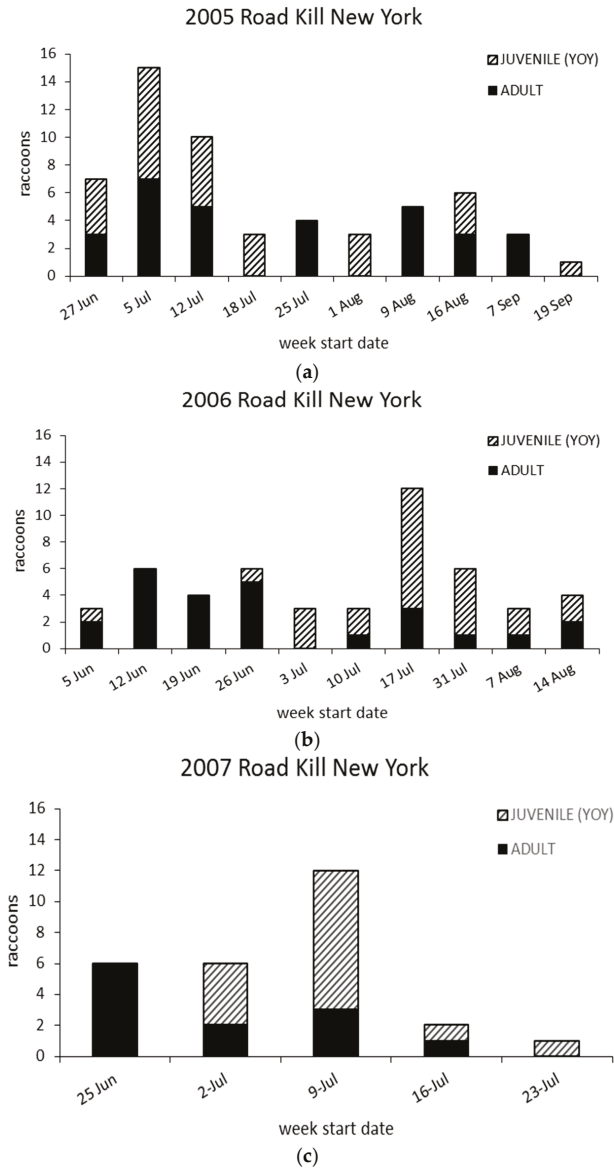


Figure 7. Adult and juvenile (young of the year) raccoons collected deemed suitable for submission for rabies testing (a) 2005, (b) 2006 and (c) 2007.

Road kill surveys required a commitment of two trained wildlife staff for 25 weeks over the survey period. Cumulative mean weekly driving distances ranged from 2372 km in 2005 to 3169 km in 2007, requiring a mean range of 35 to 50 h driving and sample preparation time. Annual variation in survey routes and adaptive changes resulted in more kilometers (km) driven over five days in 2007 than 10 days in 2005. The survey area was larger in 2005 and 2007 than 2006, but the 2005 survey area was split in half and each half only driven two days/week.

Costs associated with road kill surveys from 2005 to 2007 in 2016 USD in decreasing order of importance were: salaries and benefits (\$22,778; 61%), fuel (\$8142; 22%), vehicle depreciation (\$5274; 14%), and sample shipping (\$924; 3%) (Table 2). The cost/road kill collected to be submitted for testing was: \$176/all species, \$224/raccoon, and \$1160/skunk. While we did not estimate rabies diagnostic and associated laboratory costs for testing road kills, the NYSDOH Rabies Laboratory approximates the cost for rabies testing/specimen at \$150.00 in 2016. This cost does not include equipment depreciation, training, safety measures, administrative burden, database management, and other related costs that would be included in a comprehensive laboratory cost analysis.

Table 2. Estimated annual cost of formal road kill surveys in Northern New York State in 2005–2007 (all values are shown in 2016 USD).

Year	Salary and Benefits ¹	Fuel ²	Vehicle Depreciation ³	Sample Shipping ⁴	Totals
2005	8306	3104	1812	389	13,611
2006	8322	3043	1755	351	13,471
2007	6150	1995	1707	184	10,036
Totals	22,778	8142	5274	924	37,118

¹ Salary hourly rates for 2016 for the employee grades (GS 5₁, 6₁, 6₃, 7₁, and 7₂) for two personnel who conducted almost all of the road kill surveys and prepared samples for overnight pick-up and shipping, plus a mean benefit rate of 0.35 [14]. ² Includes five month late spring-early fall mean for the price of a US gallon of gasoline plus taxes based on 2006 (\$302.40) and 2007 (\$304.80) pump rates at an estimated 7.2 km/L (17 mi/gal). [15]. The 2006 rate was also applied for 2005, which was not readily available. ³ A simple linear annual rate based on Blue Book value for the vehicle with 150,000 mi “life expectancy” in 2016. ⁴ Based on \$14.50/shipment from Potsdam, NY to the NYSDOH Rabies Laboratory, Wadsworth Center, Slingerlands, NY, where total heads submitted/2.5 shipments ≥ 2 then an estimated 2.5 shipments occurred/week; otherwise 1 or no shipments occurred/week.

4. Discussion

The stated goals of surveillance include: (1) detection of the potential emergence of a disease as early as possible; (2) characterization of the spatial-temporal distribution of a disease once it is detected; (3) determination of factors that contributed to its emergence and distribution; and (4) determining whether the disease has disappeared or been controlled or eliminated through intervention [1].

Rabies surveillance in the U.S. has been and continues to be predominantly based on human and domestic animal exposures as a priority to ensure that public health is protected against this invariably fatal disease once clinical signs manifest. With the integration of ORV into rabies control in wild meso-carnivores, there is a management need for a more comprehensive near real-time view of the spatial-temporal distribution of specific rabies virus variants. Road kills as well as other sources of samples (e.g., strange acting animals not involved in human or domestic animal exposures, animals found dead in addition to road kills) not traditionally tested for rabies provide greater spatial-temporal surveillance scope and intensity than public health exposure-based samples alone for improved ORV decision making [4]. None of the 175 wild meso-carnivores tested from road kill surveys during 2005–2007 was rabid by dFA. During the same period, no meso-carnivores were tested or reported rabid by the NYSDOH Rabies Laboratory within the road kill survey zone; a single rabid raccoon was reported by NYSDOH near the edge of the road kill zone in 2005, northeast of Watertown, NY, USA (Figure 1). That no rabid terrestrial mammals were reported during the road kill survey period may have reflected suppression or elimination of raccoon rabies at the multiple county level, which may not be unexpected given that ORV intervention with RABORAL V-RG[®] baits (Merial Ltd., Athens, GA, USA) had been annually in place in portions of northern Jefferson and southern Clinton counties since

1995; portions of St. Lawrence and Franklin Counties were added in 1997 and 1998, respectively, along with a portion of northern Clinton County in 2007.

However, reemergence of raccoon rabies within northernmost Jefferson and portions of St. Lawrence counties in 2008 (Figure 8), where ORV continued annually but where road kill surveillance ceased as a complement to public health surveillance points to two potential issues, independently or in combination.

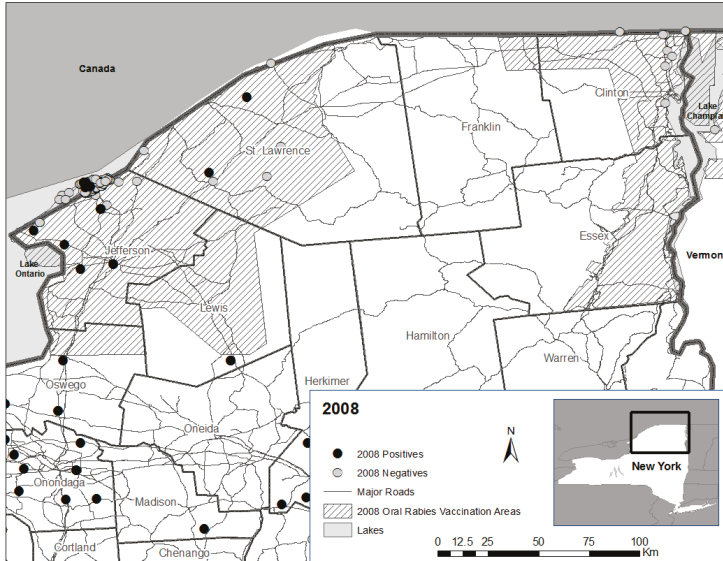


Figure 8. Distribution of raccoon rabies cases in 2008 in northern New York within and outside of long standing oral rabies vaccination (ORV) zones.

(1) It is not known if raccoon rabies had been transiently eliminated as suggested by enhanced surveillance through road kills in combination with public health surveillance, or if enhanced surveillance in combination with public health surveillance was insufficient to detect raccoon rabies presence that may have been suppressed by ORV or other factors; (2) Also, it is not known if vaccine-baits or implemented strategies (or their interaction), given generally low rabies virus neutralizing antibody seroprevalence observed in raccoon populations post-ORV [5], were adequate to contribute to lasting raccoon rabies suppression or perhaps elimination at this county scale given the perpetuation of rabies cases within the contiguous ORV zone that extended south beyond road kill survey areas in Jefferson County (Figures 1–3 and Figure 5).

The ultimate goal of the NRMP is elimination of specific terrestrial rabies variants, including raccoon rabies [18]. The detection of rabies within this specific survey area beginning in 2008 does not alter this goal or negate the value of road kills as a form of enhanced rabies surveillance or ORV as a means of controlling and eliminating specific rabies variants. Rather, this outcome underscores the need to continually seek improvements in enhanced rabies surveillance approaches by emphasizing detection of strange behaving raccoons and other meso-carnivores not involved in human or domestic animal exposures to facilitate improved ORV decisions to achieve national wildlife rabies management goals of control and elimination [19].

Road kill collection was not inexpensive at \$176/road kill and \$224/road-killed raccoon over the three-year survey period. However, we are not aware of systematic economic analysis for rabies

surveillance in the U.S. to formally compare the relative cost of road kill samples to other rabies surveillance sample categories (e.g., suspect strange behaving raccoons).

Opportunistic road kill sampling, in lieu of more structured road kill surveys, represents an alternate if areas of interest can be adequately sampled through routine travel to and from the office and field work sites by experienced personnel over regular routes. Such sampling may better integrate into surveillance budgets without compromising area coverage and effort. Also, eliciting cooperation from personnel within transportation departments who may have the responsibility of removing road kills should be explored [20], so long as protective measures are taken to ensure against rabies exposure from carcass collection. In some instances, state wildlife conservation officers or game wardens may be an option for road kill sampling, given the extensive amount of travel required to accomplish their job duties.

This study served in part as a basis for future enhanced rabies surveillance planning. While it accomplished that goal, study limitations of note included the lack of consistency across years in road kill survey routes and effort due to changing staffing needs for rabies control in New York and the eastern U.S. Also, we did not attempt to predict road kill frequency based on estimated raccoon population density indices, traffic volume, or road type (i.e., primary, secondary, tertiary). The roads driven in these surveys were largely state routes and some county connecting roads, with small segments of Interstates 81 and 87 in 2005 and 2007, respectively (Figure 5a,c). For context, estimated daily traffic volume (number of vehicles) for select roads within the St. Lawrence County portion of road kill surveys in 2006 was: 4900 for the end of Route 812 at Route 11; 620 at the end of Route 58 at Route 37; 2530 from Route 37 west of the City of Ogdensburg; and, 11,770 at the start of Route 11 in Canton [21]. Raccoon population density indices were available only from 1998 to 2000 from three sites in St. Lawrence County that ranged from 2 to 8 animals/km² (USDA unpublished data). Given these examples of variation in estimated traffic volume and raccoon densities, their potential value in determining expected sample size/effort for future road kill surveys should be explored. We also did not record animals detected and discarded that were deemed unsuitable to be submitted for rabies testing in 2005. That additional year would have better characterized variation in the proportion of road-killed animals submitted for testing relative to the total detected. The differential between road kills detected and collected serves as a measure of efficiency and is particularly informative for road kills detected on day one that may be unsuitable because they were killed during the weekend and were cleared from the highway by WS on Monday because of extended exposure to road traffic, heat, scavenging, or other factors. At more southern latitudes where road kills quickly degrade in high temperatures, the ratio of animals submitted:detected may be useful to delineate times when road kill surveys may not be a wise commitment of resources. Finally, there is a strong suspicion that the abnormal behaviors commonly associated with clinically rabid raccoons and other animals increase their likelihood of being killed on highways above that observed for healthy subjects. However, to test this hypothesis would require additional well-designed field studies.

In 2005, 2006, and 2007, during a raccoon rabies epizootic in northeast Ohio, road kills ranked third among five enhanced rabies surveillance categories for detection of rabies (2/399, 0/159, 1/481). Strange behaving raccoons not involved in human or domestic animal exposures had the highest probability of rabies detection (17/578, 5/263, 15/191), followed by animals found dead not as road kills (2/142, 1/97, 3/106). NWCO-captured, apparently healthy animals ranked last at (0/1680, 0/17, 0/12) for the three years (no rabies positives in 2005 and direct rapid immunohistochemistry test (dRIT) WS laboratory burden led to reduced testing of NWCO derived samples in 2006 and 2007), with WS trapped and tested animals from within or near the epizootic ranking number four (1/738, 0/296, 0/90) [22]. While all of these enhanced rabies surveillance categories may be valuable during rabies emergence requiring ORV intervention, road-killed raccoons and other meso-carnivores represents a dependable source of samples that appears to add value to enhanced rabies surveillance in combination with strange acting animals and animals found dead. Collectively, enhanced, and

public health rabies surveillance provides a more complete spatial-temporal view of rabies distribution on which to make the most informed ORV decisions for controlling rabies in wildlife.

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Abbreviations

APHIS	Animal and Plant Health Inspection Service
dFA	direct fluorescent antibody
NRMP	National Rabies Management Program
NYSDOH	New York State Department of Health
NWCO	Nuisance Wildlife Control Operator
ORV	oral rabies vaccination
USDA	U.S. Department of Agriculture
WS	Wildlife Services

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Article

Enhanced Rabies Surveillance to Support Effective Oral Rabies Vaccination of Raccoons in the Eastern United States

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Abstract: Enhanced rabies surveillance (ERS) is essential for sound oral rabies vaccination (ORV) decisions to prevent the spread of specific rabies virus variants in meso-carnivores and to achieve disease elimination. Use of a direct rapid immunohistochemistry test (dRIT) in North America for timely, accurate rabies diagnosis in the field has facilitated greater ERS emphasis since 2005. ERS used in tandem with exposure-based public health surveillance provides a comprehensive understanding of the geographic distribution of rabies as an aid to formulate effective management strategies for raccoons and other meso-carnivores. In 2015, best management practices were implemented for improving, reinvigorating, and standardizing ERS. A point system for weighing ERS sample categories was evaluated, to determine whether sampling emphasis should be focused upon ill or strange-acting animals, the highest quality category. During 2016, 70.7% of rabid animals detected through ERS in raccoon rabies management states were obtained from strange-acting animals, followed by animals found dead (14.1%), road kills (9.1%), and nuisance-collected specimens (6.1%). Sample category weights may be adjusted based on additional evaluation to ensure continued emphasis on the highest value samples. High quality ERS, in conjunction with serologic evidence of population-based immunity, form the backbone for ORV decisions in the elimination of raccoon rabies.

Keywords: enhanced rabies surveillance; oral rabies vaccination; rabies elimination; raccoon; wildlife; zoonosis

1. Introduction

Surveillance is the cornerstone in providing actionable data for effective wildlife disease management. Knowing specifically when and where disease occurs is vital to formulate prevention, control and elimination strategies. The World Organisation for Animal Health (OIE) has endorsed prevention of rabies in source populations [1], which includes terrestrial meso-carnivore species in North America such as raccoons (*Procyon lotor*) and striped skunks (*Mephitis mephitis*). Oral rabies vaccination (ORV) has enabled a shift in rabies management focus to the source of disease in wildlife reservoirs. The use of ORV has proven successful in eliminating rabies in European foxes [2], and multiple terrestrial reservoirs in Canada [3–5]. In the U.S., considerable emphasis has been focused on

preventing the spread of the raccoon rabies virus variant in the eastern part of the country by creating vaccination barriers ahead of the disease front [6].

To achieve the goal of controlling and eliminating rabies in terrestrial wildlife, ORV is indispensable. Yet, without near-real time surveillance to delineate where ORV should be applied, its effectiveness at a landscape scale is doubtful. Diverse meso-carnivore hosts for many of the documented rabies virus variants in the U.S. further underscore the value of prompt rabies virus detection, variant typing and timely, accurate mapping of cases by virus variant and species.

In the U.S., rabies surveillance continues to be largely exposure-based in relation to public health [7]. Public health surveillance, often used interchangeably with passive surveillance, implies rabies detection without an active effort targeting a specific area or species to search for the disease [8]. While this system has been, and continues to be, effective in protecting human and animal health, exposure-based surveillance often may not adequately characterize the spatial-temporal distribution of rabies in wild meso-carnivores in real-time for effective intervention. Public health surveillance tends to be biased by human population density, and often discounts potentially rabid animals if no human or domestic animal exposure has occurred [6].

In recognition of this limitation in relation to ORV, a paradigm shift took place in the U.S. during 2004, where enhanced rabies surveillance (ERS) was initiated to complement public health surveillance to provide a more comprehensive picture of rabies distribution by virus variant [6,9]. ERS is planned targeted surveillance that emphasizes a special effort to meet specific management needs. ERS is frequently used interchangeably with active rabies surveillance, is targeted, preferably based on risk factors, and is typically initiated with a designed sampling scheme to meet specific disease management needs [8]. This shift was facilitated largely through the application of a field-efficient, direct immunohistochemical test (dRIT) [10,11] by trained wildlife biologists in collaboration with the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia, GA, USA); the World Health Organization (WHO) Collaborating Centre, Wistar Institute (Philadelphia, Pennsylvania, PA, USA); and state health departments.

The United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Wildlife Services (WS), National Rabies Management Program (NRMP; hereafter WS) conducts ERS in support of national rabies management goals that are focused on preventing the spread of, and ultimately eliminating specific rabies virus variants in meso-carnivores. The range of ERS samples collected includes the following categories: suspect, ill or strange-acting animals without a rabies virus exposure record (hereafter, strange-acting), animals found dead and not associated with highway mortality, road kills; animals captured from focal areas where rabies cases have occurred recently (hereafter, surveillance-trapped), and apparently healthy animals collected through nuisance wildlife control operators (NWCO) or submitted by homeowners [6].

The value of ERS to raccoon rabies management was illustrated in highly urban-suburban northeastern Ohio during 2007. Public health surveillance rabies cases had been declining from 2004–2006 in an area where emergency ORV and trap-vaccinate-release rabies management strategies were ongoing, as part of a contingency response to an epizootic that began in 2004 [6,9]. All rabid animals were detected through ERS during 2007, highlighting its importance as a complement to public health surveillance to make informed rabies control decisions [9].

With a programmatic transition toward the elimination of raccoon rabies in the eastern U.S., the absence of cases will serve as the key metric to measure success in tandem with rabies virus neutralizing antibody (rVNA) sero-prevalence as an index to vaccine-induced herd immunity. These metrics form the basis to make decisions to move ORV zones farther into raccoon rabies enzootic areas. During 2015, a new U.S. program initiative was developed to standardize best management practices (BMPs) and reinvigorate ERS. The program was implemented in four pilot states to expand the diversity of surveillance collaborators and to enhance detection of strange-acting animals, the highest valued ERS sample category [9]. The program was expanded during 2016 to 16 rabies management states that implemented some components of the new ERS initiative with full ERS implementation

by these states in 2017. This revitalized ERS system includes well-defined categories to track sources of samples combined with a stratified point value system to weigh the quality of samples collected and tested. The point system and a quarterly point threshold were established to provide incentive to collect the highest quality ERS samples for detection of rabid animals. This system also provides a platform for evaluating and adjusting ERS performance in participating states, and serves as a means for making adjustments in surveillance sampling emphasis over time.

The objective of this paper is to summarize the first year of categorized ERS data from 16 eastern states seasonally and annually. In addition, we categorized ERS retrospective data from the four pilot states during 2015, for comparison to 2016, within the context of the value of ERS data for planning and implementing raccoon rabies elimination.

2. Materials and Methods

During 2015, WS developed BMPs for ERS to integrate four primary components into an improved, adaptive approach to surveillance: (1) an established diverse, cooperative surveillance networking matrix; (2) an algorithm for strange-acting animals, sample prioritization, and freezer support for temporary sample storage; (3) rabies laboratory support; and (4) data entry, monitoring and analyses.

Alabama, Maine, Ohio and West Virginia were selected as pilot states to lead implementation of the ERS initiative in 2015, with a focus on establishing a diverse, cooperative surveillance networking matrix. Potential network links were represented by federal, state, county, and municipal agencies, special interest groups, the private sector, national organizations having regional and state affiliates, and international collaborators (Table 1). A recommended minimum number of contacts for each cooperator level was established to ensure diversity of cooperators within the network. Pilot states were selected, in part, on the range of their strategic rabies management history and current value to national ORV goals. In addition, these states were selected because their existing ERS strategies were not standardized prior to 2015. The purpose of establishing and maintaining a diverse, cooperator-based network was to increase the likelihood of collecting high quality samples from suspect animals that displayed behaviors suggestive of rabies.

Table 1. Examples of expanded enhanced rabies surveillance cooperative network contacts.

Cooperator Level	Cooperator	Recommended Minimum Contacts
Federal	United States Department of Agriculture (USDA) Forest Service, USDA Veterinary Services, U.S. Fish and Wildlife Service, U.S. Department of Energy, U.S. Department of Defense, Tribal Nations	2
State	Health, Agriculture, Transportation, Wildlife/Natural Resources, Parks/Recreation, Police	3
County	Animal Control, Health, Police, Municipal Waste, Transportation, Agriculture Extension, Parks/Recreation	4
City/Town/Hamlet	Local Police, Fire Department, Community Clubs, Homeowners Associations,	3
Special Interest	Nuisance wildlife control operators (NWCO), Hunting/Trapping, Wildlife Rehabilitator, Veterinarians, Hiking/Backpacking Clubs	3
Other	4-H, U.S. Animal Health Association, Zoos, Wildlife and Public Health Professional Societies	No minimum but highly encouraged

Historically, ERS emphasized areas within and on the leading edge of ORV zones, where the risk of rabies spread beyond existing barriers was assumed to be high [12]. During 2015, the ERS area was formally designated to include the western half (20 km) of ORV zones, and the area extending 80 km west from the edge of current ORV zones along the Appalachian Ridge mountains, a 100 km-wide

priority ERS focus area (Figure 1). In states bordering Canada (Maine, ME, New Hampshire, NH, New York, NY, and Vermont, VT, USA), the highest priority areas were identified as the existing ORV zones, which represented a ≥ 40 km wide vaccination zone along the USA-Canadian border, and 80 km south of the existing ORV zones. Other critical areas were also defined, including peninsular Cape Cod, Massachusetts, and potential risk corridors with a perceived increased likelihood of rabies spread in Alabama, Kentucky, Louisiana, Michigan, and Mississippi.

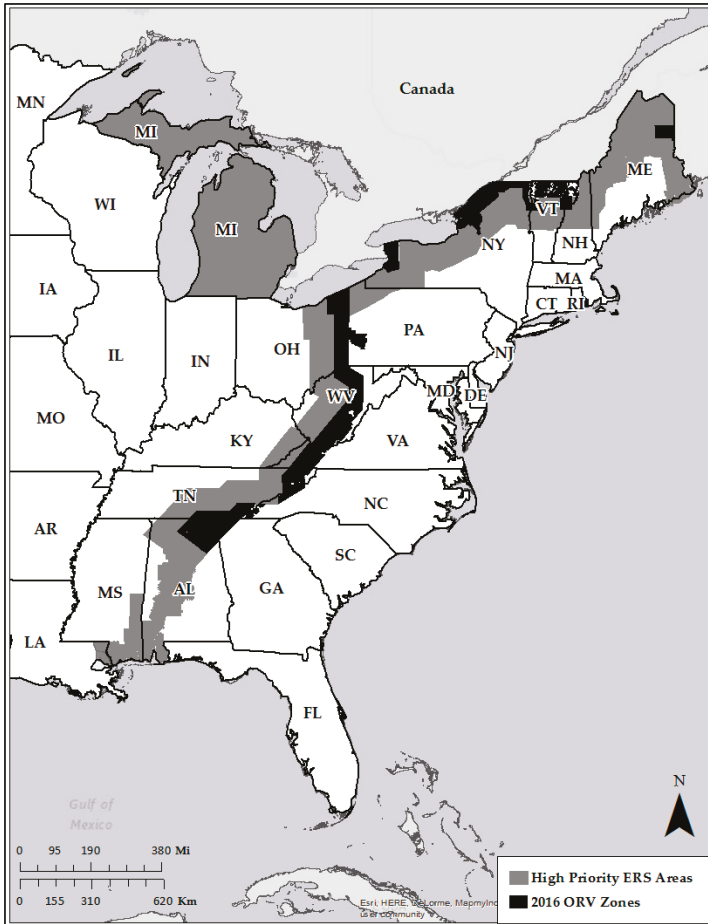


Figure 1. Highest priority enhanced rabies surveillance areas that extended 80 km west (Appalachian Ridge) or generally south (northeastern states) of current oral rabies vaccination (ORV) zones in the eastern U.S. and other critical risk areas.

We defined 6 standardized ERS sample categories within a stratified point system to value samples according to a preliminary expectation of meso-carnivore specimens that might test positive for rabies antigen (Table 2) [9]. Initial point values were devised, based on an interpretative review of ERS category data from Ohio during 2005–2007, which illustrated that strange-acting animals had a higher likelihood of being rabid compared to all other ERS categories by 6–35 times [9]. Thus the point value assigned to this category was three-fold greater than the next most valuable category, and 15 times greater than the least valuable categories. In lieu of mandating sample sizes for ERS, we initially

established a minimum threshold value of 100 points/quarter beginning in January 2016. This initial baseline threshold was designed to provide incentives for the collection of high quality surveillance samples evenly through time, as well as a guide to ensure a minimum consistent effort. It was not designed to account for seasonal spikes in rabies incidence; rather, it was designed to refocus ERS efforts on more consistent sample collection throughout the entire year.

Table 2. Six standardized sample categories and associated stratified point values (i.e., weights) for enhanced rabies surveillance.

Category	Point Value	Description
Strange-acting	15	Suspect behavior suggestive of neurological disease
Found dead	5	Unexplained with no obvious signs of trauma; not road kill
Road kill	3	Formal survey or opportunistically; 1 additional point/mile driven
Surveillance-trapped	2	Active trapping in specified raccoon rabies risk areas/response to an outbreak
NWCO/other	1	Nuisance-trapped or homeowner-derived; apparently healthy
Unknown	1	Behavior not observed; fate not determined

We evaluated the stratified point system by assigning one of the six defined sample categories to all ERS samples collected by WS in 16 raccoon rabies management states, 1 January 2016–19 March 2017 (Figure 1). We evaluated data by calendar year (1 January–31 December 2016) and by calendar season (20 March 2016–19 March 2017). Calendar seasons were defined as follows: spring 2016 = 20 March–19 June 2016, summer 2016 = 20 June–21 September 2016, fall 2016 = 22 September–20 December 2016, and winter 2017 = 21 December 2016–19 March 2017. For each sample acquired through ERS, the minimum data collected included: date of sample collection, species, location (county-level or finer spatial scale), and behavior (if observed).

We used a univariate regression analysis to evaluate the relationship between rabid animal detection and each of the six sample categories for 16 states in calendar year 2016. To compare the categorized data in the four pilot states (Alabama, AL; Maine, ME; Ohio, OH; West Virginia, WV; USA) that implemented this surveillance system during calendar year 2015, we retrospectively assigned sample categories to all ERS samples collected in the four pilot states from 1 January–31 December 2015.

An algorithm was established to define the procedure for response to telephone calls received by cooperators regarding strange-acting animals. The algorithm provides a call-tree system to triage incoming calls from the general public about potentially rabid wildlife. Call algorithms were adapted in each state, dependent on cooperative entities within county or municipal areas responsible for responding to calls from the public. For example, a county health department that fielded a call about a suspect animal with no exposure might refer the caller to their respective game warden, whom in turn would dispatch the animal and submit it to WS.

A network of freezers was established in states or expanded in several states (e.g., Ohio, OH and Tennessee, TN, USA) that had previously distributed freezers in strategic locations for sample submission and temporary sample storage prior to rabies testing. Freezer locations included county health departments, municipal animal control offices, and department of transportation facilities. The BMPs recommendation was for freezer samples to be collected a minimum of twice monthly and subsequently tested within a week when practical, with an expectation that high priority samples would be tested within 48 hours of collection.

Laboratory support outlined through the program initiative included state-level reaffirmation of cooperative relationships with state rabies diagnostic facilities. At the national programmatic level, WS engaged with the CDC, The Wistar Institute and the New York State Department of Health (NYSDOH) to ensure continued diagnostic support for confirmation of WS-tested dRIT samples using the gold standard direct fluorescent antibody (dFA) test, variant-typing for all rabid animal specimens, and availability of non-commercial monoclonal antibodies for the dRIT [6]. All dRIT positive and indeterminate specimens, in addition to 10% of negatives, were confirmed using the dFA test. Once

confirmed and variant-typed, all rabid specimen reports were provided to the appropriate cooperators in the surveillance network, though only raccoon rabies virus variant cases had implications for ORV.

The BMPs also outlined improved mechanisms for data entry, monitoring and analyses, including structured timelines for entering data into the WS Management Information System, quality assurance practices for data management, and guidelines for logging information to track cooperator network contact events to maintain a viable network over time. Improvements to data management also included assigning and recording ERS sample categories for every specimen collected and tested, which were not recorded formally prior to 2016.

3. Results

From 2005–2016, approximately 99,991 ERS samples were collected and tested through WS surveillance in 26 states and Puerto Rico, including 19 eastern states and seven states west of the Mississippi River. Rabid animals detected through ERS comprised 2.1% of all samples ($n = 2107$). Approximately 82.0% of all ERS samples were tested by WS using the dRIT and 72.1% of all ERS positives were detected using this field diagnostic method.

From 1 January–31 December 2016, ERS resulted in collection and testing of 6852 ERS samples from 16 raccoon rabies management states (Table 3), and 99 rabid animals were identified. These ERS samples generated a total of 27,851 ERS points (Table 3), with rabid animals representing 1153 ERS points. The sample assigned the highest point value, strange-acting wildlife, accounted for 18.1% of all samples collected and represented 66.7% of all weighted samples (Table 3). Strange-acting animals accounted for 70.7% of rabid animals detected through ERS, followed by 14.1% found dead, 9.1% road kills, and 6.1% nuisance-collected specimens. No rabid animals were detected in the surveillance-trapped or unknown categories. Rabies-positive samples from strange-acting animals represented 5.7% of all samples collected within this respective category, followed by 6.2% for animals found dead, 0.7% for road kill, none for surveillance-trapped, 0.2% for NWCO/other, and none for the unknown category.

Table 3. Total enhanced rabies surveillance samples collected and tested by Wildlife Services, and total category points assigned, 1 January–31 December 2016.

Sample Category	Point Value	Total Samples (%)	Total Points (%)
1 = Strange-acting	15	1239 (18.1)	18,585 (66.7)
2 = Found dead	5	225 (3.3)	1125 (4.0)
3 = Road kill	3	1370 (20.0)	4110 (14.8)
4 = Surveillance-trapped	2	13 (0.2)	26 (0.1)
5 = NWCO/other	1	3924 (57.3)	3924 (14.1)
6 = Unknown	1	81 (1.2)	81 (0.3)
Totals		6852	27,851

Simple linear regression revealed a highly significant relationship ($p < 0.0000002$) between road kill samples collected and rabid meso-carnivores detected in 2016 ($r^2 = 0.86$). A significant relationship ($p < 0.03$) also occurred between animals found dead (not as road kills) and rabid meso-carnivores when data for Michigan (42 meso-carnivores found dead, with none testing rabies positive) were removed as a potential outlier, although the found dead sample category was not highly predictive ($r^2 = 0.32$). The strange-acting sample category was weakly related to rabid meso-carnivores with New York data removed (63 ill or strange acting, with 19 testing rabid) as a potential outlier ($0.07 < p < 0.05$; $r^2 = 0.23$). We suspect that a high ratio of rabid meso-carnivores were detected in New York in this category because WS may more carefully screen suspect animals submitted to the NYSDOH based on their recommendations for dFA testing rather than conducting dRIT.

Twelve of 16 states achieved the ≥ 100 point minimum quarterly threshold except during the first quarter of 2016, where 10 of 16 states met or exceeded the minimum sample size target. Strange-acting

samples accounted for 56.8–73.7% of threshold points by quarter, followed by road kill (14.3–18.7%), NWCO (8.2–18.9%), and animals found dead other than road kill (1.9–4.8%) (Figure 2).

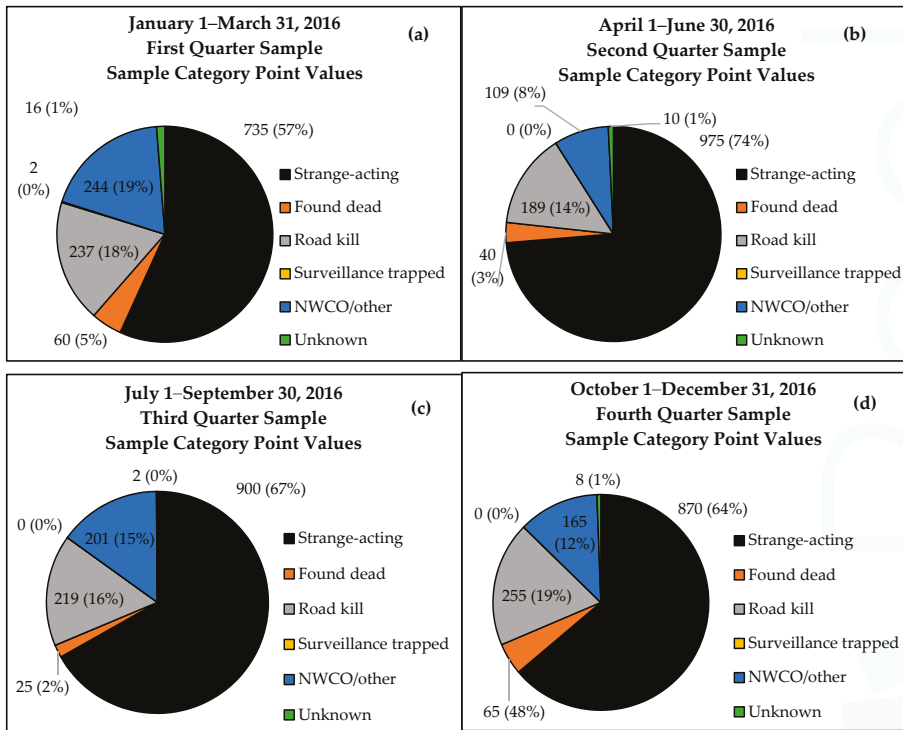


Figure 2. Minimum threshold weighted points and percentages by sample category for 16 states during (a) Quarter 1; (b) Quarter 2; (c) Quarter 3; and (d) Quarter 4 in 2016.

The highest volume of samples were collected during summer 2016 ($n = 2284$), followed by spring 2016 ($n = 2135$), fall 2016 ($n = 1486$), and winter 2017 ($n = 936$). Samples from strange-acting animals were collected most frequently during spring 2016, followed by summer 2016, fall 2016, and winter 2017 (Figure 3a). The greatest proportion of rabid animal samples across all categories was detected during spring 2016 ($n = 37$ of 2135 samples), followed by summer 2016 ($n = 25$ of 2284), winter 2017 ($n = 19$ of 1486), and fall 2016 ($n = 18$ of 936; Figure 3b). The strange-acting category comprised the greatest proportion of rabid animals detected in all seasons except for winter 2017, during which road-killed animals represented the greatest proportion of rabies positives (Figure 3b). During fall 2016, animals found dead, road kills, and NWCO/other collected samples each represented 17% of rabid animal specimens. The NWCO/other positive samples represented a greater proportion of rabid animal samples during winter 2017 than those found dead, surveillance-trapped, and unknown categories. No rabid animals were detected from the surveillance-trapped or unknown categories during any season.

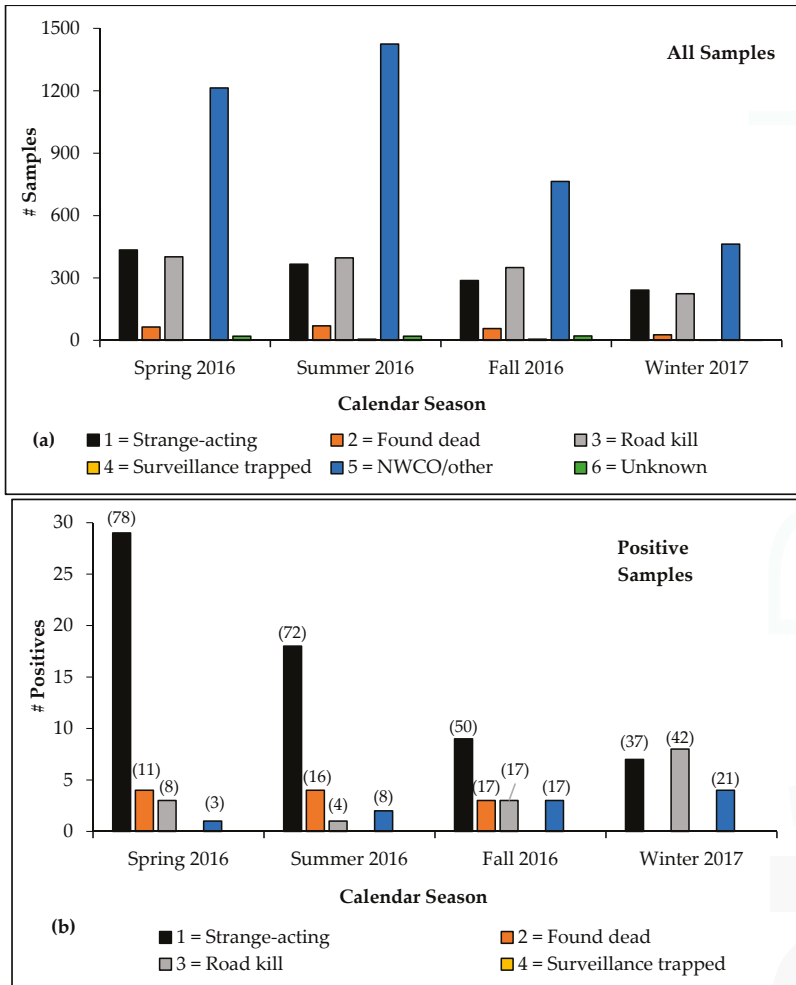


Figure 3. Comparison of enhanced rabies surveillance samples collected by season and sample category by Wildlife Services, spring 2016–winter 2017: (a) All samples and (b) Rabies-positive samples (value represents percent of all rabid animal samples detected according to sample category each season).

Alabama, Maine, Ohio and West Virginia, which implemented the new ERS initiative during 2015, increased ERS sample collection by 29.6% (and category points by 27.7%) following full implementation in 2016. Specimens from strange-acting animals increased in two of four of these pilot states in 2016 compared to 2015, representing a 36.1% increase in sample collection within this category (Figure 4a,b). Ohio collected 33 fewer samples from strange-acting animals during 2016 than 2015, and Maine collected two fewer samples from strange-acting animals during 2016 than 2015. During 2015, rabid animals ($n = 13$) were reported in all pilot states except Alabama; all four pilot states reported rabid animals ($n = 19$) during 2016. Strange-acting animals represented 38.5% and 47.4% of positive specimens during 2015 and 2016, respectively, in the pilot states.

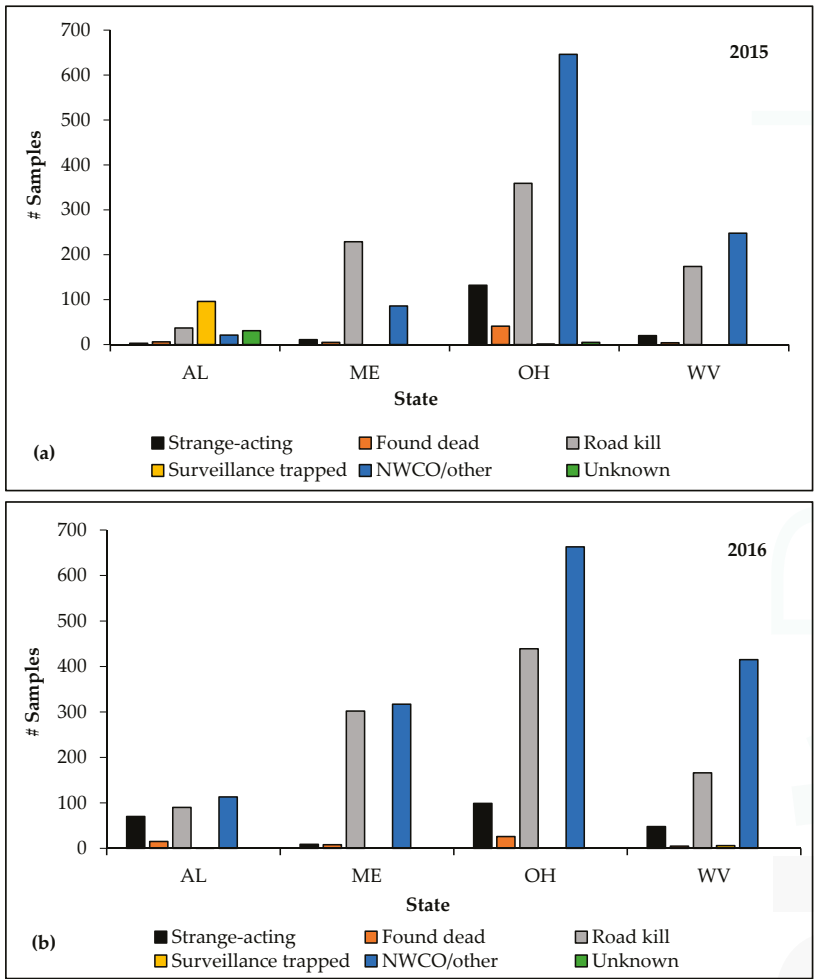


Figure 4. Comparison of enhanced rabies surveillance samples collected in 4 pilot states by Wildlife Services by sample category: (a) 1 January 2015–31 December 2015 and (b) 1 January 2016–31 December 2016. AL = Alabama; ME = Maine; OH = Ohio; WV = West Virginia.

Maine had the greatest increase in ERS samples among the four pilot states in 2016 compared to 2015, at 92.1%. Alabama, West Virginia, and Ohio increased total sample collection by 49.0%, 43.5%, and 3.1%, respectively. Alabama had the largest increase in ERS in total points and collection of the highest quality specimens among the four pilot states in 2016 compared to 2015. In Alabama, category points increased by 251%, from 430 in 2015 to 1510 in 2016 and collection of strange-acting specimens increased by 2233%, from three (2015) to 70 in 2016. Categorical points increased in Maine and West Virginia by 59.4% and 53.2%, respectively, but decreased in Ohio by 8.3%. Maine and West Virginia increased collection of the highest quality specimens by 72.7% and 140.0%, respectively, while Ohio decreased collection of specimens from strange-acting animals by 25.0%.

4. Discussion

An ERS program has been in place in the U.S. since 2004, and has been recognized as an effective means to increase sampling intensity and geographic scope for rabies virus detection when applied in tandem with public health surveillance [9]. A routine annual finding of approximately 2% ($n = 176$ /year) of rabid animals detected directly through ERS from 2005 to 2016 near ORV zones is evidence of the value of ERS as a complement to public health surveillance. These rabid animals would not likely have been detected through exposure-based surveillance, and help to provide a more complete spatial and temporal picture of rabies virus distribution at a landscape level for improved management decision-making. A basic tenet of this surveillance paradigm shift beginning during 2004 was to promote more effective management of rabies at its source in reservoir species by focal detection, rather than mere increased testing of inappropriate or low-value specimens. Through the strategic application of ORV, there has been demonstrated success in preventing appreciable spread of the raccoon rabies virus variant in the eastern U.S. Clearly, ERS serves as a foundational component for managing rabies in wildlife reservoirs given that the absence of cases represents the ultimate measure of ORV success.

Experimental access to an additional oral rabies vaccine, capable of producing higher indices of population immunity based on rVNA sero-prevalence post-ORV, has prompted WS to shift from containment to elimination strategies, underscoring the need to develop a more effective, formalized categorical sampling regime for ERS. Limitations associated with historic ERS implementation strategies were identified in 2015, including non-standardized tactics among state programs. Not all states participating in raccoon rabies management were focused on the collection of high quality samples, and instead collected primarily nuisance or otherwise apparently healthy animals. Furthermore, sampling did not occur uniformly in all ERS emphasis areas within and on the leading edge of ORV zones, potentially resulting in critical spatial and temporal 'surveillance holes' through which rabies could spread without timely detection. One such temporal surveillance gap observed was associated with a limited number of samples collected during winter months, despite programmatic evidence to suggest that meso-carnivores that are strange-acting, found dead, or killed along highways during winter in northern latitudes may have a higher likelihood of being rabid. Implementation of standardized ERS with BMPs that emphasize establishment and maintenance of a diverse cooperator network and that employs a stratified point system for weighing the quality of sample sources, served as the foundation for revitalizing the ERS system.

Prior to 2015, WS coordinated primarily with the state departments of health, agriculture and wildlife on ERS. Through this ERS initiative, a diversity of other sources of suspect samples have been added to the historic suite of state agency collaborators, through strategic in-person meetings, telephone calls, mass mailings, and email blasts. A recommended minimum number of each contact type (e.g., federal, state, city/town, special interest, etc.) was provided as part of the BMPs (Table 1). Sample categories have been considered in ERS by WS since 2004, however, they were not formally defined for evaluation or put into a point system framework until the implementation of this ERS initiative during 2016. A weighted surveillance approach employing a stratified point system has been developed for other wildlife diseases, such as chronic wasting disease [13]. Point systems may improve ERS efficiency by stratifying sample collection according to biologically-relevant categories that may increase the probability of detecting a rabid animal [14]. A primary emphasis for the ERS point system was to create incentive by giving higher weight to higher quality ERS samples that have a greater chance for rabies virus detection. Initial point values were established based on an evaluation of ERS category data from Ohio [9] as well as historical WS ERS data collected over time.

An initial quarterly point minimum threshold of 100 was established, based on historic ERS data trends. The threshold was designed to provide additional motivation to reach the 100 point mark more easily by focusing on the collection of high quality samples, but states were advised that this was only a minimum threshold and once reached, sampling should continue at the same pace. The one-year categorized snapshot of data suggests that the 100 point minimum was exceeded in most participating

state programs through emphasis on higher quality ERS samples. A comparison of the point values for the four pilot states from 2015 to 2016 further highlights that the minimum threshold provided incentive to focus sample collection efforts on high quality specimens. Points within this sample category increased in all pilot states except for Ohio, and collection of strange-acting animals improved overall. These initial values serve as a baseline from which further modifications can be made to refine the responsiveness of this system over time, including raising the minimum point thresholds and developing mechanisms to assist state programs that have difficulty meeting those thresholds.

For the 16 states, samples from animals categorized as strange-acting and found dead had a greater chance of being rabid, similar to the analysis of Ohio ERS data for 2005–2007 [9]. The strange-acting sample category resulted in detection of the greatest number of rabid animals; however, due to considerable variability in the ratio among rabid animals and samples tested by category across states, there was a lower predictive value by simple linear regression than expected. Rabid animal predictability for this category may be confounded because strange-acting behaviors may be the result of other neurological diseases. In the future, we will promote an improved and standardized interpretation among WS staff and cooperators as to what constitutes a strange-acting animal. While there was a significant relationship between the category found dead and rabid meso-carnivores detected, additional data over time will be required to determine the ability to predict rabid meso-carnivores beyond the $r^2 = 0.32$ generated for 2016 with Michigan data removed as a potential outlier.

In Ohio during 2005–2007, all other ERS categories resulted in detection of rabid animals, except for samples provided predominantly through NWCO sources (i.e., healthy animals). While our results were consistent with Ohio findings, NWCO-derived positive cases were detected at a slightly higher rate than expected during 2016. Thus, the value of NWCO-collected or otherwise apparently healthy animals should not be totally discounted as a means to find rabid animals. Use of NWCO-provided samples could be important in helping to define the extent of disease spread in a newly emerging rabies focus, in high-risk spread corridors, such as urban-suburban environments that support larger reservoir populations, or in areas where rabies is enzootic, even though only six of 3926 (0.2%) animals within this category were rabid.

Although rabies-positive samples from road-killed animals represented only <1% of all specimens collected in this category, this source of samples remains important because opportunistic and formal road kill surveys may help to fill spatial and temporal ERS gaps. Moreover, the strong relationship between road-killed samples collected and rabid meso-carnivores detected in 2016 suggests future predictive value from this sample category. Road kills typically occur in proportion to population density of the target species and often increase or decrease seasonally relative to activity patterns [15–17]. In our seasonal analysis, road-killed animals were the most important source of rabies positive samples during winter, a time of year when rabies case detection is generally lower [18]. Future refinements to the ERS initiative will include improved definitions for opportunistic versus formal road kill surveys, and will better delineate a tiered structure for evaluating road types (e.g. county road, state highway, interstate) that should be surveyed at higher rates based on traffic volume and in areas where samples from other ERS categories are difficult to obtain.

No rabid animals were detected from surveillance-trapped animals during 2016 ERS efforts. However, there were no rabies outbreaks identified where focal trapping efforts were warranted. Targeted surveillance trapping has served as a source of rabid animals in other outbreak situations, but it is highly variable, costly and labor intensive [14,19,20]. In the U.S., the rabies detection rate in targeted surveillance-trapped animals has ranged from 0% in New York and Ohio to an unusually high detection rate of 2.2% in Alabama. Only 0.1% of targeted surveillance-trapped animals in Ohio were detected during 2005, with no rabid animals captured during 2006–2007 [9]. Trapping in response to an index or early identified cases may serve as a useful ERS tool to better define an outbreak focus. However, this form of ERS may be most effective for a transient period of a few weeks in close proximity to such cases in new outbreak locations, as observed in Quebec, Canada [14].

Seasonally, rabies was detected most frequently during the spring, followed by summer, which is consistent with reported seasonal peaks in other regions [14,18]. Peaks of rabies activity during spring may result from increased denning and mating contacts in winter [21]. Secondary summer peaks may be related to increased contact during juvenile dispersal [14,18,22]. Although total sample collection was similar in spring and summer, samples declined through the fall and winter months. This observation may be related in part to lapses in ERS due to other rabies management priorities in some states during the late summer-fall months (e.g., ORV baiting and post-ORV monitoring efforts). In some northern latitudes, lower sample size during winter months may also be influenced by reduced animal activity during extreme cold and high snowfall.

To target ERS seasonal activities better, it may be more appropriate to consider biological seasons in future analysis, though pregnancy/parturition, young-rearing, dispersal, and breeding roughly coincide with spring, summer, autumn, and winter, respectively [23]. However, surveillance should not completely cease even at northern latitudes. Strange-acting animals during cold, snowy periods may have a high chance of being rabid, as observed in Franklin County, NY during the winter of 2015, as well as a recent rabies incursion near Hamilton, Ontario, Canada [24]. Latitudinal variation should be considered, as seasonal behavior patterns in raccoons found in more northern latitudes may result in differences in both sample collection and disease detection rates, compared to more moderate winter conditions that occur in the southeastern U.S. [25].

This initial study suggests that qualitative stratification of sampling categories provides a more meaningful way to evaluate ERS. However, we expect that data for the first three consecutive years should provide an improved analytical milestone to determine if this ERS system warrants modifications. The value of a revitalized ERS network has been realized in Ohio, where two strange-acting raccoons with no known exposure history submitted through a cooperator network freezer tested positive for the raccoon rabies virus variant during March–April 2017. This prompted a contingency action response because the cases were >8 km (5 miles) beyond the established ORV zone. An emergency ORV spring baiting occurred to eliminate this focus and prevent raccoon rabies from gaining a stronger foothold in east-central Ohio and beyond. Without an established ERS network, both cases would have gone undetected through traditional public health surveillance until a local epizootic emerged that resulted in human or pet exposures.

Applying point values to public health surveillance data may represent a logical progression to help refine sample category weights, because as expected, exposure-based suspect animals have a greater chance of being rabid based on the Ohio data for 2005–2007. Also, a better evaluation of ERS efforts through space and time may require more refined sample targets using defined spatial resolution and habitat type [14]. Among the pressing surveillance challenges is the need to identify significant risk corridors in relation to ORV and geophysical ‘barriers’ through which rabies has a higher probability of spreading to naïve areas. Existing models have illuminated potential raccoon rabies spread scenarios [26,27] as well as the value of targeted surveillance to formulate control plans, but developing models that possess predictive sensitivity to improve appropriate rabies management for a common, often ubiquitous, ecological generalist such as the raccoon remains a daunting task. Therefore, continuing to develop effective stratified sampling regimes to guard against spatial-temporal surveillance gaps in relation to a suite of variables, including epidemiologic facets, ecological differences, local host abundance, species behavior, rabies virus variant distribution, viral spillover potential, spatial distribution of rabies collaborators on the landscape, and other factors, will remain critical for successful implementation of wildlife management strategies to eliminate rabies.

Supplementary Materials: The following is available online at www.mdpi.com/2414-6366/2/3/34/s1: Figure S1: Call algorithm template for enhanced rabies surveillance.

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Author Contributions: J.D.K. served as corresponding author, prepared, cleaned, and validated the data from the database for analysis, and wrote the manuscript. R.B.C. co-conceived the enhanced rabies surveillance program initiative and reviewed the manuscript. K.M.N. reviewed and proofread the manuscript, and validated data. C.E.R. collaborated on dRIT diagnostics and reviewed the manuscript. T.P.A. conducted statistical analysis and reviewed the manuscript. J.D.B. collaborated on dRIT diagnostics and reviewed the manuscript. D.S. co-conceived the enhanced rabies surveillance program initiative, conducted data analysis, and wrote and reviewed the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Rabies Virus Antibodies from Oral Vaccination as a Correlate of Protection against Lethal Infection in Wildlife

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Abstract: Both cell-mediated and humoral immune effectors are important in combating rabies infection, although the humoral response receives greater attention regarding rabies prevention. The principle of preventive vaccination has been adopted for strategies of oral rabies vaccination (ORV) of wildlife reservoir populations for decades to control circulation of rabies virus in free-ranging hosts. There remains much debate about the levels of rabies antibodies (and the assays to measure them) that confer resistance to rabies virus. In this paper, data from published literature and our own unpublished animal studies on the induction of rabies binding and neutralizing antibodies following oral immunization of animals with live attenuated or recombinant rabies vaccines, are examined as correlates of protection against lethal rabies infection in captive challenge settings. Analysis of our studies suggests that, though serum neutralization test results are expected to reflect *in vivo* protection, the blocking enzyme linked immunosorbent assay (ELISA) result at Day 28 was a better predictor of survival. ELISA kits may have an advantage of greater precision and ability to compare results among different studies and laboratories based on the inherent standardization of the kit format. This paper examines current knowledge and study findings to guide meaningful interpretation of serology results in oral baiting monitoring.

Keywords: rabies; wildlife; vaccination; antibodies; serology

1. Introduction

Rabies is caused by infection with lyssaviruses, which are a group of single-stranded negative-sense RNA viruses in the family Rhabdoviridae. Lyssaviruses principally infect the nervous system (NS) of mammals, and this infection is nearly 100% fatal in both reservoir and incidental hosts after a prolonged incubation period of 1–3 months [1]. It is well recognized that both cell-mediated and humoral immune effectors are important in combating rabies infection [2], although the humoral response receives greater attention with regard to prevention of rabies. Rabies virus neutralizing antibodies comprise an important part of the humoral response and are able to block virus entry into cells, thus preventing or limiting infection, and entry of the virus into the NS. While the administration of post-exposure prophylaxis (PEP) is restricted to humans, preventive prophylaxis is recommended or required for domestic animal hosts in many parts of the world, and the principle of preventive

vaccination has been adopted for strategies of oral rabies vaccination (ORV) of wildlife reservoir populations for decades to control circulation of rabies virus in free-ranging hosts [3,4]. Efficacy of the biologic in protecting against a lethal rabies infection in a significant proportion of vaccinated animals (e.g., >86%) must be demonstrated in a captive setting prior to product licensure [5]. There remains much debate about the levels of rabies antibodies that confer resistance to rabies virus infection and, although antibody levels are one key index of resistance to challenge in animal models [6,7], no single cutoff level of rabies antibody is recognized as being invariably protective [8]. This is due to repeated observations that small fractions of animals presenting detectable levels of antibody prior to challenge can still succumb to rabies infection, and conversely some seronegative animals survive challenge [8,9].

The immunogenicity of the vaccine, measured by induction of rabies binding and/or neutralizing antibodies, generally correlates well with survival to lethal challenge, although there may also be significant yet unmeasured cell-mediated responses involved during infection and replication of the live vaccine virus in the oral mucosa and tonsils. An inverse relationship between the strength of the humoral versus cell-mediated immune responses to inactivated rabies vaccination in humans was documented in one study [10], and it is unclear whether animals demonstrate a similar inverse relationship following infection with live oral rabies vaccines. Along with other host factors, this in part also contributes to the inability of rabies antibodies alone to serve as a perfect correlate of protection against lethal infection. Studies have demonstrated the level of immune response to vaccination is related to an individual's polygenetic major histocompatibility complex (MHC) phenotype [11–13]. Such genetic variation in peptide binding regions may also play a role in the induction of rabies virus antibodies [14]. Depending on the length of the experiment, rabies antibodies can be measured at several time points pre- and post-challenge, and are important for a greater understanding of antibody kinetics post-vaccination and anamnestic response induced following challenge. Despite this, two time periods appear to yield the critical information needed for predicting the survival of most vaccinated animals in efficacy studies: the initial induction or Days 15–30 post-vaccination antibody level, and the level of antibody present immediately prior to rabies virus challenge. Studies need only establish that the animals were naïve prior to vaccination, as ultimately the survival data alone following challenge determine vaccine efficacy. The gold standard in measurement of rabies antibodies has typically involved serum neutralization tests (SNT) to detect rabies virus neutralizing antibodies (rVNA) (i.e., the rapid fluorescent focus inhibition test (RFFIT) or fluorescent antibody virus neutralization test (FAVN)), although enzyme-linked immunosorbent assays (ELISAs) are now also recognized as acceptable tests to detect binding rabies antibodies. Few studies measure both by SNT and ELISA methods but, where they have, a strong but not strict correlation in levels is observed between these two different antibody detection methods [15–18].

Part of ORV post-campaign monitoring is assessing the vaccination coverage by demonstrating an immune response in the target species (population level antibody prevalence); animals are sampled during a pre-determined interval after bait distribution. The application of rabies serology for monitoring the immune response through sampling demands an understanding of the relationship of antibody measured and survival upon challenge. To date, no study has examined whether this sample measured by the prescribed assays with a set cutoff value is actually suitable in terms of predicting protective immunity in wildlife populations (population level immunity). Similarly, reliability of the prescribed assays has not been comprehensively established. The objective of this paper is to quantitatively examine the induction of rabies binding and neutralizing antibodies following oral immunization of animals with live attenuated or recombinant rabies vaccines, as correlates of protection against lethal rabies infection in captive challenge settings by reviewing both published literature and our own unpublished animal studies. Using these data, we challenge the notion of a single antibody cutoff level being used to indicate complete protection against lethal rabies challenge across animal species.

2. Materials and Methods

2.1. Literature Review

Published peer-reviewed journal articles concerning oral rabies vaccination in wildlife that included rabies antibody measurement after oral vaccination and challenge were reviewed in an attempt to identify a protective level per rabies antibody. These were obtained through a search on PubMed with the terms “rabies oral vaccination”, “rabies challenge studies in wildlife”. Of the 42 articles initially identified by description in the abstracts, 6 were removed for lack of serological data leaving 35 articles [19–53]. Six of the articles included studies on more than one species. The number of articles by species included fox (17), skunk (9), raccoon, (7), dog (6), and one each for mongoose and raccoon dog. The articles were reviewed for information about vaccination construct and challenge virus doses and strains, serological assay type, and sampling time periods, as well as correlation of antibody measurement with survival/death outcomes and are summarized in Table 1. In particular, identification of discrepancies and sources of potential errors as well as studies that could be comparable due to consistent study designs were targeted.

2.2. Animal Studies

Empirical data from 28 different animal studies were analyzed (Table 2). All vaccinated animals received vaccine constructs by oral route; either by direct oral instillation or by offering a bait containing a vaccine-loaded blister. Hence, the animal species included in Table 2 are all considered target species for oral vaccination.

2.3. Animals

Animals described in Table 2 were obtained from commercial sources, except for the small Indian mongoose (*Herpestes auropunctatus*). Mongooses were wild-caught on a rabies-free island off the coast of Croatia. All studies were carried out according to prevailing guidelines. All experimental protocols had been reviewed and approved by the German and American Ethics Committees. All studies at IDT were approved by “Landesverwaltungsamt Sachsen—Anhalt, Referat Verbraucherschutz, Veterinär-angelegenheiten”. All studies at Friedrich-Loeffler-Institut (FLI) were approved by “Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern”. Studies at NWRC were approved by NWRC institutional animal care and use committee (IACUC), see Table 2 for approval information (approval board, number, date).

2.4. Vaccine

Five different vaccine constructs were used in the empirical studies. The four rabies virus constructs were modified by site-directed mutagenesis using reverse genetics. The live recombinant virus construct expresses the rabies virus glycoprotein SPBN GAS [54], SPBN GASGAS [55,56], SAD dIND [57], ORA-DPC [58,59], and HAdVRG1.3 [60,61].

Table 1. Animal vaccine efficacy and challenge studies referenced for serology data as part of the literature review. MICLD₅₀: Mouse intracerebral lethal dose 50%. See key for facility below.

Study	Animal Species	Number Vaccinated	Number Controls	Challenge After ... Days	Challenge Dose (MICLD ₅₀ log)	Challenge Virus Strain	Facility Where Research Was Done	Reference
1	arctic fox	6	5	56	5.7	fox	5	30
2	arctic fox	8	4	112	5.7	canine/fox	5	27
3	arctic fox	10	4	56	3.9	fox	5	28
4	arctic fox	6	6	56	5.7	fox	5	29
5	domestic dog	18	6	30	3.8	dog	10	38
6	domestic dog	21	10	109	6.5	dog/Ariana	10	39
7	domestic dog	40	10	180	6.5	dog	11	41
8	domestic dog	12	6	28	7.4	dog	11	51
9	domestic dog	30	12	35	7.4	dog	11	48
10	domestic dog	12	7	35	3.7	unknown	13	52
11	mongoose	5	5	28	5	skunk	11	46
12	raccoon	5	5	30	4.9	raccoon	11	44
13	raccoon	7	7	28	4.9	raccoon	11	45
14	raccoon	14	5	56	4.9	raccoon	11	47
15	raccoon	61	19	442	6.7	raccoon	4	49
16	raccoon	30	10	350	5.9	raccoon	4	24
17	raccoon	10	5	60	*	raccoon	11	49
18	raccoon	6	13	28/65	5.5	dog	12	53
19	raccoon dog	6	9	124	2.8	coyote	10	38
20	red fox	36	6	34	4.7	fox	1	19
21	red fox	84	30	31	4	fox	2	20
22	red fox	66	25	730	4	fox	2	21
23	red fox	27	4	28	3.8	fox	1	22
24	red fox	13	4	28/180/360	3.2	fox	3	23
25	red fox	25	9	547	4.9	fox	4	26
26	red fox	18	6	2490	6.7	fox	4	31
27	red fox	25	0	28	5.7	fox	6	32
28	red fox	27	14	190	5.3	canine/coyote	7	33
	red fox	8	2	45	5.1	canine/coyote	9	37



Table 1. *Cont.*

Study	Animal Species	Number Vaccinated	Number Controls	Challenge After... Days	Challenge Dose (MICLD ₅₀ log)	Challenge Virus Strain	Facility Where Research Was Done	Reference
16	red fox	6	14	97	3	fox	10	38
29	red fox	16	8	107	3.3	fox	8	40
30	red fox	16	8	16	3.2	fox	8	35
28	striped skunk	3	2	45	5.1	canine/coyote	9	37
31	striped skunk	54	10	247	4.9	arctic fox	4	25
29	striped skunk	32	8	90/107	7.8	skunk	8	40
32	striped skunk	6	9	16	5.5	skunk	11	42
33	striped skunk	24	6	116	6.3	skunk	11	43
12	striped skunk	5	5	30	5	skunk	11	44
13	striped skunk	17	6	35	4.2	skunk	11	45
34	striped skunk	26	8	90	6.3	skunk	8	36
35	striped skunk	32	8	90	5.3	skunk	8	34

* Challenge dose given as ≥ 5 LD₅₀. Facility Key: 1—Ministere de l'Agriculture, Centre National d'Etudes sur la Rage et la Pathologie des Animaux Sauvages, Malzeville, France; 2—Connaught Laboratories Limited, Ontario, Canada; 3—Department of Virology, Faculty of Veterinary Medicine, University of Liege, Brussels, Belgium; 4—Ontario Ministry of Natural Resources, Wildlife Research and Development Section, Trent University, DNA Building, Peterborough, Ontario, Canada; 5—Institute of Arctic Biology, University of Alaska, Fairbanks, AK, USA; 6—Laboratoire de Genetique, Dedex, France; 7—Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institute-Federal Research Institute for Animals Health, Germany; 8—Agriculture Canada, Animal Diseases Research Institute, NEPEAN, Ontario, Canada; 9—IDT Biologika GmbH, Am Pharmapark, 06855 Dessau-Kosslau, Germany; 10—AFSSA, National Research Laboratory on Rabies and Wildlife Diseases, WHO Collaborating Centre for Research and Management in Zoonoses Control, OIE Reference Laboratory on Rabies, EU Reference Institute for Rabies Serology, Malzeville, France; 11—Centers for Disease Control and Prevention, Atlanta, GA, USA; 12—Wistar Institute of Anatomy and Biology; 13—Department of Pathology, University of Georgia, Athens, GA, USA.

Table 2. Animal vaccine efficacy and challenge studies referenced for serology data as part of the empirical data analysis.

Study	Animal Species	Vacc.	CtIs.	Challenge after . . . Days	Challenge Dose (MITCD50 log)	Challenge Virus Strain	Facil.	Approval	Date (dd/mm/yy)
1	domestic dog	16	4	56	3.6	dog	IDT	42502-3-710 IDT	2/26/2014
2	mongoose	15	4	56	4.2	dog	IDT	42502-3-693 IDT	24/06/2013
3	mongoose	11	3	42	4	dog	IDT	42502-3-693 IDT	6/24/2013
4	raccoon	5	3	49	4.7	coyote	IDT	42502-3-582 IDT	28/10/2009
5	raccoon	3	-	56	5	coyote	IDT	42502-3-582 IDT	28/10/2009
6	raccoon	20	4	56	4.6	dog	IDT	42502-3-669 IDT	11/23/2012
7	raccoon	-	12	-	4.2-6.2	raccoon	IDT	42502-3-726 IDT	29/07/2014
8	raccoon	11	6	180	6.2	dog(fox)	NWRC	QA2278A	4/21/2014
9	raccoon	23	6	180	5.9	dog(fox)	NWRC	QA2278B	4/21/2014
10	raccoon dog	6	2	56	5	coyote	IDT	42502-3-669 IDT	11/23/2012
11	raccoon dog	30	12	190	0.7	fox	FLI	7221.3-2-007/14	4/1/2014
12	raccoon dog	-	22	-	2.3-3.0	fox	IDT	42502-3-741 IDT	8/17/2015
13	raccoon dog	16	2	28	2.0-2.7	fox	IDT	42502-3-761 IDT	17/08/2015
14	raccoon dog	-	20	-	1.7-2.7	fox	IDT	42502-3-761 IDT	17/08/2015
15	raccoon dog	30	12	183/184	3	fox	FLI	7221.3-2-018/15	17/06/2015
16	red fox	12	3	98	5	coyote	IDT	Unknown *	Unknown *
17	red fox	6	2	62	5	coyote	IDT	42502-3-582 IDT	28/10/2009
18	red fox	6	2	56	5	coyote	IDT	42502-3-669 IDT	11/23/2012
19	red fox	6	4	56	6.7	dog	IDT	42502-3-669 IDT	11/23/2012
20	red fox	9	2	56	4.6	fox	IDT	42502-3-669 IDT	11/23/2012
21	red fox	-	18	-	2.0-4.7	fox	IDT	42502-3-735 IDT	17/08/2015
22	red fox	20	4	50	3	fox	FLI	7221.3-2-005/16	1/28/2016
23	red fox	30	12	190	0.7	fox	FLI	7221.3-2-007/14	4/1/2014
24	striped skunk	8	2	42	5	coyote	IDT	42502-3-582 IDT	28/10/2009
25	striped skunk	5	3	58	4.7	coyote	IDT	42502-3-582 IDT	28/10/2009
26	striped skunk	6	3	56	5	coyote	IDT	42502-3-582 IDT	28/10/2009
27	striped skunk	16	4	28/56	4.6	dog	IDT	42502-3-669 IDT	11/23/2012
28	striped skunk	20	4	335	5.9	dog(fox)	NWRC	QA2278	8/15/2014

* At that time, a different procedure was followed (2007). Facility key: IDT—IDT Biologika GmbH, Germany; FLI—Friedrich Loeffler Institute, Germany; NWRC—National Wildlife Research Centre, Fort Collins, USA.

2.5. Serum Samples

Individual serum samples were used from experimental efficacy and challenge studies involving animals that were captive raised and never had prior vaccination against rabies, or that had been collected from a rabies-free area. In efficacy studies, depending on the experimental design, serum samples were taken at baseline and different time points post-vaccination prior to and post-challenge. For the purpose of this study, only serum samples collected at baseline (Day 0) and post-vaccination on Days 28–30 (referred to later as Day 28) and the day of or immediately prior to challenge virus administration were considered in the analysis. In challenge virus titration studies, blood samples were only taken at baseline prior to infection (Day 0). All sera were stored at $-20\text{ }^{\circ}\text{C}$ and heat inactivated after thawing for 30 min at $56\text{ }^{\circ}\text{C}$ prior to testing. The samples were tested using a RFFIT (SNT) and ELISA assays. Sometimes no absolute value could be determined for the level of rVNA. In this case, and for the purpose of quantitative analyses, the upper limit was halved. For example, values less than 0.5 and 0.13 International Unit per milliliter (IU/mL) were converted to 0.25 and 0.07 IU/mL, respectively.

2.6. Serology

At the FLI, rVNA for each sample was determined by using a modification of the RFFIT (Smith et al., 1973) essentially as described [62] using the standard rabies (RABV) challenge strain, challenge virus standard (CVS-11), as test virus. For titration, a two-fold Ig2 dilution series of each serum sample (1:10 to 1:5120) in Dulbecco minimum essential medium (DMEM) was prepared. Subsequently, 50 μL of the serum dilution was mixed with 50 μL of a constant dilution of the test virus (adjusted to induce about 40 infected cells in a well of a Terasaki plate). After incubation for 90 min at $37\text{ }^{\circ}\text{C}$, 0.1 mL of a 10^6 /mL concentration of baby hamster kidney (BHK)-21 C 13 cells in DMEM containing 10% (wt/v) fetal calf serum was added. Subsequently, 10 μL of the serum-virus-cell suspension was pipetted into wells of a Terasaki plate (Greiner, Germany) and incubated for 24 h in a controlled humidity, CO_2 incubator at $37\text{ }^{\circ}\text{C}$. After fixation with acetone (80%) for 30 min at $4\text{ }^{\circ}\text{C}$, cells were stained with a fluorescein isothiocyanate (FITC) anti-rabies conjugate (Fujirebio Diagnostics, Malvern, PA, USA) for 30–60 min and examined with an inverse immunofluorescence microscope. In each test run, the World Health Organization (WHO) international standard immunoglobulin (2nd human rabies immunoglobulin preparation, National Institute for Biological Standards and Control, Potters Bar, UK) adjusted to 0.5 IU/mL, and a naïve serum, served as a positive and negative control, respectively. The rVNA titer was defined as the dilution of the test serum showing a 50% reduction in the number of infected cells per microscopic field (50% neutralizing dose, ND50) compared to the virus control. The exact titer was calculated using inverse interpolation as described [63] and subsequently converted into concentrations expressed in international units (IU/mL) using the calibrated positive control. As a national and OIE reference laboratory and WHO Collaborating Center for rabies the FLI is certified by the German accreditation body (DAKks) for human and animal rVNA testing and recognized by the German Ministry of Agriculture for performance of rVNA measurement of animal sera for pet travel purposes. The RFFIT assay described here has been internally validated per OIE guidelines for the purpose of rVNA measurement and per governmental approval, as it had been officially used for monitoring of oral vaccination campaigns of foxes in Germany for many years. Sensitivity and specificity was 95% and 98%, respectively.

At Kansas State University, rVNAs were measured in serum samples following the RFFIT method [64] as published in the WHO and the World Organisation for Animal Health (OIE) manuals [5,65]. Briefly, 100 μL of each serum sample, in duplicate, was diluted in serial five-fold dilutions in 10% fetal bovine serum supplemented Eagles minimum essential media (EMEM) and loaded into 8-well lab-tek chamber slides after which 100 μL of the challenge virus, at a concentration of 50 tissue culture infective dose 50% (TCID₅₀), was added. Slides were incubated at $37\text{ }^{\circ}\text{C}$ for 90 min after which 200 μL of a suspension of 5×10^5 BHK-21 C 13 cells in 10% FBS supplemented EMEM was added to each well. Slides were placed in a 2–5% CO_2 incubator at $37\text{ }^{\circ}\text{C}$ for 24 h. After incubation,

the slides were washed and fixed in 80% cold acetone, dried and stained with FITC conjugated anti-rabies antibody (Chemicon, Temecula, CA, USA). Twenty fields/well were examined under 100× magnification using a fluorescence microscope for the presence of rabies virus and rVNA titers were calculated using the Reed and Muench method [66]. In each test run, an international standard rabies immunoglobulin (SRIG) (WHO 1st human rabies immunoglobulin preparation Lot R-3, FDA/CBER, Rockville, MD, USA or WHO 2nd human rabies immunoglobulin preparation, National Institute for Biological Standards and Control, Potters Bar, UK) adjusted to 2.0 IU/mL, internal rVNA standards, and naïve serum served as controls. The SRIG was also used as a calibrator to calculate the test sample IU/mL value by the following formula: SRIG titer/Sample titer × SRIG potency in IU/mL. The RFFIT assay has been internally validated for rVNA measurement at Kansas State University (KSU) to international standards for human samples [67] and per OIE guidelines and United States Department of Agriculture (USDA) recognized for the purpose of pet travel and wildlife serosurveys. Sensitivity and specificity was 98% and 98%, respectively.

Sera were examined in a commercial blocking ELISA (BioPro Rabies ELISA, BioPro, Prague, Czech Republic) [68] for the presence of rabies-specific binding antibodies, following instruction of the manufacturer essentially as described [69]. Briefly, 100 µL of a 1:2 dilution of each serum was pipetted into wells of full RABV antigen coated microplate. After overnight incubation at 2–8 °C, in two consecutively-following steps 100 µL of a biotinylated anti-rabies antibody and 100 µL horseradish peroxidase (HRP)-conjugated streptavidin was added to each wells and incubated at 37 °C for 30 min followed by four times rinsing to remove unbound conjugate. Color change was developed by adding 100 µL of 3,3', 5,5-tetramethylbenzidine (TMB) chromogen solution to each well for 15–20 min at room temperature and the quantity of the analyte measured at 450 nm. According to the manufacturer, a 40% inhibition of the test serum compared to the negative controls was considered a cutoff for positivity and a 70% inhibition considered equal to 0.5 IU/mL. The performance characteristics were evaluated by the manufacturer and stated in the kit insert. The BioPro ELISA kit has been internally validated at FLI for humans, foxes, raccoon dogs, mongooses and raccoons using defined experimental (vaccinated/unvaccinated) as well as field sera.

A subset of sera was examined with a commercial indirect ELISA (Bio-Rad Platelia Rabies Kit II Ref: 355 1180, Marnes-la-Coquette, France) using the Bio-Rad Evolis instrument per the manufacturer's instructions. The kit contains strips of wells coated with rabies glycoprotein (G protein). The secondary (detection) system is an enzyme conjugated *Staphylococcus aureus* protein A/substrate color reaction. The results were reported in equivalent units (EU)/mL (anti-rabies glycoprotein level) calculated by comparison of the sample optical density reading against a standard curve of positive standards supplied in the kit. The kit control is calibrated against the 2nd WHO (World Health Organization) international rabies immunoglobulin reference serum. The performance characteristics were evaluated by the manufacturer and stated in the kit insert [70]. The BioRad ELISA kit has been internally validated for use with raccoon sera at KSU.

2.7. Statistical Analysis

Data were analyzed using statistical software GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, California, USA. Test agreement was calculated using the GraphPad QuickCalcs Web site: <http://graphpad.com/quickcalcs/kappa2/>.

3. Results

3.1. Literature Review

3.1.1. Serological Methods and Study Design Summary

The vaccines studies covered a combination of live attenuated and/or genetically altered rabies viruses: SAD, SAG, and ERA; and live recombinant constructs incorporating only the rabies virus

glycoprotein: VRG, HAdV5RG1.3, Ad5RG1, and CAV2-RVG, as well as mutants of these. Most are linked with the original SAD isolate in 1935. In general, the challenge virus strain used was derived from the species under study, with seven exceptions (Table 1). The challenge dose varied between 1×10^3 and 1×10^8 MICLD₅₀. The site of challenge was mostly the masseter and temporal muscles, but also targeted were the biceps, gluteal, abductor digiti quinti, and cervical musculature. The interval between vaccine and challenge ranged from 28 to 2490 days.

In these studies (Table 1), the RFFIT was the most frequently employed assay for measurement of rabies antibody levels in response to vaccination, and the majority (but not all) referenced the original method in Smith, 1973. The remaining studies, besides three using a competitive ELISA (cELISA), used other serum neutralization assays: four used FAVN method as described in the OIE manual; five used a fluorescence inhibition microtest (FIMT) that essentially represents a modified RFFIT [71], three used other modified RFFIT methods, and three used undefined serum neutralization assays. Only 54% (19 of 35) of the papers reported a cutoff level of seroconversion or seropositivity. In ten of the 19 studies, 0.5 IU/mL was the level defined as rVNA-positive; these included studies using RFFIT (4), FAVN (4), and modified RFFIT (2) assays. Three of the studies using FIMT applied 0.13 IU/mL as the level of seropositivity. Three referred to evidence of neutralization at a 1:5 dilution of the serum, but also reported the result in IU/mL. The three studies using the cELISA assay defined seroconversion as $\geq 25\%$, 26% and 28% inhibition, and one further defined it as three consecutive weeks of measurements above the cutoff, and measured antibody response by cELISA in weekly samples, but the pre-challenge samples by SNT.

3.1.2. Serological Results Summary

Due to the heterogeneity of experimental design and serological methods used, after review of the published studies listed in Table 1, the relationship of vaccinated status to serology and survival following challenge could only allow a general conclusion: the majority of the subjects in the studies that developed detectable rabies antibodies post-vaccination were also more likely to survive challenge. Those individual subjects with the highest titers in each separate study had the best likelihood of survival regardless of the range in titers produced in the vaccinated subjects, demonstrating that, though the assays used in these various studies had not been formally correlated, and indeed could vary in cutoff level and range, the results were generally consistent in identifying the likely survivors in a group by the level of antibody measured. Vaccinated status did not guarantee survival, and neither did demonstration of circulating rabies antibodies post-vaccination.

Thirty-five studies reported rabies antibody level in individual values, either IU/mL, titer (for SNT assays), or percent inhibition (for cELISA assays). In regards to correlation of serology results to survival from challenge, in 18 of the 39 studies (46%) the relationship was absolute: all subjects with detectable rabies antibodies survived challenge and all with no detectable antibody succumbed. For 66% (12/18) of those, the effective cutoff level was 0.5 IU/mL. Animals with undetectable or below cutoff rVNA levels survived challenge in 14 of the 39 studies (36%). However, in 6 of the 39 studies the opposite trend occurred where rVNA “positive” animals succumbed (e.g., levels of 0.65 IU/mL, 0.57 IU/mL, >0.5 IU/mL, 1.07 IU/mL, 1.3 IU/mL, and >1:5 titer); in many of these events, the animal that succumbed had the lowest rVNA level among the group of vaccinated subjects in the experiment. Only in one of the 39 studies was the relationship between serological response and survival weak; subjects that succumbed had rVNA levels at day of challenge between 0.06 and 2.2 IU/mL and, for the ones that survived, the range was 0.04 to 12.2 IU/mL. In this study, the subjects were considered seroconverted if they had detectable rabies antibodies for more than three consecutive weeks; there was no correlation of seroconversion to survival, with the range of consecutive weeks for both the survivors and died encompassing 1–15 weeks.

The ability of the rabies serology results to predict percent survival is partially predicated on the day of sampling and the day of challenge. In the 26 studies where both Days 14–30 and day of challenge (within seven days) rabies antibody results were given, Days 14–30 antibody levels were

higher or essentially the same as at day of challenge in 69% of the studies; the levels higher at day of challenge in 31% of the studies. For the remaining studies where the results were higher at the earlier time-point, the day of challenge occurred later than 90 days post-vaccination, thus in extended sampling it appears the determination of early seroconversion was a better predictor of survival than the level at day of challenge.

3.2. Animal Studies

3.2.1. Correlation between Blocking ELISA and RFFIT Values and Test Agreement

For pooled data independent of species (Figure 1) there was a poor correlation ($R^2 = 0.0189$) between ELISA and RFFIT values, although a significant positive trend is observed ($p = 0.0002$; 95% CI of slope 0.1792 to 0.5760, Figure 1A). The correlation remains poor even if different subgroups, i.e., baseline versus day of challenge sera, are considered. For baseline samples, there was no detectable trend (Figure 1B–D). Only for the red fox ($n = 212$), was a significant positive trend detected ($p < 0.0001$; 95% CI of slope 5.569 to 9.850, Figure 1D).

In studies 8 and 9 (Table 2), an indirect ELISA (Bio-Rad) was used. A better correlation between the RFFIT and the indirect ELISA values was demonstrated overall, although not linear (Figure 2A). However, when the correlation was determined for RFFIT values ≤ 1.0 IU/mL, the correlation of this subset is relatively poor (Figure 2B). While it appears that the Bio-Rad ELISA is quite suitable in identifying true negatives, the test had difficulties in identifying vaccinated animals, particularly at Day 28 (Figure 2D). In total, 12 of 29 (41.4%) vaccinated raccoons in this study tested “negative” (≤ 0.125) in the Bio-Rad ELISA on day of challenge but subsequently survived the challenge infection (Figure 2C).

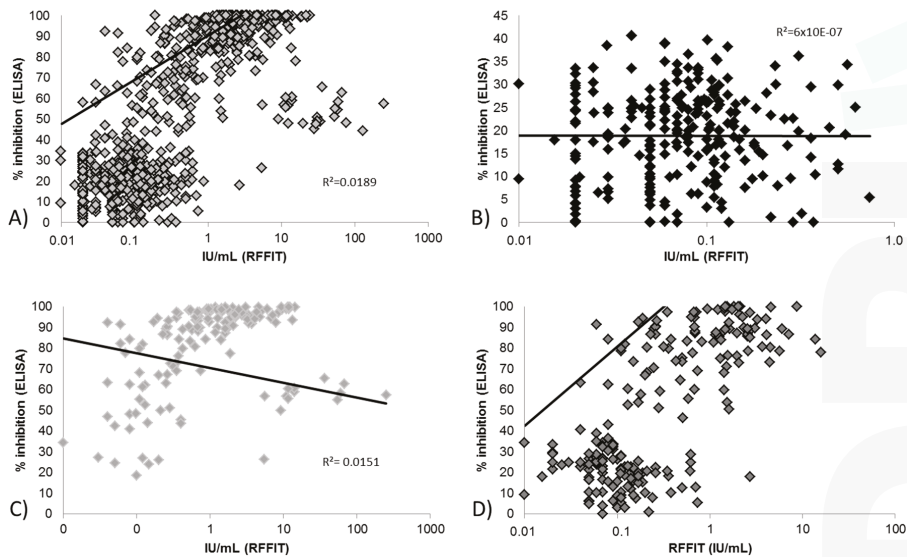


Figure 1. Correlation between the rapid fluorescent focus inhibition test (RFFIT) (IU/mL) and blocking enzyme linked immunosorbent assay (ELISA) (% inhibition) results of: (A) pooled time points and species ($n = 725$); (B) pooled species baseline samples ($n = 255$); (C) pooled species day of challenge samples ($n = 178$); and (D) pooled time points fox samples ($n = 212$). Blocking ELISA values of $<0\%$ and $>100\%$ were set at 0% and 100% , respectively.

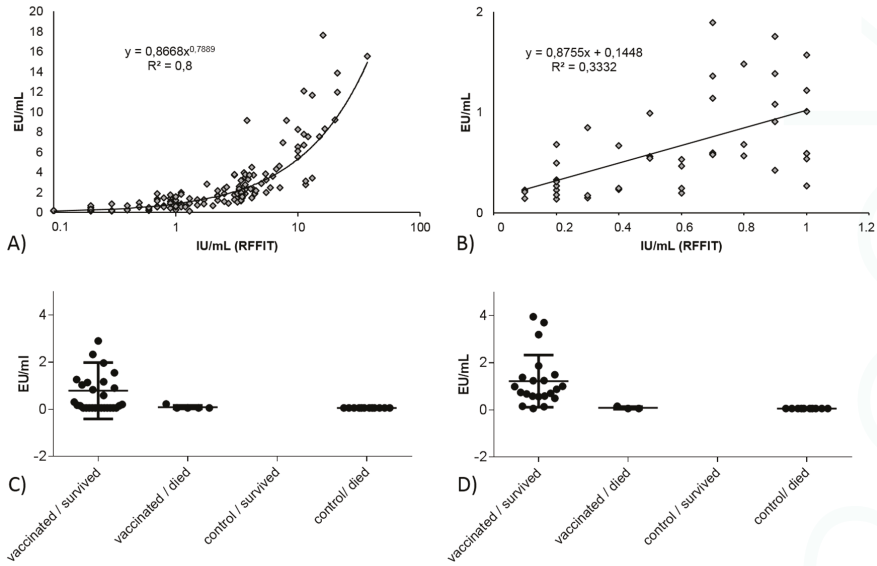


Figure 2. The correlation between RFFIT and indirect ELISA (Bio-Rad) for study 8 (Table 2): (A) the complete data set of absolute values, one outlier (indirect ELISA—48.78) was removed; (B) same data set but only for values with a RFFIT-result of <1.0 IU/mL; (C) indirect ELISA results at day of challenge for the vaccinated and control animals per outcome of challenge; and (D) indirect ELISA results on Day 28 post-vaccination for the vaccinated and control animals per outcome of the challenge infection. EU/mL is Equivalent Unit per milliliter.

3.2.2. Limit of Detection for Blocking ELISA and RFFIT

For both assays, the lower limit of detection was assessed by analyzing the immune response measured in naïve (unvaccinated) animals. For the blocking ELISA the equation of best fit was $y = 8.44 + (1 - e^{-0.0476X})$, resulting in 95% of all samples from unvaccinated animals showing an inhibition of <43% (Figure 3A). When the same analysis was done for RFFIT results using the same sample set, the equation of best fit was $y = 100(1 - e^{-11.79X})$. In this case, 95% and 70% of all samples from unvaccinated animals had rVNA-levels of <0.25 and <0.10 IU/mL, respectively. Hence, the lower limit of detection for RFFIT was <0.25 IU/mL (Figure 3B).

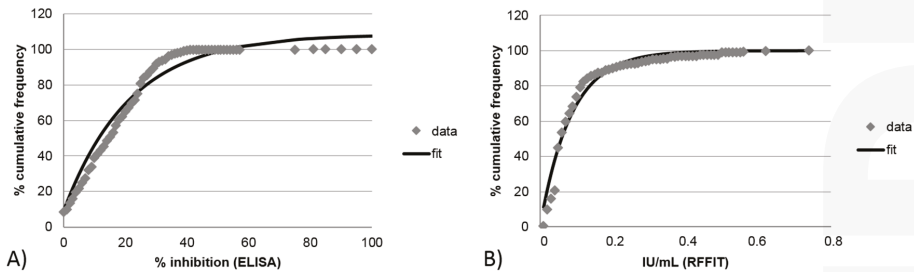


Figure 3. Cumulative frequency of rabies antibody test values in samples from unvaccinated animals (i.e., baseline samples of vaccinated animals and control unvaccinated animals) for the: blocking ELISA (A); and the RFFIT (B). For the latter, only absolute values were used and relative values of <0.5 or <0.1 IU/mL were omitted.

3.2.3. Correlation and Agreement of Test Results Obtained in Blocking ELISA and RFFIT with Survival/Death

Based on the currently used thresholds of seropositivity (blocking ELISA: cutoff >40% inhibition; RFFIT >0.5 IU/mL), the agreement between the assays was high (85.66%) resulting in a Kappa value of 0.71 (95%CI: 0.66 to 0.76). When the RFFIT cutoff was set as >0.25 IU/mL, the agreement slightly increased to 0.75 (95% CI: 0.70 to 0.80). In both cases, the strength of agreement was considered to be “good” (Table 3).

When the immune response measured at Day 28 post vaccination, day of challenge, and the outcome of the challenge were analyzed using RFFIT values, a Michaelis Menten model demonstrated the best fit (Figure 4A,B). This model predicts a survivorship of 95% and 99% of the animals with rVNA levels of ≥ 0.43 and 0.65 IU/mL at Day 28 post vaccination, irrespective of day of infection, respectively. In contrast, for the same time points and outcomes but using blocking ELISA values, a Boltzmann sigmoidal model demonstrated the best fit (Figure 3C,D). Here, survivorship of 93% and 57% are predicted when animals have inhibition values of $\geq 40\%$ at Day 28 and at the day before challenge, respectively.

When comparing different thresholds of seropositivity for both assays, the relative chance of a seropositive animal to survive a challenge compared to a negative animal was highest for the blocking ELISA with the currently defined cutoff of 40% inhibition. In addition, the sensitivity was highest for this cutoff setting, while still demonstrating sufficient specificity (Table 3). While for all cutoff settings the positive predictive value was higher than 95%, the negative predictive value was again highest for the blocking ELISA with the cutoff of 40% inhibition. Although the agreement between test result and outcome of infection varied across species, the best agreement (i.e., “good”) was achieved for the 40% inhibition test setting.

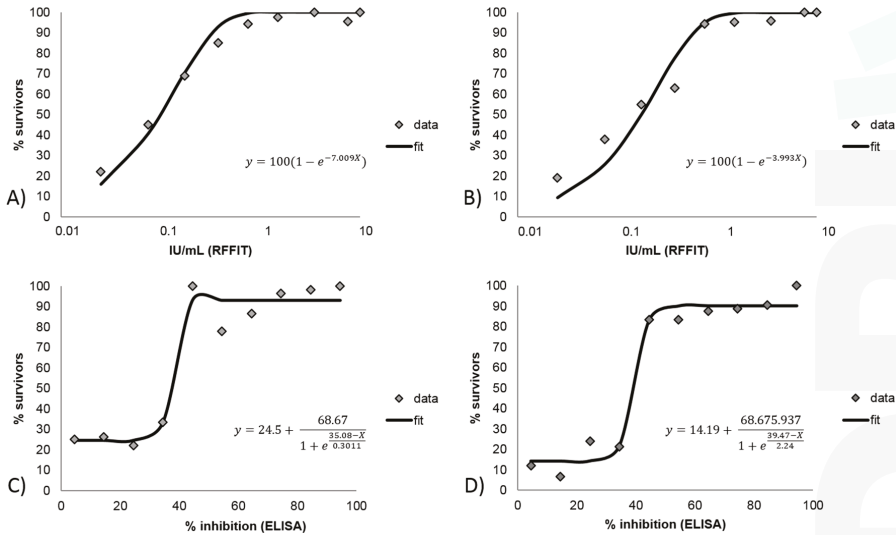


Figure 4. Relation between survivorship and immune response at: Day 28 post-vaccination (A,C); and the day of challenge (B,D). For RFFIT (A,B) the Michaelis Menten model is shown, while for blocking ELISA (C,D) the Boltzmann sigmoidal model is shown.

Table 3. Test characteristics and test agreement between the respective tests and the outcome of infection (survival vs. death), with two different cutoff values for the blocking ELISA (BioPro) and for RFFIT. For all results (OR = odds ratio, Se = sensitivity, Sp = specificity, PPV = positive predictive value, NPV = negative predictive value), the 95% confidence interval (CI) is provided.

Diagnostic Test	OR	(95% CI)	Se	(95% CI)	Sp	(95% CI)	PPV	(95% CI)	NPV	(95% CI)	p Value	Kappa	(95% CI)	Agreement
blocking ELISA (70% Inhib)	18.54	4.982–78.59	0.4873	0.4486–0.5263	0.9512	0.8386–0.9913	0.9935	0.9768–0.9989	0.1074	0.07959–0.1435	<0.0001	0.016	0.062–0.126	Poor
blocking ELISA (40% Inhib)	56.39	21.5–148	0.9063	0.8567–0.9399	0.8537	0.7156–0.9312	0.9667	0.9292–0.9846	0.6604	0.5259–0.7731	<0.0001	0.681	0.564–0.799	Good
Fox	126	14.48–1383	0.9825	0.9071–0.9991	0.6923	0.4237–0.8732	0.9333	0.8407–0.9738	0.9	0.5958–0.9949	<0.0001	0.741	0.527–0.955	good
Raccoon dog	∞	12.29–∞	0.8795	0.7922–0.9332	1	0.6756–1	1	0.95–1	0.4444	0.2456–0.6628	<0.0001	0.562	0.332–0.792	moderate
Mongoose	∞	9.259–∞	1	0.7225–1	1	0.6457–1	1	0.7225–1	1	0.6457–1	<0.0001	1.000	1.000–1.000	perfect
Skunk	6	0.8095–41.37	0.8	0.5481–0.9295	0.6	0.2307–0.9289	0.8571	0.6006–0.9746	0.5	0.1876–0.8124	0.1313	0.375	0.071–0.821	fair
Dog	N/A	N/A	0.375	0.1848–0.6136	N/A	N/A	1	0.6097–1	0	0–0.2775	>0.9999	0.000	0.000–0.000	poor
Raccoon	∞	2.432–∞	0.6364	0.3538–0.8483	1	0.6756–1	1	0.6457–1	0.6667	0.3906–0.8619	0.0128	-0.390	-0.676–-0.104	worse
RFFIT (0.5 IU/mL)	32.1	10.2–101.1	0.7171	0.6519–0.7743	0.9268	0.8057–0.9748	0.98	0.9429–0.9945	0.3958	0.3038–0.4958	<0.0001	0.419	0.312–0.526	Moderate
Fox	16.25	3.735–60.62	0.8298	0.6986–0.9111	0.7692	0.4974–0.9182	0.9286	0.8099–0.9754	0.5556	0.3372–0.7544	0.0001	0.526	0.285–0.767	moderate
Raccoon dog	∞	7.174–∞	0.7952	0.6962–0.868	1	0.6756–1	1	0.945–1	0.32	0.1721–0.5159	<0.0001	0.406	0.201–0.611	moderate
Mongoose	∞	1.121–∞	0.3793	0.2269–0.56	1	0.6457–1	1	0.7412–1	0.28	0.1428–0.4758	0.0756	0.192	0.037–0.348	poor
Skunk	∞	4.573–∞	0.8571	0.6536–0.9502	1	0.5655–1	1	0.8241–1	0.625	0.3057–0.8632	0.0009	0.698	0.391–1.000	good
Dog	N/A	N/A	0.5	0.28–0.72	N/A	N/A	1	0.6756–1	0	0–0.3244	>0.9999	0.000	N/A	poor
Raccoon	∞	0.6622–∞	0.2941	0.1328–0.5313	1	0.6756–1	1	0.5655–1	0.4	0.2188–0.6134	0.1399	0.211	0.013–0.408	fair
RFFIT (0.25 IU/mL)	24.83	9.924–59.72	0.8098	0.7505–0.8576	0.8537	0.7156–0.9312	0.9651	0.926–0.9839	0.473	0.3634–0.5852	<0.0001	0.502	0.382–0.622	Moderate
Fox	5.415	1.584–18.03	0.7719	0.6479–0.8616	0.6154	0.3552–0.8229	0.898	0.7824–0.9556	0.381	0.2075–0.5912	0.0152	0.313	0.072–0.554	fair
Raccoon dog	∞	9.542–∞	0.8434	0.7502–0.9061	1	0.6756–1	1	0.948–1	0.381	0.2075–0.5912	<0.0001	0.486	0.265–0.708	moderate
Mongoose	∞	2–∞	1	0.7412–1	0.8571	0.4869–0.9927	0.9167	0.6461–0.9957	1	0.64697–1	0.0004	0.880	0.653–1.000	very good
Skunk	∞	5.995–∞	0.9048	0.7109–0.9831	1	0.5655–1	1	0.8318–1	0.7143	0.3589–0.9492	0.0003	0.785	0.506–1.000	good
Dog	N/A	N/A	0.875	0.6398–0.9778	N/A	N/A	1	0.7847–1	0	0–0.8223	>0.9999	0.000	N/A	poor
Raccoon	∞	1.618–∞	0.4706	0.2617–0.6904	1	0.6756–1	1	0.6756–1	0.4706	0.2617–0.6904	0.0261	0.363	0.106–0.619	fair

The good agreement with a cutoff of 40% inhibition for the blocking ELISA was also evident when species were analyzed separately, where except for raccoon dogs, very few animals with values <40% survived a challenge infection (Figures 5 and 6). In contrast, low rVNAs (0.05 to 0.25 IU/mL) were obtained in both survivors and animals that succumbed to rabies challenge. Some species-level differences were evident, with most survivors among mongooses having RFFIT titers ≥ 0.5 IU/mL, while this proportion is lowest for dogs and raccoons. Except for raccoon dogs, where the proportion of RFFIT positives among survivors remains the same, a lower cutoff value of 0.25 IU/mL increases the proportion. The same applies to the day of testing, where samples taken 28 days post-vaccination have a greater proportion of RFFIT positive results than samples taken at the day of challenge (Figure 6A). This effect is not evident for the blocking ELISA, which generally provides a better match between survivorship and a positive result. Again, similar to the RFFIT, raccoons had the highest proportion of blocking ELISA negatives among survivors (Figure 6B).

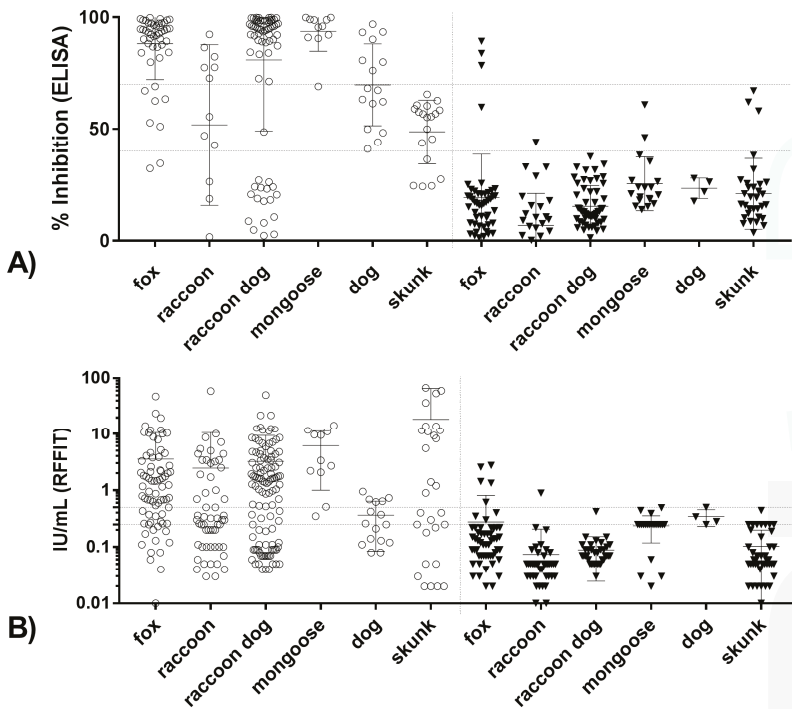


Figure 5. Scatter dot plot for: blocking ELISA (A); and RFFIT (B), for the individual species. Values are presented for survived (open circles) as well as for animals that succumbed to the infection (black triangle). Mean and standard deviation are indicated. Horizontal dotted lines indicate the thresholds used for blocking ELISA (40% and 70% inhibition) and for RFFIT (0.5 and 0.25 IU/mL).

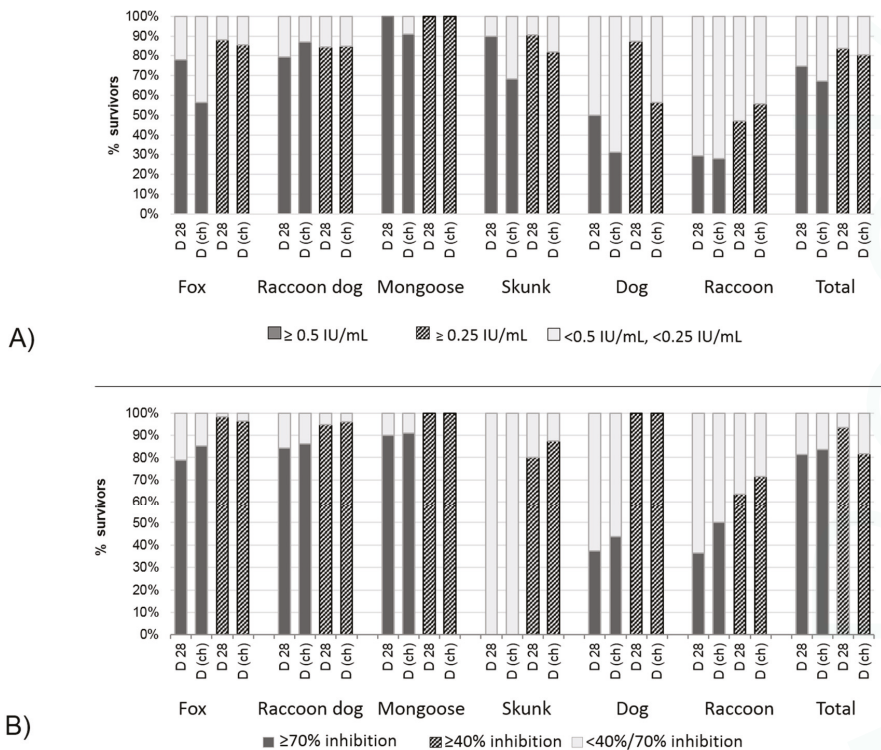


Figure 6. (A) Percentage of RFFIT positives and negatives per species among survivors, at different time points and using different cutoff values; and (B) percentage of blocking ELISA positives ($\geq 40\%$ inhibition) and negatives per species at Day 28 post-vaccination (D 28) and before challenge (D (ch)).

4. Discussion

Challenge studies demonstrating oral rabies vaccine efficacy in animals follow the same study design standards as parenterally-administered live or inactivated rabies vaccines. However, for oral vaccines, two efficacy studies are recommended by OIE guidelines [5], one involving direct instillation of the vaccine into the oral cavity, and the other involving bait delivery of the vaccine. However, U.S. and European specific guidelines appear to only require demonstration of bait efficacy for licensing [72,73]. The duration of vaccine immunity may vary according to product label intent and applicable guidelines. The minimum recommended duration standard for wildlife oral rabies vaccines is 180 days in the European Union [5] and 365 days in the U.S. [72]. Other differences include blood sampling at different time points and serological definition of population-level rabies immunity. For example, the US standards require several serum antibody measurements post-vaccination and prior to challenge [72], whereas the European Union does not [73]. Additionally, the proportion of vaccinated animals surviving challenge must be 87% or greater in the U.S. and with 80% or greater mortality of unvaccinated controls, but the European Union requires 92% or greater survival among vaccinates and 90% or greater mortality of unvaccinated controls [72,73]. These differences in acceptance standards pose difficulty for industry and end-users in defining adequate efficacy monitoring levels.

Tools to assess the effectiveness of ORV baiting programs to control rabies in an area are currently limited to measurement of rabies antibodies produced in response to vaccination, or case reductions or elimination of rabies cases following successive campaigns as the most definitive proof of baiting

program effectiveness. However, particularly in rabies-free vaccination zones implemented to stop further spread, there is no other tool to verify effectiveness than to test population-based immunity. To this end, population-level antibody prevalence still is an imperfect means for assessing ORV baiting efforts, as antibodies are not the only informative measure of immunity against rabies infection. Clearly, the cellular as well as humoral immunity has been shown to play an important role in preventing disease, as well as innate immunity. However, the key to control rabies in susceptible wildlife is pre-exposure vaccination. This is due to unique viral mechanisms of the rabies virus. Pathogenic wild type rabies virus likely limits replication in nerves to lower the expression of glycoprotein, and downregulates receptors and signaling for infected nerve apoptosis by the immune system [74–76]. The virus also appears to downregulate the inflammatory response and enhance destruction of infiltrating lymphocytes to actively suppress the immune system [74]. Prior to accessing the NS, humoral and cell-mediated immune effector cells may have an opportunity to detect and clear the virus, which may permit host survival. In this regard, the type 1 helper T cell pathway of response, signaled by increased production of interferon gamma (IFN- γ) and inflammation, is critical to activation of cytotoxic T cells for clearance of the peripheral virus infection. However, nerve cells do not express class I MHC for presentation to activated T cells, and once rabies virus enters the immune-privileged central nervous system (CNS), the immunosubversive and evasive strategies of wild type RABV make it nearly impossible for the host to effectively combat the NS infection [2,74]. Vaccination prior to virus challenge induces immune effectors (B and T cells) for establishment of rabies-specific antibody producing plasma cells. Circulating rabies antibodies will then be present to neutralize virus at the time of exposure as well as memory cells, which are primed and ready to respond and expand the response on repeated activation [77]. This effectively eliminates immunosubversive and evasive mechanisms of the rabies virus.

Oral vaccination of wildlife has led to the elimination of rabies in the target species in large parts of Europe and North America [78]. Application of serological monitoring as means of evaluating vaccination efficacy demands good understanding of the relationship of measured antibody level and survival from challenge. Certainly there are host factors that affect individual response to vaccination (e.g., genetic variation in MHC molecules) in addition to variable bait uptake interactions, but in strictly considering the means of predicting successful immunization (protection), establishing the reliability of serological monitoring as a tool is critical. The review of previous studies in which serological testing was performed in rabies challenge studies presented several difficulties in deducing what rabies antibody level was associated with a strong probability of survival. Analysis of the serological results in the literature review indicated a general positive correlation between level of rabies antibody and survival, but also revealed several variations in measurements, and in some studies, no solid relationship between antibody level and survival was demonstrated [24]. In regard to the assays used, understanding the abilities and limitations of the assay as well as use of quality control measures aids in interpretation of results [79]. While it is true that each study evaluated in the literature review did not use the same vaccine, same strain and dose of challenge virus, or same day of challenge, it is also true that use of a consistent method to measure rabies antibody would have allowed more robust comparison of antibody protection elicited by vaccination across studies. There are critical components of serum neutralization assays that will directly affect the accuracy of the antibody measurements. Key among them are the virus dose, virus strain, and reference serum [80]. Use of a high dose of virus may not allow detection of low antibody levels and too low a dose may overestimate the level. Use of a challenge strain that is not closely related antigenically to the vaccine strain may underestimate the antibody level [81]. Additionally, if the reference serum used to calibrate the titer results is not an international standard or qualified against one, the resulting values may be skewed [8,82]. Therefore, at minimum, the specificity, sensitivity, and measurable range of the assay employed should be defined and identified when reporting results to allow discernment as to the comparability of the findings with other studies.

In the publications reviewed, the majority of serological measurements were performed with serum neutralization assays, which functionally should translate most clearly to protection. However, serum neutralization assays are less likely to be standardized when compared to ELISA assay kits, due to the many manual steps and component parts that can be independently sourced, thus less strictly controlled between laboratories. Therefore, while ELISA kits measure binding antibodies, not necessarily neutralizing antibodies, they have the advantage of ease of standardization and greater assurance of consistent results [8]. Several studies have demonstrated a good correlation between SNT and ELISA methods with human and animal sera at specific cutoffs [15,17,18,69,83,84]. In particular, a comparison of SNT and blocking ELISA in wild-caught raccoons and skunks from ORV areas demonstrated the utility and comparability of both assays at a cutoff of 0.5 IU/mL and 40% (or 25% as defined in some studies) respectively [83,85]. Overall, correlation between blocking ELISA and RFFIT values in this study for the all samples was poor, indicating that the two assays are measuring partially different antibody functions. This remains true even when the samples are analyzed in subgroups (survivors, died, Day 0, day of challenge, etc.) (Figure 1). Using the threshold for seropositivity (per BioPro, >40% inhibition; per RFFIT, the commonly used level of 0.5 IU/mL) good agreement between tests was obtained (Table 3). If a lower level was used for RFFIT seropositivity (0.25 IU/mL) the agreement was marginally improved. Correlation of BioRad ELISA and RFFIT results varied by day of draw with better correlation at day of challenge compared to Day 28, due to the fact that the ELISA only detects IgG antibodies, thus under-measures the total antibody response in the earlier time-point (Figure 2). Due to the simplicity, better precision, and repeatability of an ELISA kit compared to a complex, manual serum neutralization test, the functional test may not be the best test for the purpose of monitoring ORV campaigns. However, it is important to note ELISA techniques can vary in terms of ability to estimate protective (neutralizing) antibodies.

The key motivation for this study was to estimate a cutoff level associated with survival in animals vaccinated orally with live constructs. As can be surmised from the lack of correlation between seroconversion as assigned by cELISA results and challenge survival in one of the studies in the review [85], this is not an easy task. One of the complicating factors is species-associated matrix differences that can cause false positive results if the lower limit of detection has not been identified with a specific species serum, if there are interfering factors that prevent binding of competitive rabies antibodies in the cELISA or that non-specifically bind in the indirect ELISA [8,86]. The timing of blood draw for evaluation of antibody level appears to be critical in assessing protection conveyed by vaccination. Antibody development after vaccination has been well studied: a general pattern of detectable antibody level followed by a rise in neutralizing antibody, peaking between 14 and 30 days, followed by a slow decrease to a stable level; and if a booster dose is received a faster rise in level is expected followed by stabilization of a higher level [26,87]. While there are studies in dogs and cats that show a clear association between detectable rVNA before challenge and survival [6,7], review of available published studies in wildlife and our own empirical studies included here indicate that detection of rVNA at Days 28–30 is also predictive of survival, and usually more so the later the day of challenge was in relation to the day of vaccination. In a study where the challenge day was 83 months from vaccination, 60% of the survivors had detectable rVNA at challenge and 40% did not; of those 40%, all had detectable rVNA 66 months but only half of those had a level above 0.5 IU/mL at every sampling time point beyond the initial measurement at one month, indicating that measurable initial response was the better predictor of survival at challenge [31].

Determination of applicable cutoff level is important, as stated previously, for a specific assay given a specific purpose. Evaluation of background signal of negative samples provides information regarding a lower limit of detection. Ideally, negative samples will have levels significantly below the determined effective level to allow discernment between “positive” and “negative” samples. The same is true for determination of vaccination success and failure. Evaluation of Day 0 (unvaccinated subjects) samples (all species) revealed that 95% had a blocking ELISA result of $\leq 43\%$ inhibition and a RFFIT result of ≤ 0.25 IU/mL. Species differences were noted, with raccoons having less of a variation in

result range than other species (Figure 5), indicating that further evaluation of negative samples by species is necessary for assignment of cutoffs for lower limit of detection of an assay. Figure 4 illustrates that the blocking ELISA “negative” and “positive” results are more clearly delineated in scale compared to the RFFIT data, which shows a more gradual relationship between rVNA level and survival. For example, the statistical probability for survival increases dramatically between blocking ELISA values of 35% (30–40%) and 45% (40–50%) inhibitions, whereas the same increase in probability occurs between an order of magnitude difference (i.e., 0.06 and 0.5 IU/mL) for RFFIT values. For some species this may make assignment of a cutoff more difficult for RFFIT results than for the ELISA results (Table 1). This may indicate that the RFFIT is more sensitive at lower levels of antibody, causing poor delineation between “negative” and “positive” samples. Use of a specific challenge virus strain or increasing the challenge dose in the RFFIT procedure, possibly could correct this weakness. For RFFIT at >0.50 IU/mL, the probability of survival reaches 95% and at levels >2.0 IU/mL it becomes 100% using a best fit curve of the data. For the blocking ELISA, >80% probability of survival is obtained at >40% inhibition and at >70% inhibition over 90% probability of survival is attained. This analysis is remarkably similar whether the result used is from Day 28 or day of challenge, and our empirical results also demonstrated that Day 28 post-vaccination titer levels were a better predictor of survival than titers measured immediately prior to challenge. As expected, the higher the antibody level measured, whether by indirect ELISA, cELISA, blocking ELISA or RFFIT, the higher the probability of survival, to a robust point where the level is associated with 100% survival; beyond that point, higher titer levels do not translate into “better protection”.

Oral vaccination has proven to be an effective tool in rabies prevention and control. The target species varies per endemic region and vaccines are developed that are of highest efficacy in their targeted species. The specific G protein, copies presented, and other details of the vaccine construct in combination with the specific assay used for antibody measurement theoretically can affect the relationship of rabies antibody level and probability of survival. An analysis of the results by vaccine construct indicates constructs are not the reason for the effect seen in raccoons and skunks, and that the limited data for dogs do not allow for a robust conclusion (data not shown). It is not unusual therefore to see various correlations of serological results and survival among different species. Indeed, the results of this study demonstrate that a single cutoff level of seropositivity is not universally applicable. As mentioned previously, the unvaccinated raccoon samples had a lower mean percent inhibition by blocking ELISA and lower mean IU/mL by RFFIT compared to the other species (Figure 5A,B), while unvaccinated fox samples had a small number of samples with results above the cutoffs for both methods. For all the species, except fox, the 70% inhibition cutoff for blocking ELISA was robust; the lower threshold (40% inhibition) allowed misidentification of unvaccinated fox, raccoon, mongoose, and skunk samples. For RFFIT, the 0.25 IU/mL cutoff was robust for raccoons, raccoon dogs, and skunks, and the higher cutoff of 0.5 IU was better at identifying unvaccinated mongoose and dog samples (Figure 5A,B). The Bio-Rad ELISA results appeared to be better correlated to RFFIT results overall; however, for results below 1.0 IU/mL, the correlation is poor, indicating that it may not be as useful in situations where low antibody levels are expected. The Bio-Rad ELISA was quite suitable in identifying true negatives, while the test had difficulties in identifying vaccinated animals (Figure 2C,D). It is possible that increasing the sensitivity of this assay by adjustment of conjugate or antigen could improve performance for wildlife species.

These findings illustrate the importance of evaluation of the appropriate cutoff by species and by assay. Similarly, relationship of rabies antibody level and challenge outcome varied by species, assay used, and cutoff level. For RFFIT the best predictor of survival having a result of ≥ 0.25 IU/mL at Day 28 across species, although for raccoon dogs and raccoons the best predictors were ≥ 0.25 IU/mL at day of challenge (Figure 6A). For the blocking ELISA results, the 40% inhibition cutoff was an excellent predictor of survival for fox, raccoon dog, mongoose, and dog, with 90% or more subjects at that inhibition level surviving challenge. Across species, the 70% inhibition level was a less accurate

predictor of survival (Figure 6B). Overall, the >40% inhibition blocking ELISA result at day of challenge determined survival most often, with the majority of results from fox and raccoon dog studies.

5. Conclusions

Acceptance standards for challenge studies determining an associated protective antibody level with challenge survival in the review of previous challenge studies in wildlife were not feasible (Table 1).

Assays must be evaluated for purpose (for example, to identify vaccinated animals, to identify vaccinated animals with protective immunity, and to identify free-ranging wild animals previously exposed to rabies infection) and generalizations should not be made to other purposes for which an assay was never evaluated.

Specificity (based on the vaccine antigen and assay antigen relationship), sensitivity, accuracy (including linear range) and precision of different assays may vary among species. Standardization and quality control of reagents and procedures, whether kits or manual procedures are established, is absolutely essential. These criteria should be evaluated before assigning the assay as fit for purpose.

Timing of sampling for antibody response to oral vaccine bait uptake should be optimized to target the initial seroconversion peak period, because this point predicts (given the appropriate cutoff and assay for the target species) survival as well as or even better than the level measured just before challenge.

In our study, positive blocking ELISA results are a better predictor for survival than the various SNTs applied overall. If the ELISA kits used have sufficiently good lot to lot quality control, the results from one study can be directly compared to another separated by time and space, with high confidence. Monitoring of ORVs based on seroconversion rates of the target population should make use of blocking ELISA tests instead of SNTs, for both strength as a survival predictor and ease of standardization.

Whatever assay is selected, it should have a defined (by validation) relationship to protection and robustly meet the minimum requirements for the purpose.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2414-6366/2/3/31/s1>.

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Conflicts of Interest: A.V. is a full-time employee of IDT Biologika, Germany, a company that also manufactures oral rabies vaccine baits. T.M. and C.M.F. received funding from IDT for research into mechanisms of oral rabies vaccination and serological response. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Article

Comparison of a Micro-Neutralization Test with the Rapid Fluorescent Focus Inhibition Test for Measuring Rabies Virus Neutralizing Antibodies

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Abstract: The rapid fluorescent focus inhibition test (RFFIT) is routinely used in the United States to measure rabies virus neutralizing antibodies (rVNA). RFFIT has a long history of reproducible and reliable results. The test has been modified over the years to use smaller volumes of reagents and samples, but requires a 50 μ L minimum volume of test serum. To conduct pathogenesis studies, small laboratory animals such as mice are regularly tested for rVNA, but the minimum volume for a standard RFFIT may be impossible to obtain, particularly in scenarios of repeated sampling. To address this problem, a micro-neutralization test was developed previously. In the current study, the micro-neutralization test was compared to the RFFIT using 129 mouse serum samples from rabies vaccine studies. Using a cut-off value of 0.1 IU/mL, the sensitivity, specificity, and concordance of the micro-neutralization test were 100%, 97.5%, and 98%, respectively. The geometric mean titer of all samples above the cut-off was 2.0 IU/mL using RFFIT and 3.4 IU/mL using the micro-neutralization test, indicating that titers determined using the micro-neutralization test are not equivalent to RFFIT titers. Based on four rVNA-positive hamster serum samples, the intra-assay coefficient of variability was 24% and inter-assay coefficient of variability was 30.4%. These results support continued use of the micro-neutralization test to determine rabies virus neutralizing antibody titers for low-volume serum samples.

Keywords: rabies; virus neutralizing antibodies; diagnostic test; assay development; assay validation

1. Introduction

Measurement of rabies virus neutralizing antibody (rVNA) is essential to evaluating pre- or post-exposure prophylaxis and rabies diagnosis in humans and vaccination status in domestic animals [1–3]. The rapid fluorescent focus inhibition test (RFFIT) is one WHO-recommended test for measuring rVNA [3]. The RFFIT is used widely, primarily in the US, due to the standardized and functional results produced [4]. Developed in 1973 as a replacement for the mouse neutralization test, the RFFIT represented a major advance in cost, time, and replacement of animal use [5]. When compared to the mouse neutralization test, the RFFIT was 95% concordant, 100% sensitive, and 83% specific [5]. Over the years, the RFFIT method has been modified to use mouse neuroblastoma cells in place of BHK cells [6], to use a 96-well format similar to the tissue culture serum neutralization test or more widely used fluorescent antibody virus neutralization (FAVN) test [7–9], and to use half the volume of reagents. Despite these modifications, the RFFIT method requires a minimum volume of 50 μ L of serum per test [10].

Kuzmin, et al. (2008) developed a micro-neutralization test based on the RFFIT to measure rVNA in serum samples with limited volume, e.g., from bats [11]. The micro-neutralization test has numerous advantages compared to the standard RFFIT, including the need for only 3 μL of serum per test. Additionally, instead of using 8-well, chamber slides, the micro-neutralization test uses 4-well, Teflon-coated slides, which decreases the reagent content by ~90% representing a cost savings compared to the traditional RFFIT. Also, the dilutions are simplified starting with 10^{-1} , and only 10 fields in each well are scored for results rather than 20 fields, representing a time saving compared to the RFFIT. Furthermore, because the first dilution is higher than RFFIT, the micro-neutralization test is less susceptible to cytotoxicity. Overall, the micro-neutralization test is less labor intensive than the Terasaki plate method [12], but similar to the RFFIT, the micro-neutralization test still requires a 20–40 h incubation period, highly skilled personnel, and appropriate biocontainment and biosafety risk mitigation associated with use of live rabies virus.

The objective of the current study was to compare the rVNA measured in the same test serum using the RFFIT and micro-neutralization test. Mouse serum generated for other rabies immunization experiments was curated for volume and quality. The sample set was run with both tests using the same lots of reagents and rabies virus strain CVS-11. Results were compared based on sensitivity and specificity.

2. Materials and Methods

Approved animal use protocols were established with CDC's Institutional Animal Care and Use Committee (protocols 2330SMIMOU, 2332SMIMOU, 2622SMIHAMC). For mouse serum, adult female CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All animals received experimental or commercial rabies vaccine on day 1. Approximately 0.2 mL of blood was collected using the submandibular technique on days 0, 15, and 30. Serum was separated and stored at ≤ -10 °C. After the primary studies were completed, a convenient set of 129 serum samples were selected based on sufficient volume, previous RFFIT result (positive or negative), and lack of cytotoxicity. Cytotoxic samples were excluded because they could not be accurately classified as positive or negative in the RFFIT. Of the convenient sample, 55 had detectable rVNA (>0.05 IU/mL) and 74 had no detectable rVNA (<0.05 IU/mL) by RFFIT.

For hamster serum, adult female LVG Syrian hamsters were purchased from Charles River Laboratories (Wilmington, MA, USA). All animals were challenged with rabies virus on day 0. Some animals received post-exposure prophylaxis with commercial human rabies vaccine and human rabies immune globulin or experimental monoclonal antibody product on day 1, followed by additional doses of vaccine on day 4 and 8, while some animals received no post-exposure prophylaxis. Approximately 0.2 mL of blood was collected using the subclavicle technique on days 0, 4, 8 and at termination. Serum was separated and stored at ≤ -10 °C. After the primary studies were completed, four serum samples were selected to determine assay variability.

The RFFIT was completed according to a standard protocol [10]. The micro-neutralization test was modified slightly from the previous report [11]. The test was set up in a humidity chamber made from a petri dish and wet paper towel to prevent evaporation from the wells. An amount of 3 μL of test serum or standard rabies immune globulin (SRIG, US FDA lot R-3) was serially diluted in 12 μL of Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum in each well of a Teflon-coated, 4-well slide. An amount of 12 μL of rabies virus CVS-11 (CDC lot V-404) diluted to 50 FFD₅₀/mL ($50 \times 50\%$ fluorescing foci doses/mL) was added to each well. Back titration of the rabies virus CVS-11 and cell-only control were completed in a separate 4-well slide. Slides were incubated 60 min at 37 °C with 0.5% CO₂. 24 μL of mouse neuroblastoma cells diluted to approximately 5×10^5 cells/mL was added to each well and slides were incubated 20 h at 37 °C with 0.5% CO₂. Slides were fixed with acetone for 30 min at -20 °C and stained with FITC-anti-rabies virus antibodies (Fujirebio Diagnostics, Malvern, PA, USA) with additional 0.001% Evan's Blue for 30 min at 37 °C with 0.5% CO₂. Slides were washed twice with 0.1 M PBS pH 7.5 and observed with a fluorescence

microscope. In each well, 10 fields at 20× magnification were scored based on presence/absence of fluorescent foci and endpoint titer was calculated using the Reed-Muench method. Endpoint titers were converted to international units per milliliter (IU/mL) based on comparison to the SRIG diluted at 2 IU/mL.

Results for the same sample from two independent tests were compared. The cut-off of 0.1 IU/mL was chosen empirically based on approximately 50% neutralization in the first dilution of the micro-neutralization test. The same cut-off was chosen for RFFIT based on equivalency which is approximately complete neutralization in the first dilution. Sensitivity, specificity, predictive values, and 95% confidence intervals were calculated using GraphPad Prism v6. The correlation between quantitative titers determined by either test was evaluated using the Pearson test with $\alpha = 0.05$. Coefficient of variability was calculated by dividing the standard deviation in IU/mL by the mean in IU/mL.

3. Results

Based on the 0.1 IU/mL cut-off, 127 (98%) of 129 mouse serum samples tested had concordant results between the two tests. The remaining two samples were above the cut-off based on RFFIT but fell below the cut-off in the micro-neutralization test (Table 1). This results in a sensitivity of 100% (CI = 92.6–100%) and specificity of 97.5% (CI = 91.4–99.7%) for the micro-neutralization test. The positive predictive value was 96% (CI = 86.3–99.5%), the negative predictive value was 100% (CI = 95.4–100%), and the false negative rate for the new test was 2.5%.

Table 1. Sensitivity, specificity, as well as positive and negative predictive value of the micro-neutralization test compared to the rapid fluorescent focus inhibition test (RFFIT).

Test	Microneut. ¹ ≥0.1 IU/mL	Microneut. <0.1 IU/mL	Total	PPV NPV ²
RFFIT ≥0.1 IU/mL	48	2	50	96%
RFFIT <0.1 IU/mL	0	79	79	100%
Total	48	81	129	
SN/SP ³	100%	97.5%		

¹ Micro-neutralization test; ² Positive predictive value (PPV), Negative predictive value (NPV); ³ Sensitivity (SN), Specificity (SP).

Using the micro-neutralization test, 37% of samples were positive for rVNA compared to 39% with RFFIT. While the number of positive samples in a set may be slightly underestimated, the individual titers of positive samples appear higher using the micro-neutralization test (Figure 1). The geometric mean titer of rVNA positive samples was 3.4 IU/mL with the micro-neutralization test and 2.0 IU/mL with the RFFIT. Because of this difference in the individual rVNA titers, the correlation between the two tests was poor ($R^2 = 0.55$).

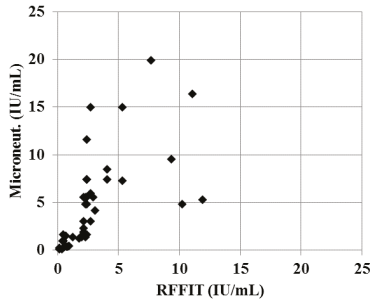


Figure 1. Comparison of rVNA titers (IU/mL) measured by the micro-neutralization test (microneut.) and RFFIT. Rabies virus neutralizing antibody was measured in individual serum samples by the micro-neutralization test (Microneut.) and RFFIT. Positive rVNA titers in IU/mL were plotted on a linear scale. The axis limits exclude four samples that had very high rVNA titers by both tests. The geometric mean titer was 3.4 IU/mL with the micro-neutralization test compared to 2.0 IU/mL with the RFFIT.

Intra- and inter-assay coefficient of variability was calculated using IU/mL from six statistical replicates, from two biologic replicates, for four rVNA-positive hamster serum samples. The intra-assay coefficient of variability ranged from 21–24% for the statistical replicates, and the median inter-assay coefficient of variability was 30.4% for the biologic replicates.

4. Discussion

A rabies virus micro-neutralization test is necessary to measure rVNA from small volume samples. The method described herein has been developed over time to meet this need. For purposes of this study, only serum from vaccinated animals was used, which is different, in terms of immune profile, from serum collected from wildlife to estimate prevalence of rabies virus natural infection/exposure. The above comparison to the RFFIT demonstrates that the method has utility for measuring rVNA in samples from vaccinated animals. This will be extremely beneficial in small animal challenge studies validating efficacy of medical countermeasures, where serum volumes are often limited.

Additional validation of the micro-neutralization test for study of natural rabies virus exposure in different taxa, geographic regions, and divergent lyssaviruses is still an important consideration. For purposes of validation, this study would fall under stage one: comparison to a standard test method [13]. Based on the sensitivity and specificity calculated here, 2% error, and 95% confidence, a panel of 95 rVNA positive samples, ideally from naturally infected/exposed animals, and 279 rVNA negative samples from the same species, would be required to validate the diagnostic sensitivity and specificity of the assay [13].

Endpoint titers, including for SRIG, were higher in the micro-neutralization test than RFFIT. Paradoxically, the micro-neutralization titers in IU/mL were also higher, meaning the endpoint titers of test serum increased more than the endpoint titer of SRIG. This trend holds true even when a subset of 20 samples with rVNA <1 IU/mL (RFFIT) is analyzed; although the difference is less for this subset. Because of the difference in individual titers, results from the micro-neutralization test should not be aggregated with results from RFFIT. The differences in titers may be due to differences in the evaporation, contact surface area, or fluid dynamics in the small drop used in the micro-neutralization test. Using a humidity chamber as described in the methods section is recommended to prevent evaporation especially when a large number of samples are being tested. In the future, the method could be further optimized to address the differences in the titers.

The 0.1 IU/mL cut-off provided high levels of agreement between the micro-neutralization test and RFFIT. Using a higher or lower cut-off of 0.05 IU/mL or 0.5 IU/mL resulted in 95%

and 93% concordance, respectively, compared to 98% concordance at 0.1 IU/mL. Based on these results, the 0.1 IU/mL cut-off is recommended for determining positive and negative rVNA in the micro-neutralization test.

The coefficient of variability shows that the micro-neutralization test is consistent and reliable for measuring rVNA. The variability is consistent with published variability for the RFFIT of 18–26% intra-assay and 28–30% inter-assay [4]. Given the advantages of the micro-neutralization test including sample/reagent sparing and time/labor saving, this assay should be used when the RFFIT is not feasible. Overall, the favorable comparison between the micro-neutralization test and RFFIT supports its continued use for measuring rVNA in small-volume samples.

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Author Contributions: A.G. and T.S. conceived and designed the experiments; T.S. performed the experiments; T.S. analyzed the data; T.S. and A.G. wrote the paper.

Conflicts of Interest: The authors declare no conflicts of interest.

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Article

Sero-Surveillance of Lyssavirus Specific Antibodies in Nigerian Fruit Bats (*Eidolon helvum*)

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Abstract: The aetiological agent of rabies is a member of the *Lyssavirus* genus (*Rhabdoviridae* family, order *Mononegavirales*). The disease (rabies) is endemic in many parts of Asia and Africa and still remains an important public and veterinary health threat. In Nigeria, there is a dearth of information on the natural infection and/or exposure of bat species to lyssaviruses. Therefore, this study was undertaken to assess the prevalence of rabies virus (RABV) neutralizing antibodies in sera obtained from bats from the central Plateau and North-East Bauchi States in Nigeria. Two hundred serum samples were collected from Nigerian fruit bats from six different locations and tested for anti-RABV antibodies using a commercial blocking ELISA. Of the 200 bat serum samples collected, one batch consisting of 111 samples did not meet the validation criteria and hence was not included in the final analysis. Of the remaining 89, only three (3.4%) contained anti-lyssavirus antibodies, demonstrating a low prevalence of lyssavirus antibodies in the study population. In order to further understand the exposure of bat species to phylogroup II lyssaviruses (Lagos bat virus and Mokola virus), the same panel of samples will be tested for neutralizing antibodies to phylogroup II members, viruses that do not cross-neutralize with members of phylogroup I.

Keywords: lyssavirus; rabies; *Eidolon helvum*; Nigeria; blocking ELISA; phylogroup I; phylogroup II

1. Introduction

Rabies is a fatal zoonotic disease and the aetiologic agent belongs to the genus *Lyssavirus* (*Rhabdoviridae* family) and order *Mononegavirales*. Despite rabies being a very old disease, it still commands great global veterinary and public health importance. There are presently fourteen recognised lyssavirus species in the genus and these include: rabies lyssavirus (RABV), Lagos bat lyssavirus (LBV), Mokola lyssavirus (MOKV), Duvenhage lyssavirus (DUVV), European bat 1 lyssavirus (EBLV-1), European bat 2 lyssavirus (EBLV-2), Australian bat lyssavirus (ABLV), Aravan lyssavirus (ARAV), Khujand lyssavirus (KHUV), Irkut lyssavirus (IRKV), West Caucasian bat lyssavirus (WCBV), Bokeloh bat lyssavirus (BBLV), Shimoni bat lyssavirus (SHIBV), and Ikoma lyssavirus (IKOV) [1,2]. Furthermore, two putative lyssavirus species, Lleida bat lyssavirus (LLEBV) [3] and Gannoruwa bat lyssavirus (GBLV) [4], have been identified in bat species and are still awaiting official classification. The viral species of this genus have been further sub-divided into three phylogroups based on their genetic distances, serologic cross-reactivity and/or pathogenicity studies in mouse model [5–7].

RABLV is found worldwide, and is responsible for the overwhelming majority of reported animal and human rabies cases [8–10]. Other lyssaviruses appear to have more restricted geographical and host range, with the majority having been isolated from bats with limited public and animal health implications. However, all lyssaviruses tested cause clinical disease indistinguishable from RABLV. In Nigeria, LBV was first isolated from the straw-coloured fruit bat *Eidolon helvum* in 1956 on Lagos Island [11]. A seroprevalence of 19% was demonstrated in this species (in Nigeria) by a different research group [12], whereas almost double that seroprevalence (37%) was observed in the same bat species in Ghana [13]. In contrast, other studies found an even higher seroprevalence, for instance in Kenya (40–67%), albeit from different bat colonies [14]. In Nigeria and throughout most of the African continent where bat populations are found, the natural infection of bat species with lyssaviruses is either unknown or poorly understood.

2. Materials and Methods

Ethical clearance (AEC/02/04/14) was granted by the Animal Ethical Committee (AEC) of the National Veterinary Research Institute, Vom, Nigeria, for the collection of blood samples from bats. Two hundred *E. helvum* were captured using mist nets from six different roosting sites in the central Plateau and North East Bauchi states of Nigeria with a view to assess the seroprevalence of RABV antibodies in this bat species. The bats were restrained manually and anesthetized with ketamine hydrochloride as described previously [15]. Blood samples were then collected (0.1–1 mL) through the jugular vein using 2 mL needle and syringe. The blood samples were then transferred into 5 mL sterile serum separator tubes via cold chain to the laboratory, where all the blood samples were centrifuged at 4000 rpm for 3 minutes, and thereafter serum samples were dispensed into sterile 2 mL screw capped cryovial tubes and stored at -80°C until required.

This panel of serum samples was tested with an ELISA kit (BioPro rabies ELISA Ab kit, Prague, Czech Republic), a blocking ELISA for serological diagnostic of rabies lyssavirus antibody in serum or plasma of domesticated and wild animals [16]. In brief, the serum samples and controls (rabies positive control serum, rabies control serum 1, rabies control serum 2, rabies control serum 1 and rabies negative control) were diluted two-fold using sample diluent buffer provided in the kit. At least 100 μL of each dilution were distributed into the respective well of the plate. Thereafter, the plate was sealed with plate sealer and incubated at $4-8^{\circ}\text{C}$ overnight (O/N) with gentle shaking on orbital shaker. The plate was washed six times with washing solution using automated washer (BioTek, Winooski, VT, USA) and excess buffer was absorbed on paper towel. The biotinylated anti-rabies antibody was diluted to a working dilution of 1:100 and 100 μL was distributed into each well. The plate was covered with a plate sealer and incubated at $37 \pm 2^{\circ}\text{C}$ for 30 minutes with gentle shaking as described before, then washed four times. Excess buffer was removed by tapping on paper towel. The streptavidin peroxidase conjugate was diluted 100-fold and 100 μL was dispensed into each well. The plate was sealed and incubated as previously. The plate was washed four times with washing solution and excess buffer was removed as before. At least 100 μL of ready-to-use TMB substrate was added to each well and the plate was incubated at room temperature for 15–30 min, with gently shaking on an orbital shaker away from direct sunlight. Thereafter, the reaction was stopped with 50 μL of stop solution per well and the results were read at 450 nm using an ELISA reader. The optical density (OD) values were expressed as percentage blocking by using the following formula:

$$\text{PB\%} = [\text{OD NC} - \text{OD SAMPLE} / \text{OD NC} - \text{OD PC}] \times 100 \quad (1)$$

The results were accepted only when the OD of a negative control serum of higher than 1 was obtained and the difference between the means of OD of negative and positive control serum samples was equal or higher than 0.8.

3. Results and Discussion

Using the set cut-off values (of 40% and 70%) for this ELISA antibody kit, with the latter corresponding to the gold standard method fluorescent antibody virus neutralization test (FAVNT) cut-off value of 0.5 IU/mL, it could be shown that only two serum samples were on the borderline and an equal number just above the cut-off value (Figure 1). Of the 200 serum samples analysed, only 89 met the validation criteria specified by the manufacturer and included for analysis. Three serum samples (sample numbers 2, 24, and 67), i.e., 3.4% of the study sample, contained lyssavirus-specific antibodies with percentage blockings (PBs) of 70%, 81% and 75% respectively (Figure 1).

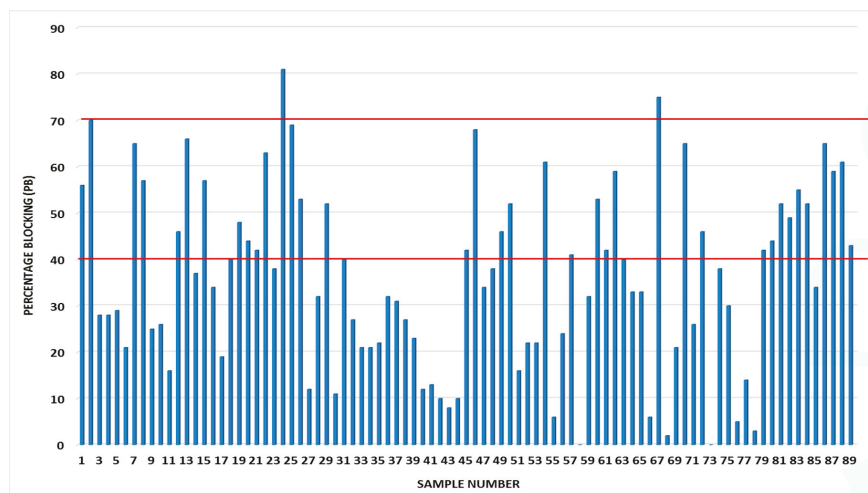


Figure 1. The distribution of percentage blocking values obtained from some of the bat sera obtained from *Eidolon helvum* from the central Plateau and North East Bauchi States of Nigeria. The red bars are the cut-off values (40% and 70%) with the former considered positive for rabies antibodies, and 70% considered as serum sample with antibody level equal to or higher than 0.5 IU/mL based on the FAVN test.

The three serum samples were subsequently shown to contain RABV-neutralising antibodies just below the 0.5 IU/mL cut-off using a laboratory strain of rabies virus (challenge virus standard). The 70% cut-off value demonstrates adequate seroconversion for international movement of pets using a fluorescent antibody neutralisation test [17]. In order to further understand the exposure of bat species to phylogroup II lyssaviruses (Lagos bat virus and Mokola virus), the same panel of samples (particularly those above 40%) can be tested for neutralizing antibodies to phylogroup II members. Data from a study carried out in Ibadan (western Nigeria) in 1990 showed the presence of RABV neutralizing antibodies in the sera of fruit bats [18]. However, given *Eidolon helvum* has been associated with a reservoir of LBV, it is crucial that the panel of samples be tested against this (LBV) lyssavirus [12,13]. These data therefore indicated possible cross-reactivity of antibodies amongst specific phylogroups within the *Lyssavirus* genus.

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Author Contributions: D.A.T.—collection of samples and performing of experiments; B.B.B.—collection of samples and writing of manuscript; T.A.W.—collection of samples and performing of experiments; E.N.—performing of experiments and writing of manuscript; C.T.S.—supervising postgraduate student and writing manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Lagos Bat Virus Infection Dynamics in Free-Ranging Straw-Colored Fruit Bats (*Eidolon helvum*)

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Abstract: Bats are key species for ecological function, but they are also reservoirs of zoonotic agents, such as lyssaviruses that cause rabies. Little is known about the maintenance and transmission of lyssaviruses in bats, although the observation of clinically sick bats, both in experimental studies and wild bats, has at least demonstrated that lyssaviruses are capable of causing clinical disease in bat species. Despite this, extensive surveillance for diseased bats has not yielded lyssaviruses, whilst serological surveys demonstrate that bats must be exposed to lyssavirus without developing clinical disease. We hypothesize that there is endemic circulation of Lagos bat virus (LBV) in the straw-coloured fruit bat (*Eidolon helvum*) in Ghana, West Africa. To investigate this further, longitudinal blood sampling was undertaken quarterly between 2012 and 2014 on wild *E. helvum* at two sites in Ghana. Serum samples were collected and tested for LBV-neutralizing antibodies using a modified fluorescent antibody virus neutralisation (FAVN) assay ($n = 294$) and brains from moribund or dead bats were tested for antigen and viral RNA ($n = 55$). Overall, 44.7% of the 304 bats sampled had LBV-neutralising antibodies. None of the brain samples from bats contained lyssavirus antigen or RNA. Together with the results of an earlier serological study, our findings demonstrate that LBV is endemic and circulates within *E. helvum* in Ghana even though the detection of viral infection in dead bats was unsuccessful. Confirmation that LBV infection is endemic in *E. helvum* in Ghana is an important finding and indicates that the potential public health threats from LBV warrant further investigation.

Keywords: bat; rabies; *Eidolon helvum*; Lagos bat virus; seroprevalence; lyssavirus; Ghana

1. Introduction

Wildlife populations constitute a large and largely undefined reservoir of infectious agents, some of which are zoonotic [1–3]. Infectious pathogens that originate in wild animals have become increasingly important globally, as they have had substantial impacts on human health, agricultural production, wildlife-based economies and wildlife conservation. Bats (*Chiroptera*) represent a large group of wildlife, comprising ~20% of the 4600 mammalian species recognized to date [4]. Bats have several key ecological functions, including pollination, seed dispersal and insect control [5]. However, bats have also been implicated as the reservoir hosts for numerous emerging infectious diseases. Increasingly, bats have been recognized as important reservoir hosts for viruses that have the potential to cross species barriers to infect humans, domestic and wild mammals [4,6].

Lyssaviruses (family *Rhabdoviridae*, genus *Lyssavirus*) are the aetiological agents that cause the disease rabies—an acute, progressive, viral encephalitis that is almost invariably fatal [7]. Most lyssaviruses are carried by bats, but the association of these viruses with bats is complex and poorly understood [8]. Rabies virus (RABV) is found in terrestrial carnivores globally, but it is only found in bats in the Americas, where it is endemic in frugivorous, insectivorous and hematophagous bats [8]. In contrast, the other 13 known lyssavirus species have only been detected in the Old World, predominantly in bat species although infection of terrestrial carnivores has been documented for some lyssavirus species [9]. Globally, the most significant public health and veterinary burden caused by lyssaviruses is dog-mediated transmission of RABV, but instances of other lyssavirus infection of humans or domesticated animals have been reported on rare occasions following spill-over events [8,10].

One bat lyssavirus that has been associated with the infection of terrestrial carnivores is Lagos bat virus (LBV), a virus most commonly associated with infection of African fruit bats. Fruit bats of several species have been identified as reservoir hosts for LBV, with spill-over infections reported in dogs, cats and a mongoose [11–14]. Notably, LBV has never been reported in human cases of rabies or any other case of human meningoencephalitis. Diagnostic tests that can differentiate between lyssaviruses are not available in the majority of rabies endemic areas, therefore clarity surrounding the impact of non-RABV lyssaviruses, such as LBV, is lacking. High seroprevalence against lyssaviruses has been reported in apparently-healthy bats, including for LBV in the straw-colored fruit bat (*E. helvum*) in Ghana [15]. While the ecology, pathogenesis and pathology of RABV in terrestrial animals and humans is partially understood [7], knowledge on the other lyssavirus species is scant. In this study, investigations into the presence of LBV infection in a natural host, *E. helvum*, were made through post-mortem sampling of moribund and dead bats as well as through serological assessment of samples for LBV neutralizing antibodies.

2. Material and Methods

2.1. Ethics Statement

The study was approved by the Institutional Review Board of the Nuguchi Memorial Institute for Medical Research of the University of Ghana (NMIMR-ITB CPN 002/13-14 IORG0000908) and the Ethics Committee of the Zoological Society of London (WLE/0638).

2.2. Study Sites

This study was undertaken at two *E. helvum* roost sites in Ghana, West Africa. The roosts comprised between approximately 3000 to 1,000,000 bats each, depending on year and season. One roost was located in Greater Accra (05°35.192' N, 000°11.053' W), within the grounds of a large general infirmary (37-Military Hospital) in the center of the city. The other roost was in Tanoboase in the Brong-Ahafo region (07°38' N, 01°11.54' W), within a protected forest area (Figure 1). The site in Tanoboase, known locally as Tano Sacred Grove, was developed for ecotourism in 1996 and is visited

by both local and international tourists. The site also hosts an annual Apoo festival (a time of spiritual cleansing held in April–May).

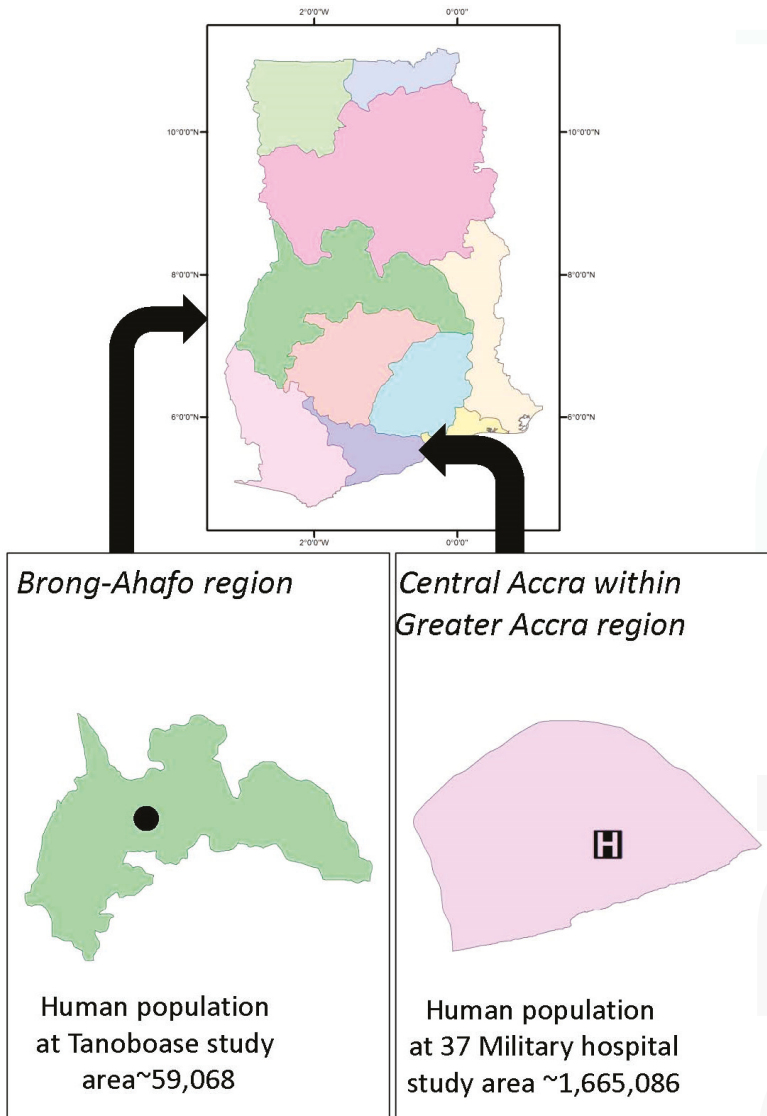


Figure 1. Locations at which wild straw-colored fruit bats (*Eidolon helvum*) were captured and sampled.

2.3. Study Design

Sera Collection for Lagos Bat Virus Antibody Prevalence in Wild *Eidolon helvum*

Wild bats were captured using mist nets from 2012 to 2014 inclusive, as detailed in Table 1. Sampling in June/July was aimed to correspond with late-stage pregnancy. In July, the majority of the bats in each roost had migrated to alternative, as yet unknown, sites. By November, most

bats had returned to each roost. Captured bats were housed individually in cloth bags for short time periods before being processed. The sex and sexual maturity of each bat were assessed based on body size (body mass and forearm length were measured routinely) and observation of external genitalia and mammary tissue. Approximately 1 mL of blood was collected in micro-tubes (SARSTEDT, Germany) from the propatagial vein of each individual, as described previously [15]. Blood samples were centrifuged at $6000 \times g$ for 15 min and the separated sera were stored at -70°C until required. Each bat was micro-chipped (using the Trovan animal identification system, Electronic Identification Systems, Germany) to enable identification in case of recapture. Bats were released at the site of capture immediately following sampling, after ensuring haemostasis at the site of venipuncture.

Table 1. Sample data collected.

		37-Military Hospital			Tanoboase		Totals
Sampling Date		10-06-2013	04-09-2013	14-07-2014	17-12-2012	01-07-2014	
Age	Adult	34	87	32	57	26	236
	Juvenile	11	6	3	0	2	22
	Sub-adult	0	25	11	0	0	36
	Not determined	0	1	3	5	1	10
Sex	Male	34	88	30	33	25	210
	Female	11	30	16	24	3	84
	Unsexed		1	3	5	1	10
Serostatus	Positive	17	53	24	29	13	136
	Negative	28	66	25	33	16	168

2.4. Collection and Examination of Dead Bats

Members of the public who frequented the areas under the roosts were asked to report any dead or sick bats they found. Also, during each sampling visit, searches for dead or sick bats were undertaken in the immediate vicinity of the roosts. Sick bats were euthanised using intramuscular ketamine and medetomidine, followed by exsanguination via cardiac puncture. Bat carcasses were stored in a freezer at -70°C until necropsy. At necropsy, a range of tissues, including brain, was collected and stored fixed or frozen for later investigation.

2.5. Laboratory Investigations

2.5.1. Antigen Detection Using the Fluorescent Antibody Test (FAT)

Fluorescein isothiocyanate (FITC)-conjugated anti-rabies nucleocapsid polyclonal antibody conjugate (Fujirebio Diagnostics, Malvern, PA, USA) was used diluted 1:40 in 0.1 M phosphate buffered saline (PBS) pH 7.2 to stain virus antigen in acetone-fixed impression smears taken from the hippocampus, cerebellum and brain stem of bats found dead or sick (following euthanasia), as described previously [16]. Slides were examined using a fluorescence UV microscope. LBV-infected mouse brain material was used as a positive control for comparison of antigen distribution.

2.5.2. Detection of LBV-Neutralizing Antibodies (mFAVN)

The presence of antibodies capable of neutralizing LBV in collected bat sera was assessed using a modified version of the fluorescent antibody virus neutralization assay (mFAVN), as described previously [2,15]. Briefly, lineage B LBV [17] was prepared to a working concentration of 100 tissue culture infective doses (TCID)₅₀/50 μL . Both negative sera from an unvaccinated dog and positive anti-LBV Nig56 rabbit serum samples were used as serological controls for the assay. Each test serum sample was diluted three-fold (starting with a 1:9 dilution) in minimum essential medium (MEM), and incubated with the viral inoculum for 1 h at 37°C before being added to BHK-21 cells in suspension in a 96-well plate. Following incubation for 48 h at 37°C , the plates were fixed in 80% chilled acetone for 20 min, and stained with FITC-labelled anti-rabies polyclonal antibody (Fujirebio Diagnostics, Malvern, PA, USA). Fluorescent foci present in cells infected with LBV were visualized at 488 nm using

a fluorescence UV microscope. The lack of accurately-titred control sera meant that all neutralizing antibody levels were expressed as D_{50} values. The D_{50} for each sample is the dilution endpoint at which 50% of the wells showed the presence of virus. D_{50} values were calculated using the Spearman-Kärber method. A serological cut-off was assigned according to neutralization of control sera and a back titration of input virus.

2.5.3. Detection of LBV RNA Using Molecular Methods

Total RNA was extracted from brain tissue samples using Trizol (Invitrogen) following the manufacturer's protocol. A pan-lyssavirus SYBR Green real-time (q) RT-PCR [18] and a pan-lyssavirus conventional hemi-nested-RT-PCR [19] were used to determine the presence or absence of viral RNA.

3. Statistical Analysis

The chi-squared test was used to investigate if there were significant differences in seroprevalence according to the age, sex and roost location of bats.

4. Results

4.1. LBV Seroprevalence

Samples taken and data regarding age, sex and serology are detailed in Table 1. A total of 304 bat sera were collected and tested for LBV neutralization antibodies during the sampling periods described. Of the 304 sera tested, 44.7% ($n = 136$) were considered seropositive (Table 1; Figure 2). This comprised 213 from the 37-Military Hospital in Accra and 91 from the Tanoboase roost site, with 236 adults, 36 sub-adults, 22 juveniles and 10 bats of undetermined age (morphometric data not recorded); 210 males, 84 females and 10 of undetermined sex being tested across the two sites (Table 1). Seroprevalences were high during the three years of sampling (2012—46.8%, $n = 62$; 2013—42.7%, $n = 164$; 2014—47.4% $n = 78$). Of those bats that tested serologically positive, 45.2% were known to be male ($n = 95/210$) while 61.9% were identified as female ($n = 52/84$). Results from samples collected from bats of undetermined sex or age were not included in the statistical analyses. There was no significant difference in the different age groups that tested positive (p -value = 0.78) (Table 2). Seroprevalence in adult bats (0.46, $n = 236$) was slightly higher than in the sub-adult and juvenile bats combined (0.43, $n = 58$).

Table 2. Seroprevalence in wild-caught bats by age.

	Total Sampled	Total Positive (% Positive)
Adult	136	109 (80.15%)
Juvenile	58	25 (43.10%)
Total	294	134 (45.57%)

In June 2013, two nursing *E. helvum* dams (ID: 900 and ID: 905) were caught with their pups (ID: 901 and 904, respectively) at the 37-Military Hospital roost site. All four animals were apparently healthy with a good body condition and no outward signs of disease. Following serological assessment, the two mothers were both seronegative with a D_{50} of 5.20. In contrast, both pups were seropositive with detectable anti-LBV neutralizing antibody D_{50} of 46.77 and 81.00 for animals 901 and 904, respectively.

Overall, there was no statistically significant difference in seroprevalence between the two sites (Table 1). At 37-Military Hospital, 44.1% ($n = 94/213$) were seropositive (Figure 2a) compared to 46.1% ($n = 42/91$) of bats at Tanoboase (Figure 2b) (chi-square test = 0.04; odds ratio (OR) = 1.085; relative risk (RR) = 1.046; $p = 0.84$).

To assess potential seasonal variation, an evaluation of seropositivity between the two sites was made for each sampling time. From the total sample set obtained for the two sites, there

was no statistical significance in the seasonal difference in seroprevalence between the dry season (December–March) and the rainy season (April–November) ($p = 0.83$) (Table 3).

Table 3. Proportion of seroprevalence in wild-caught bats by season.

	Positive (+ve)	Negative (–ve)	Total Tested
Dry Season	29 (21%)	33 (20%)	62 (20%)
Rainy Season	107 (79%)	135 (80%)	242 (80%)
Totals	136 (45%)	168 (55%)	304

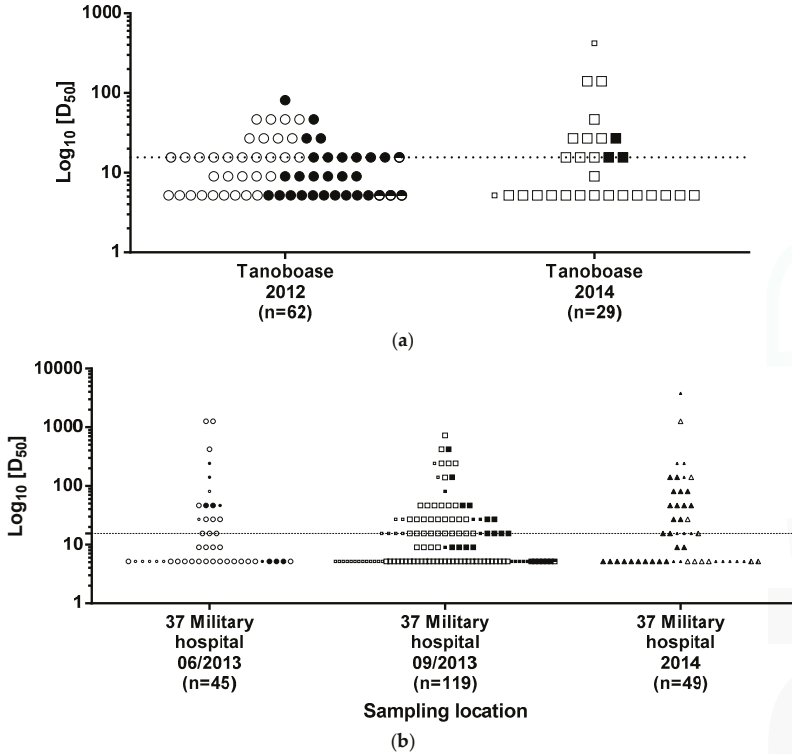


Figure 2. Serological profiles of straw-colored fruit bats sampled at (a) Tanoboase in the Brong-Ahafo region and (b) at 37-Military Hospital, Greater Accra. Open symbols represent male bats, closed symbols represent female bats; half-filled symbols represent bats of undetermined sex. Smaller symbols represent juvenile or sub-adult bats. The D_{50} value considered the cut-off for positivity is indicated by a dashed line.

4.2. Necropsy Findings in *E. helvum* Bats

Forty-six bats were examined post-mortem, of which 38 bats had been collected from the 37-Military Hospital roost and eight bats collected from Tanoboase. Thirty-three bats were found dead, while 13 bats were found moribund and were euthanized, following clinical assessment, on welfare grounds (Table 4). Most moribund bats ($n = 11$) had traumatic injuries of unknown origin. All brain samples were negative for lyssavirus antigen and RNA using FAT and the RT-PCRs, respectively.

Table 4. Necropsy findings in dead/moribund *E. helvum* bats. All bats found sick were terminated humanely and blood taken as described.

Bat ID	Location	Date Found	Condition	Comments
A1	37-Hospital	20/01/2013	Found dead	N.A.D. *
A2	37-Hospital	20/02/2013	Found dead	N.A.D.
A3	37-Hospital	20/02/2013	Found dead	N.A.D.
A4	37-Hospital	20/02/2013	Found dead	N.A.D.
A6	37-Hospital	28/10/2013	Found dead	Very enlarged spleen
A5	Accra Ridge	29/09/2013	Found dead	Found dead
A12	37-Hospital	27/11/2013	Found sick	Paralysed
A13	37-Hospital	27/11/2013	Found dead	Fractured right arm
A7	37-Hospital	07/12/2013	Found sick	Paralysis
A8	37-Hospital	07/12/2013	Found sick	Paralysis
A9	37-Hospital	07/12/2013	Found dead	Abscess on neck
A10	37-Hospital	10/12/2013	Found dead	N.A.D.
A11	37-Hospital	08/12/2013	Found dead	Fractured forearm
A14	37-Hospital	11/12/2013	Found dead	Fractured forearm
A15	37-Hospital	11/12/2013	Found dead	Severe intestinal, abdominal haemorrhages
A16	37-Hospital	11/12/2013	Found dead	Fractured head
A17	37-Hospital	13/12/2013	Found sick	Paralysis
A18	37-Hospital	13/12/2013	Found dead	N.A.D.
A19	37-Hospital	13/12/2013	Found dead	Punctured wing
A22	37-Hospital	22/12/2013	Found sick	Paralysis, severe haemorrhages on head
A23	37-Hospital	22/12/2013	Found dead	N.A.D.
A24	37-Hospital	22/12/2013	Found dead	N.A.D.
A20	37-Hospital	03/01/2014	Found dead	N.A.D.
A21	37-Hospital	03/01/2014	Found dead	Emaciated carcass
A25	37-Hospital	22/01/2014	Found dead	Fractured forearm
A26	37-Hospital	22/01/2014	Found dead	Decomposed carcass
A27	37-Hospital	22/01/2014	Found dead	N.A.D.
A28	37-Hospital	29/01/2014	Found dead	N.A.D.
A29	37-Hospital	05/02/2014	Found dead	N.A.D.
A30	37-Hospital	12/03/2014	Found dead	N.A.D.
A31	37-Hospital	12/03/2014	Found dead	N.A.D.
A32	37-Hospital	12/03/2014	Found dead	N.A.D.
A33	37-Hospital	12/03/2014	Found dead	N.A.D.
A34	37-Hospital	12/03/2014	Found dead	N.A.D.
A35	37-Hospital	12/03/2014	Found dead	N.A.D.
A36	37-Hospital	06/04/2014	Found sick	Paralysis, emaciated
A37	37-Hospital	06/04/2014	Found sick	Paralysis
A38	Achimota	09/07/2014	Found dead	N.A.D.
T1	Tanoboase	22/01/2013	Found dead	N.A.D.
T2	Tanoboase	22/01/2013	Found sick	Fractured forearm
T3	Tanoboase	22/01/2013	Found sick	Fractured forearm
T4	Tanoboase	23/01/2013	Found dead	Multiple body injury
T5	Tanoboase	23/01/2013	Found sick	Fractured forearm
T6	Tanoboase	07/03/2013	Found sick	Weak
T7	Tanoboase	07/03/2013	Found sick	Multiple body injury
T8	Tanoboase	07/03/2013	Found sick	Weak

* N.A.D.—no abnormality detected

5. Discussion

A total of 304 bat sera was collected and tested for LBV neutralizing antibodies from 2012 to 2014. Of the 304 sera tested, 136 tested positive (seroprevalence: 44.7%) (Table 1; Figure 2). Seroprevalences were high during each of the three sampling years (2012—46.8%, $n = 62$; 2013—42.7%, $n = 164$; 2014—47.4% $n = 78$). These results, taken together with similar LBV serology results reported previously [2], demonstrate the long-term endemic circulation of LBV in the Ghanaian *E. helvum* population. Previous studies have reported serological evidence of exposure to LBV in different *E. helvum* populations, including in Nigeria (14–44% seropositivity; $n = 140$), Ghana (37% seropositivity; $n = 66$), and Kenya (40–67% seropositivity; $n = 102$) [15,17,18]. This is the first time a previously-described seropositive population has been re-tested several years later and has been shown

to maintain a similar level of seroprevalence. The mechanism of maintenance for lyssaviruses in bat species with very high roost numbers and population densities is largely undefined, although several variables including population size, contact rates, species susceptibility, immunological competence, and roost structure may all contribute to virus maintenance via an as-yet-undefined mechanism [8,20].

The size of populations involved within the roosts studied and the movement between defined roosts and other roosting sites that remain undefined during migratory periods may influence virus exposure and hence seropositivity. There was no difference in seroprevalence between the two field sites when assessing data comparing sex and age of sampled bats. However, this species is migratory and individuals may travel long distances to feed [21] perhaps enabling interaction between *E. helvum* populations and possibly also those of other bat species.

Of the 294 sera from wild-caught bats tested of known age class, 134 (45.5%) were serologically positive for LBV neutralizing antibodies. There was no significant difference in the seroprevalence detected between the different age groups tested. The slightly higher proportion of seropositive adult bats (0.46) over juvenile bats (0.43) are similar to levels reported in studies addressing serological positivity to RABV in hoary bats (*Lasiurus cinereus*) [22,23]. The observation of two serologically-positive pups from dams that were serologically negative is unexplained. Maternal antibody transfer is unlikely in the absence of detectable circulating antibodies in the dams. Whilst both dams were seronegative, it is possible that their neutralizing antibodies were below the detection limits of the test applied. The pups might also have acquired the antibodies from other seropositive mothers (through allosuckling) or survived infection and thus acquired immunity.

The effect of migration and coloniality on infection dynamics is not known. Reduced migration and population connectivity has been suggested as a mechanism that could increase the amplitude of seasonal outbreaks and increase the probability for spillover of pathogens to other species, such as human beings [24]. The absence of any seasonal difference in LBV seroprevalence was surprising as increased interactions between bats, and hence an increase in pathogen transmission, might be expected during the dry season in Ghana, when there are increased numbers and an increased population density at the roosting sites. It is possible, though, that the bats have similar aggregation and colonial roosting behaviors during migration as observed during the dry season in Ghana. If so, this would account for the lack of variation in seroprevalence observed in this study.

The current understanding of lyssavirus serological positivity within bat populations is poor. Persistence of neutralizing antibodies to lyssaviruses for longer than 12 months has been described previously [25,26]. The long duration of antibodies detected in bats may reflect repeated exposure and may explain the elimination of notable seasonal serological fluctuation in wild-caught bats. Such bats will also influence the epidemiology of an outbreak in the population. From such a small sample set, however, strong conclusions cannot be made and there is a need for a more extensive study to assess serodynamics in bat populations.

The distribution of LBV lineages across Africa is diverse (reviewed in [8]). In Ghana, only serological evidence of LBV has been previously reported, and then only using neutralization assays with lineage B LBV as the test virus [2,15]. The data generated here were also based on using LBV B as the test virus for neutralization. Future studies might benefit from assessment against all four lineages of LBV as well as against other lyssavirus for the potential detection of cross-neutralizing antibodies. Indeed, interpreting serological responses to lyssaviruses is problematic due to the potential for exposure, and serological responses to, undiscovered virus(es). It is possible that further related lyssaviruses exist, confusing the interpretation of these serological outputs. Nevertheless, numerous bats demonstrated significant neutralizing antibody titres against the test virus. This must indicate prior exposure to an LBV or to an antigenically-related lyssavirus.

Where higher titres are seen, it may indicate repeated exposure to antigenically-similar viruses that drive an anamnestic response. A multiple exposure infection study with rabies virus in bats [26] demonstrated that repeated infection may confer significant immunological memory and reduced susceptibility to RABV infection. It is, therefore, unknown if high seroprevalence is driven by exposure

to sub-lethal doses of LBV via routes other than biting, such as from aerosol, grooming or food sharing. The significance of lyssavirus seropositivity in bat populations currently remains undefined.

In an attempt to detect live virus in bats at each study site, diseased and dead bats were assessed for the presence of virus antigen and nucleic acid in brain material. Clinical signs observed in moribund bats included paralysis, ataxia, weakness, vocalization and self-urination. The inability to detect LBV antigen or RNA in the brain samples of moribund or dead bats supports previous findings [27] that the infection prevalence of lyssavirus in gregarious colonial bat species is usually less than 1% of the population. The assays used for molecular detection of viral RNA in the present study are pan-lyssavirus in design and have been assessed to ensure that they are able to detect all lyssaviruses described to date. Many of the necropsied bats were found with evidence of trauma and might have been injured via intra-specific aggression, predators or human hunters; *E. helvum* is widely hunted in Ghana [28]. Many bats, however, had no evidence indicating possible cause of illness or death. A much larger sample size of dead or moribund bats may improve the chances of detecting infection with LBV or a related lyssavirus. A similar study in Ghana with a much higher sample size ($n = 567$) succeeded in isolating LBV in one *E. helvum* bat [29].

6. Conclusions

A high prevalence of antibodies against Lagos bat virus (LBV) in *E. helvum* and other bat species was previously demonstrated in Ghana [15,30]. Further follow-up studies confirmed the endemicity of LBV in *E. helvum* in Ghana [2]. The current study reports continued high antibody prevalence levels that support earlier findings. The high seroprevalence of LBV reported previously and in this study in apparently healthy *E. helvum* colonies provides evidence of long-term maintenance and circulation of LBV in this bat population. Demonstrating the maintenance of antibody titres within bat populations is important as it indicates that individual bats are being re-exposed to virus, which in turn suggests that bats are shedding virus and are exposing conspecifics. Whilst this perhaps is not surprising, demonstrating what appears to be a stable level of seroprevalence in a population is important. The mechanisms of transmission of bat lyssaviruses remain ill-defined but minimally, the data presented here reiterate that virus continues to circulate within the bat population, which is important in our understanding of the relationship between bats and lyssaviruses.

Ongoing studies have indicated the occurrence of close interactions between chiropteran, domestic animal, and human populations. Current methods used to confirm rabies virus infection in people and terrestrial carnivores are unable to differentiate between different lyssavirus species. As human consumption of bats is practiced in many parts of Ghana [30], the potential for human infection with LBV exists and the invariably fatal outcome of lyssavirus infection poses questions as to the risk from bat populations. Importantly, existing rabies vaccines do not fully protect against some African non-rabies lyssaviruses including LBV, Mokola virus, Shimoni bat lyssavirus, Ikoma lyssavirus and West Caucasian bat virus [31–35]; therefore an understanding of the potential risk of transmission of these viruses to human populations is required. The results presented here are of relevance to public health, although further information is required on LBV infection dynamics within bat populations, potential spillover mechanisms, and bat population dynamics, to further define the risk of human infection.

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Article

Pathogenicity and Immunogenicity of Recombinant Rabies Viruses Expressing the Lagos Bat Virus Matrix and Glycoprotein: Perspectives for a Pan-Lyssavirus Vaccine

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Abstract: Lagos bat virus (LBV) is a phylogroup II lyssavirus exclusively found in Africa. Previous studies indicated that this virus is lethal to mice after intracranial and intramuscular inoculation. The antigenic composition of LBV differs substantially from that of rabies virus (RABV) and current rabies vaccines do not provide cross protection against phylogroup II lyssaviruses. To investigate the potential role of the LBV matrix protein (M) and glycoprotein (G) in pathogenesis, reverse genetics technology was used to construct recombinant viruses. The genes encoding the glycoprotein, or the matrix and glycoprotein of the attenuated RABV strain SPBN, were replaced with those of LBV resulting in SPBN-LBVG and SPBN-LBVM-LBVG, respectively. To evaluate the immunogenicity of the LBV G, the recombinant RABV SPBNGAS-LBVG-GAS was constructed with the LBV G inserted between two mutated RABV G genes (termed GAS). All the recombinant viruses were lethal to mice after intracranial (i.c.) inoculation although the pathogenicity of SPBNGAS-LBVG-GAS was lower compared to the other recombinant viruses. Following intramuscular (i.m.) inoculation, only SPBN-LBVM-LBVG was lethal to mice, indicating that both the M and G of LBV play a role in the pathogenesis. Most interestingly, serum collected from mice that were inoculated i.m. with SPBNGAS-LBVG-GAS neutralized phylogroup I and II lyssaviruses including RABV, Duvenhage virus (DUVV), LBV, and Mokola virus (MOKV), indicating that this recombinant virus has potential to be developed as a pan-lyssavirus vaccine.

Keywords: rabies; vaccines; Africa; lyssavirus; pathogenesis; recombinant viruses

1. Introduction

The *Lyssavirus* genus consists of 14 viral species and three putative species, grouped into three phylogroups [1–3]. While all members of the lyssavirus genus are capable of causing a fatal encephalitic disease, rabies virus (RABV) in phylogroup I is responsible for most human and animal rabies cases and is globally distributed. Other lyssavirus species have a more restricted distribution, with Lagos bat lyssavirus (LBV), Mokola lyssavirus (MOKV), Duvenhage lyssavirus (DUVV), Shimoni bat lyssavirus (SHIBV) and Ikoma lyssavirus (IKOV) exclusively identified from Africa. LBV is associated with Pteropodidae bat species [4–6] and although no human infections have been reported to date,

fatal spill-over into dogs [7,8], cats [5] and a mongoose [9] have been reported. Although case reports of LBV are limited, pathogenicity studies in mice have indicated that the virus is pathogenic to mice when inoculated via the intramuscular (i.m.) and intracranial (i.c.) route with distinct pathogenicity profiles observed between different LBV lineages [6,10,11].

The lyssavirus genome codes for five proteins; the nucleo- (N), phospho- (P), matrix- (M), glyco (G) and RNA polymerase protein (L) and their cooperative effect in pathogenesis have been shown [12–15]. Gene exchange between RABV strains with different pathogenicity profiles has been performed in previous studies mostly focusing on the G protein [13,16–20]. Different pathogenic mechanisms have been linked to this protein, including interaction with the cell surface molecules, p75 neurotrophin receptor [21,22], nicotinic acetylcholine receptor [23] and neural cell adhesion molecule [24], to facilitate binding and entry into the cell. Controlled expression levels of the G gene are important to prevent apoptosis of neuronal cells and they allow transport of the virus to the central nervous system [25]. A number of studies have demonstrated that replacement of the G gene from a non-pathogenic RABV strain with that of a pathogenic strain results in a pathogenic RABV strain [13,16,17,20].

Replacement of the M gene of Ni-CE strain (non-pathogenic both i.c. and i.m.) with that of the Nishigahara strain (pathogenic both i.c. and i.m.) was shown to result in a pathogenic strain when mice were inoculated i.c. [15]. Pulmanasahakul et al. (2008) showed that replacement of both the M and G gene of a non-pathogenic strain with that of a pathogenic strain resulted in increased pathogenicity when compared with replacing only the G gene. Substitution of the M gene alone did not result in a pathogenic strain. In another study, replacement of the M, G, G-L intergenic region and L gene from a pathogenic strain with that of a non-pathogenic strain resulted in a non-pathogenic strain when mice were inoculated i.m., but when only the G, G-L region and L gene were replaced, the virus was pathogenic [12]. The M gene of LBV and MOKV has also been shown to induce apoptosis *in vitro* in neuroblastoma and HeLa cells [26]. Apoptosis has been reported to be inversely proportional to pathogenicity of RABV [25,27].

The recombinant RABV backbones (non-pathogenic i.m.) were used for interspecies gene or partial gene replacement between RABV and other lyssaviruses in previous studies. Interspecies G protein substitution was performed between a RABV vaccine strain (SAD B19) and European bat lyssavirus type 1 and 2 (EBLV-1 and EBLV-2) [18]. The RABV with an EBLV-1 G protein was shown to cause higher mortality when inoculated i.m. compared to inoculation with a RABV recombinant backbone. Genz et al. (2012) also generated chimeric RABV and EBLV-1 or EBLV-2 G protein in a backbone of RABV (SAD B19). The chimeric G protein consisted of the cytoplasmic tail from RABV while the transmembrane and ectodomain was from the EBLVs. The recombinant viruses had comparable growth kinetics *in vitro* and were lethal to mice when inoculated i.c. [28]. Interspecies M protein substitution between the RABV vaccine strain (SAD B19) and EBLVs (EBLV-1 and EBLV-2) were performed and it was shown that the M protein plays a role in intracellular virus accumulation [29]. Although gene exchange between different strains of RABV has been done, no study has performed gene exchange between RABV and LBV [12].

All currently licensed lyssavirus vaccines are based on a RABV backbone. These vaccines have been shown to protect against members of phylogroup I lyssaviruses [30–35]; however, there appears to be no vaccine affording protection against phylogroup II and III lyssaviruses [2,30,36–38]. Mice vaccinated with RABV vaccine produced significantly lower virus-neutralizing antibodies (VNAs) against DUVV when compared to RABV VNAs.

The generation of recombinant viruses using reverse genetics systems has led to the construction of safe and immunogenic rabies vaccines. Recombinants included the introduction of two or three G genes [39–42], mutation of pathogenic domains on the G gene [39,40], deletion of genes required for replication [43] and introduction of inflammatory cytokines [44,45], chemokines [46] and pro-apoptotic [47] genes into the RABV genome. Recombinant RABV vaccines containing two RABV G genes were shown to express high levels of the G protein, and were more immunogenic than parental strains [39,41,42]. To increase the safety of the vaccine, mutations of Asn 194 to Ser and

Arg 333 to Glu were introduced in the G gene [39,48,49]. A recombinant RABV vaccine with three G genes was investigated [40] and the triple G vaccine was shown to be non-pathogenic to juvenile mice, adult mice deficient in some immune functions, and normal adult mice when inoculated via the i.c. route. The recombinant vaccine protected mice against lethal challenge with RABV and also protected mice when administered as post-exposure prophylaxis.

The aim of this study was to determine the importance of the LBV M and G genes in pathogenesis using a recombinant RABV and replacing the G gene, as well as both the M and G gene, with that of LBV. In addition, the pathogenicity and immunogenicity of a RABV backbone with a LBV G gene sandwiched between two RABV G genes was determined. We demonstrate that the RABV backbone can express functional LBV genes, and that both the M and G genes of LBV play a role in pathogenicity. The feasibility of generating a pan-African lyssavirus vaccine that can induce neutralizing antibodies against at least phylogroup I and II lyssaviruses was demonstrated.

2. Material and Methods

2.1. Virus Isolates, Recombinant Viruses and Cell Lines

The recombinant viruses, SPBN and SPBNGAS-GAS-GAS, were constructed in previous studies [40,50]. The LBVAFR1999 [51,52] was maintained in mouse neuroblastoma (MNA) cells (C-1300). MNA and BSR-T7 (a clone of BHK-21) [53] cells were grown in an atmosphere of 37 °C and 5% CO₂ using Dulbecco's modified Eagle's medium (DMEM/F12) (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (Lonza, Verviers, Belgium) and 1% antibiotics (penicillin [100 units/mL], streptomycin [100 µg/mL] and amphotericin B [0.25 µg/mL]) (Lonza, Verviers, Belgium).

2.2. Construction of the Recombinant Viruses

Total RNA was isolated from LBVAFR1999 cell culture material using TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. To replace the G gene of SPBN with that of the LBVAFR1999, the G gene was amplified from the LBVAFR1999 RNA using *Pfu* DNA polymerase (Promega, Madison, WI, USA) and the primers LBVXmaI and LBVPacI containing restriction enzyme sites *XmaI* and *PacI* respectively (Table 1). The amplified PCR product was digested with *XmaI* and *PacI* (New England Biolabs, Ipswich, MA, USA) and then ligated to pSPBN previously digested with *XmaI* and *PacI*. The resulting plasmid was designated pSPBN-LBVG (Figure 1). To replace both the M and G genes of SPBN with those of the LBVAFR1999, the *KpnI* site was introduced upstream of the pSPBN-LBVG M gene start signal, through digestion of another pSPBN (that contains the *KpnI* site) with *AvrII* and *KpnI* (New England Biolabs, Ipswich, MA, USA) (Figure 1). The digested fragment was ligated to pSPBN-LBVG previously digested with the same restriction enzymes (*AvrII* and *KpnI*). LBVAFR1999 M gene was synthesized (GenScript, Piscataway, NJ, USA) to contain the *KpnI* and *XmaI* restriction enzyme sites. The M gene was digested with the *KpnI* and *XmaI* and then ligated to pSPBN-LBVG previously digested with *KpnI* and *XmaI*. The resulting plasmid was designated pSPBN-LBVM-LBVG (Figure 1). To construct the recombinant virus with a LBVAFR1999 G gene sandwiched between two RABV G genes, the G gene was amplified from the LBVAFR1999 RNA using *Pfu* DNA polymerase (Promega, Madison, WI, USA) and the primers LBVEcoRIBsiWI and LBVXbaIAsiSI (Table 1). The amplified PCR product was digested with *BsiWI* and *AsiSI* (New England Biolabs, Ipswich, MA, USA) and then ligated to pSPBNGAS-GAS-GAS previously digested with *BsiWI* and *AsiSI*. The resulting plasmid was designated pSPBNGAS-LBVG-GAS (Figure 1).

Table 1. Primers used for amplification or sequencing of the Lagos bat virus matrix and glycoprotein genes.

Primer	Sequence in 5' to 3' Direction *	Gene Targeted
LBVXmaI	TATCCCCCGGAAGATGAGTCAATTGTCTCAACC	G gene
LBVPacI (to be used with LBVXmaI)	CCGACCTTAATTAAGGTTAGACACTGATGTCTCTTATATG	G gene
LBVEcoRIBsiWI	GCAGAA T CCGTACGAAGATGAGTCAATTGTCTCAACC T CAT	G gene
LBVXbaIAsiSI (to be used with LBVEcoRIBsiWI)	TAA TCTAGAG CGCATCGCCGTTAGACACTGATGTCTCTTATATGA	G gene
LBV1319F (binds on position 1319 on the open reading frame of the LBVAFR1999 G gene, EF547432)	CGACTTCGTCGATGCCACATGCC	G gene
LBV255R (binds on position 255 on the open reading frame of the LBVAFR1999 G gene, EF547432)	GTTGGTGATGTGACTGCCTCG	G gene
RabC2121F (to be used with LBV255R [binds on position 2121 on the complete genome of RABV, NC_001542])	CAGTGAGGCTGAGATCGCTC	M gene

* Restriction enzyme sites are indicated in bold or underlined, start/stop codons are in italics.

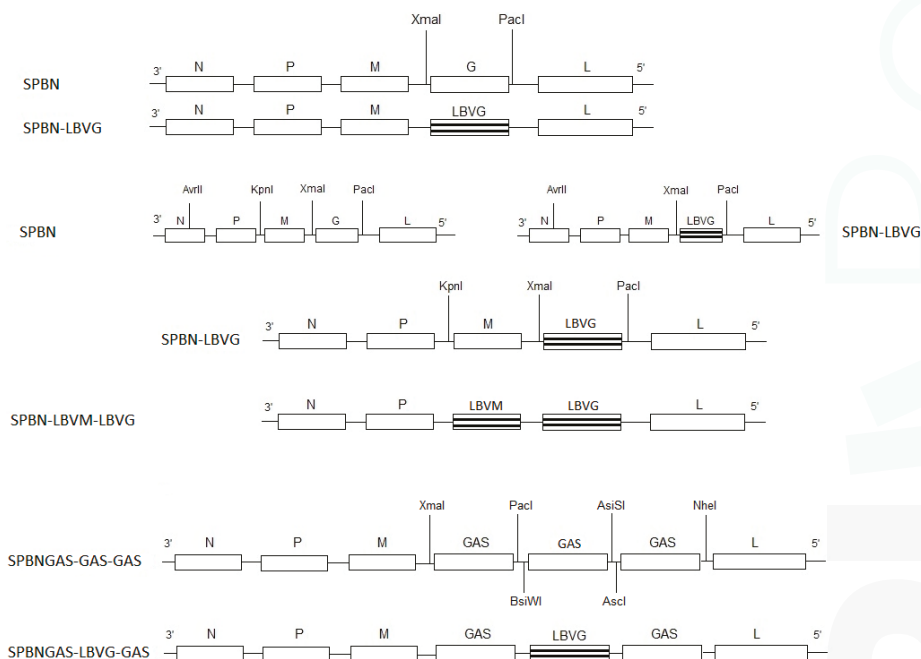


Figure 1. Schematic representation of the construction of recombinant viruses. Restriction enzyme sites are indicated (*XmaI*, *PacI*, *AvrII*, *KpnI*, *BsiWI*, *AsiSI*, *NheI* and *AscI*). GAS represents the SPBN G gene with two amino acid substitutions (Asn 194 to Ser and Arg 333 to Glu). The following abbreviations were used: N, nucleoprotein; M, matrix protein; G, glycoprotein; L, RNA-dependent RNA polymerase. LBVM and LBVG represent the LBV M and G gene respectively.

2.3. Rescue of the Recombinant Viruses

The recombinant viruses were rescued as described previously [40,50]. Briefly, 250 µL of FuGENE (Promega, Madison, WI, USA) in serum-free DMEM (Lonza, Verviers, Belgium) was used to transfect BSR-T7 cells with 10 µg of full length plasmids (pSPBN-LBVG, pSPBN-LBVM-LBVG or pSPBNGAS-LBVG-GAS), helper plasmids (5 µg of pTIT-N, 2.5 µg of pTIT-P, 2.5 µg of pTIT-L, 1 µg of pTIT-G) and 2 µg of pTIT-T7 (a plasmid expressing the T7 RNA polymerase). Supernatants were transferred onto MNA cells in 12-well plates (Greiner Bio-one, Frickenhausen, Germany) 72 h post-transfection and incubated in an atmosphere of 37 °C and 5% CO₂ for 72 h. Positive cells were determined by immunostaining [54] using fluorescein isothiocyanate conjugate (FITC)-labeled RABV

N gene specific antibody (Centocor Inc., Malvern, PA, USA). The nucleotide sequence of the inserted genes was confirmed by sequencing as described previously [10].

2.4. Amplification and Titration of the Rescued Recombinant Viruses

MNA cells in 6-well plates (Greiner Bio-one, Germany) were infected with supernatant from wells that were positive during transfection and incubated in an atmosphere of 37°C and 5% CO₂ for 72 h. The supernatant was harvested and then immunostaining was performed on the plates using FITC-labeled RABV N gene specific antibody (Centocor Inc.). Supernatant from positive wells in the 6-well plates (Greiner Bio-one, Germany) was subsequently amplified in MNA cells in T75 flasks (Corning Incorporated, Corning, NY, USA) and incubated in an atmosphere of 37 °C and 5% CO₂ for 72 h to prepare virus stocks. To determine virus yield, MNA cells in 96-well plates (Greiner Bio-one, Frickenhausen, Germany) were infected with ten-fold serial dilutions of the virus stocks and incubated in an atmosphere of 37 °C and 5% CO₂ for 48 h. All titrations were performed in triplicate. Immunostaining using FITC-labeled RABV N gene-specific antibody (Centocor, Inc.) was performed on the plates and the 50% tissue culture infectious dose (TCID₅₀) was determined according to the method described elsewhere [55].

2.5. *Lagos Bat Virus Glycoprotein Expression by the Recombinant Viruses*

Neutralization of the SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-LBVG-GAS, SPBN (as a negative control) and LBVAFR1999 (positive control) was determined by the rapid fluorescent focus inhibition test (RFFIT) [6,56] using LBV antiserum. This serum was previously collected from bats in South Africa [57] and was found to have virus-neutralizing antibodies (VNAs) against LBV but not against RABV. Four different dilutions (1:10, 1:100, 1:400 and 1:1000) of the serum were used for the neutralization assay. The titer of the challenge viruses used in the neutralization assay was 1×10^3 FFU/mL. The virus neutralization index (VNI) was calculated by subtracting the virus titer of the untreated challenge virus with the virus titer of the LBV antiserum- treated challenge virus.

2.6. Single- and Multi-Step Growth Assays

Single- and multi-step growth assays were performed to determine the growth pattern of the different recombinant viruses. MNA cells in T25 culture flasks were infected with virus inoculums at a multiplicity of infection (MOI) of 2 or 0.01 for the single- and multi-step growth curves respectively, and incubated for 2 h in an atmosphere of 37 °C and 5% CO₂. The virus inoculum was removed, the cells were washed three times with 5 mL of PBS (Lonza, Verviers, Belgium) and then 7 mL of RPMI (containing 0.2% bovine serum albumin (BSA)) (Lonza) was added to the flask. At 24, 48, 72 and 96 h post infection, 100 µL of the supernatant was collected and virus titration was performed as described above. The virus titer was expressed as focus-forming units per mL (FFU/mL).

2.7. Experimental Infections of Mice

Six-week-old mice (CrI:CD1 [ICR]) (Onderstepoort Biological Products, Pretoria, South Africa) were used for experimental infection. The experiments were performed in a biosafety level 3 (BSL-3) laboratory at the Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, South Africa. Ethical approval (EC052-13) for these experiments was granted by the University of Pretoria Animal Ethics Committee and Section 20 approval according to the Animal Disease Act, 1984, was granted by the Department of Agriculture, Forestry and Fishery, South Africa. Mice were housed in a group of five in HEPA-filtered OptiMICE cage units (Animal Care Systems, Centennial, CO, USA). For the pathogenesis study, groups of 4 to 5 mice were inoculated via the i.c. or i.m. (in the hind thigh) route with 5×10^6 TCID₅₀/50 µL of virus inoculums (LBVAFR1999, SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-LBVG-GAS or SPBNGAS-GAS-GAS) using sterile 1 mL syringes (Becton Dickinson, Franklin Lakes, NJ, USA). For the vaccine study, 10 mice were vaccinated on day 0 and boosted on day 20 with SPBNGAS-LBVG-GAS via the i.m. (in the hind thigh) route with

1×10^5 TCID₅₀/50 μ L of virus inoculum using sterile 1 ml syringes (Becton Dickinson). Mice were monitored daily for 43 days for the pathogenesis study and 30 days for the vaccine study. Death and clinical signs such as not eating, paralysis, confusion, running in circles, weight loss, ruffled fur and restlessness were recorded daily. The mice were euthanized by i.m. inoculation with a mixture of ketamine (Anaket-V) (35 mg/kg body mass) and xylazine (Chanazine or Rompun) (5 mg/kg body mass) upon development of clinical signs or at the end of the experiment in cases where clinical signs were not observed. Brains were collected and analyzed for the presence of lyssavirus antigen using anti-rabies polyclonal FITC conjugate (Rabies Unit, Onderstepoort Veterinary Institute, Agricultural Research Council, Pretoria, South Africa) in the fluorescent antibody tests (FAT) as described by Dean et al. 1996. Blood was collected in BD Microcontainer™ (Becton Dickinson) from the saphenous vein using 80 μ L capillaries (Lasec, Cape Town, South Africa) on days 13 and 28 after primary vaccination. Mice were anesthetized with isoflurane (Piramal Healthcare, India) prior to bleeding. The blood was centrifuged at 13,400 rpm for 5 min, and serum collected in 2 mL microcentrifuge tubes (Quality Scientific Plastics, San Diego, CA, USA) and stored at -20°C until use.

2.8. Rapid Fluorescent Focus Inhibition Test (RFFIT)

Virus-neutralizing antibodies (VNAs) from serum of mice vaccinated with SPBNGAS-LBVG-GAS were determined by a modification of the rapid fluorescent focus inhibition test (RFFIT) [6,56]. The following challenge viruses were used: RABV (challenge virus standard [CVS]), LBV (LBVAFR1999), MOKV (252/97) and DUVV (DUVVSA2006). Eight different dilutions were tested: 1:10, 1:25, 1:65, 1:160, 1:400, 1:1000, 1:2500 and 1:6100. The VNA titer was indicated as the highest dilution where there was a 50% reduction in the number of foci. Since there are no reference sera available for LBV, MOKV and DUVV, the VNA titer for RABV were not converted to international units.

2.9. Statistical Analyses

For the pathogenesis study, Fisher’s exact test (CI = 95%) was used to determine the difference in survival between the different groups of mice. For the single- and multi-step growth curves, as well as the vaccine study, statistical analysis was performed using SPSS version 20, licensed to the University of Pretoria. One-way ANOVA was performed followed by Tukey HSD as a post-hoc test.

3. Results

3.1. Confirmation of LBV G Protein Expression by Recombinant Viruses

A virus neutralization test (RFFIT) using LBV antiserum was employed to ascertain that the LBV G protein is expressed. The neutralization indices tests shown in Table 2 reveal that SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-LBVG-GAS and LBVAFR1999 (positive control) but not SPBN (negative control) were completely neutralized by the LBV anti-serum.

Table 2. Neutralization of recombinant viruses with the different dilutions of the anti-Lagos bat virus serum as determined by the rapid fluorescent focus inhibition test (RFFIT).

Challenge Virus *	Virus Neutralization Index (VNI) # Anti-LBV Serum Dilution			
	1:10	1:100	1:400	1:1000
LBV (LBVAFR1999) (positive control)	10 ³	10 ³	10 ³	10 ⁰
SPBN (negative control)	10 ⁰	10 ⁰	10 ⁰	10 ⁰
SPBN-LBVG	10 ³	10 ³	10 ³	10 ⁰
SPBN-LBVM-LBVG	10 ³	10 ³	10 ³	10 ⁰
SPBNGAS-LBVG-GAS	10 ³	10 ³	10 ⁰	10 ⁰

* 10³ FFU of each challenge virus was used; # VNI = virus titer of untreated challenge virus – virus titer of challenge virus incubated with anti-LBV serum.

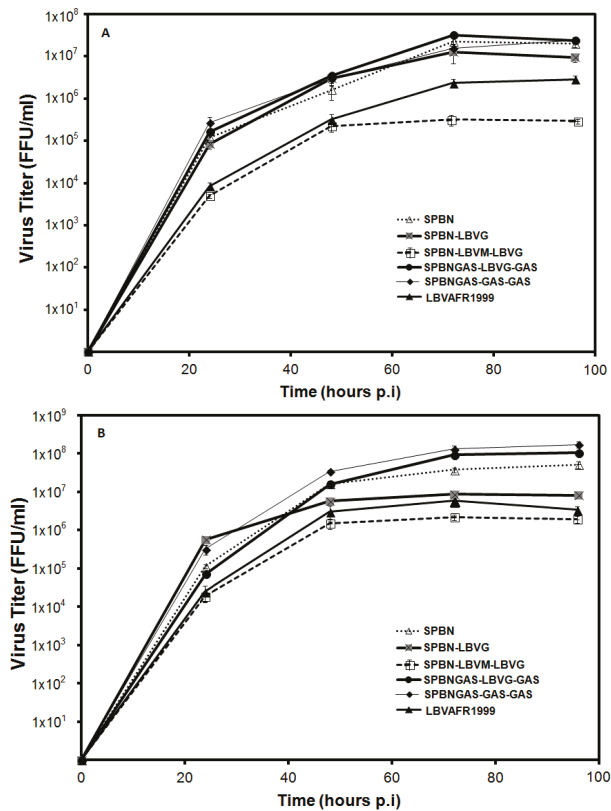


Figure 2. Multi-step (A) and single-step (B) growth curves of recombinant rabies viruses in MNA cells. MNA cells were infected with the recombinant viruses (SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-GAS-GAS or SPBNGAS-LBVG-GAS) or LBVAFR1999 at a MOI of 0.01 (A) or a MOI of 2 (B) and incubated at 37 °C. At 24, 48, 72 and 96 h post infection, the recombinant viruses were harvested and titration was performed. Data represent the mean of triplicate experiments.

3.2. In Vitro Growth of Recombinant Viruses in MNA Cells

Viral replication rate has been linked to pathogenicity of the virus [12,13]. We investigated the growth kinetics of recombinant RABVs in which the G only or M and G genes were replaced with corresponding LBV genes (Figure 2). Multi-step growth curves (Figure 2A) showed that SPBN and SPBNGAS-GAS-GAS had the highest growth rate followed by SPBN-LBVG and SPBNGAS-LBVG-GAS. While there was no statistically-significant difference between the growth rate of these viruses ($p > 0.05$), the growth rate of SPBN-LBVM-LBVG was lower than that of the other viruses including LBVAFR1999. The single-step growth curves yielded similar results, with SPBNGAS-GAS-GAS, SPBNGAS-LBVG-GAS and SPBN producing the highest titers, and SPBN-LBVM-LBVG and LBVAFR1999 again producing the lowest titers (Figure 2B). SPBN-LBVG also produced a lower titer, however it was still higher than that of SPBN-LBVM-LBVG.

3.3. Pathogenicity of Recombinant Viruses in Mice

The ability of various recombinant viruses to cause lethal encephalitis in mice was compared. Virus infection of the brain was confirmed postmortem by FAT. All viruses, except

SPBNGAS-GAS-GAS, caused mortality in mice after i.c. inoculation and all the mice that died exhibited signs of rabies including walking in circles, hind leg paralysis, weight loss and ruffled fur. The mortality rate ranged from 0% to 100% (Figure 3 and Table 3). Mice inoculated with LBVAFR1999 and SPBN-LBVM-LBVG had the highest mortality rate (100%) while those inoculated with SPBNGAS-LBVG-GAS had the lowest mortality rate (20%). After i.m. inoculation of recombinant viruses, only LBVAFR1999 and SPBN-LBVM-LBVG caused mortality (Figure 4). The rates were 40% for LBVAFR1999 and 20% for SPBN-LBVM-LBVG with incubation periods of 16 and 14 days, respectively. These results show that both the M and G proteins of LBV play a role in the pathogenesis of LBV.

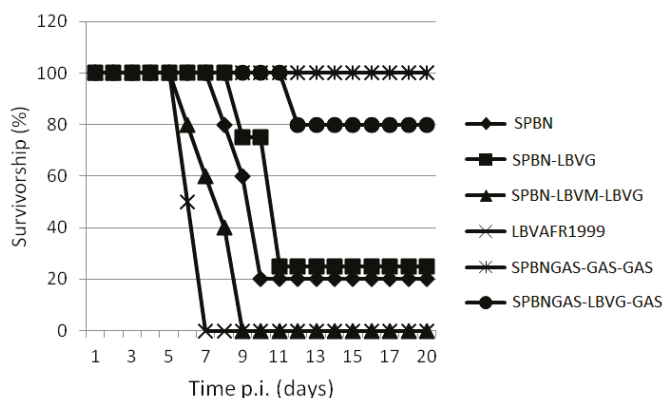


Figure 3. Pathogenicity of SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, LBVAFR1999, SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS in mice. Groups of (CrI:CD1 [ICR]) mice were inoculated via the i.c. route with 5×10^6 TCID₅₀/50 μ L of virus. The groups consisted of 5 mice except for SPBN-LBVG and LBVAFR1999 which consisted of 4 mice per group. The experiment was terminated after 43 days, but no clinical signs were observed 12 days post-infection.

Table 3. Intracranial experimental infections of mice with recombinant viruses.

Virus	Mean Incubation Period, s.d. and Range of Incubation Periods (Days)		Number of Mice that Died per Group of Mice Inoculated i.c.
	Mean and s.d.	Range	
LBVAFR1999	6.5 \pm 0.58	6–7	4/4 (100%)
SPBN	9.25 \pm 0.96	8–10	4/5 (80%)
SPBN-LBVG	10.33 \pm 1.15	9–11	3/4 (75%)
SPBN-LBVM-LBVG	7.8 \pm 1.30	6–9	5/5 (100%)
SPBNGAS-GAS-GAS	-	-	0/5 (0%)
SPBNGAS-LBVG-GAS	12 \pm NA	12	1/5 (20%)

N/A = no s.d. since only one mouse died; s.d. = standard deviation; i.c. = intracranial.

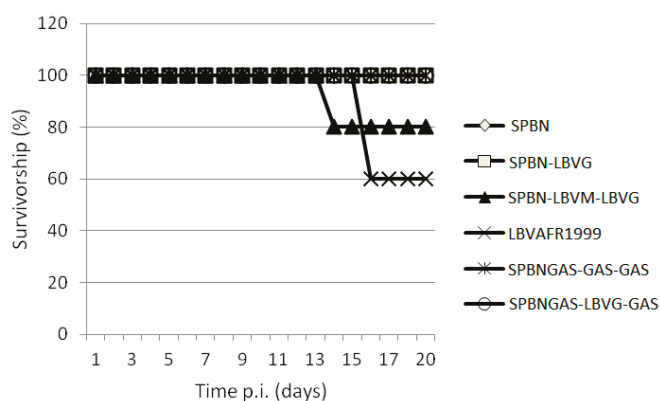


Figure 4. Pathogenicity of SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, LBVAFR1999, SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS in mice. Groups of 5 (CrI:CD1 [ICR]) mice were inoculated via the i.m. route with 5×10^6 TCID₅₀/50 μ L of virus. The experiment was terminated after 43 days, but no clinical signs were observed 16 days post infection.

3.4. VNA Response of Mice Vaccinated with SPBNGAS-LBVG-GAS

For primary vaccination (Table 4), VNA titers against LBV and MOKV were significantly lower ($p < 0.05$) compared to RABV (with the exception of VNA titer for RABV and LBV at day 28, where there were no significant differences). There was no significant difference ($p > 0.05$) in VNA titer between RABV and DUVV (Table 4). Booster vaccination resulted in significantly higher ($p < 0.05$) VNA titers against all challenge viruses used. This VNA analysis demonstrates that SPBNGAS-LBVG-GAS can induce cross-reactive antibodies capable of neutralizing different lyssaviruses.

Table 4. Virus neutralizing antibodies in mice vaccinated with SPBNGAS-LBVG-GAS as determined by the rapid fluorescent focus inhibition antibody test (RFFIT).

Mouse No.	Neutralization Dilution * (RABV-CVS) #		Neutralization Dilution * (DUVVSA2006) #		Neutralization Dilution * (LBVAFR1999) #		Neutralization Dilution * (MOKV252/97) #	
	Day 13	Day 28	Day 13	Day 28	Day 13	Day 28	Day 13	Day 28
Mouse 1	1:400	1:2500	1:160	1:1000	1:160	1:1000	1:65	1:400
Mouse 2	1:160	1:2500	1:65	1:400	1:65	1:400	1:10	1:160
Mouse 3	1:1000	1:6100	1:400	1:2500	1:400	1:6100	1:160	1:1000
Mouse 4	1:400	1:1000	1:400	1:1000	1:160	1:400	1:65	1:400
Mouse 5	1:400	1:2500	1:160	1:1000	1:65	1:400	1:25	1:400
Mouse 6	1:400	1:1000	1:160	1:1000	1:160	1:1000	1:65	1:400
Mouse 7	1:160	1:1000	1:65	1:1000	1:65	1:400	1:25	1:400
Mouse 8	1:400	1:2500	1:400	1:2500	1:160	1:1000	1:65	1:400
Mouse 9	1:400	1:2500	1:400	1:1000	1:160	1:1000	1:160	1:1000
Mouse 10	1:1000	1:6100	1:400	1:6100	1:400	1:2500	1:160	1:1000
Range	1:160–1:1000	1:1000–6100	1:65–1:400	1:400–1:6100	1:65–1:400	1:400–1:6100	1:10–1:160	1:400–1:1000

Key: * Represents the dilution where there was 50% virus neutralization by the serum. The experiments were performed in triplicate; # Indicates the challenge virus used; ~ Indicates the range of virus neutralization titer between the different mice.

4. Discussion

The successful rescue of RABV recombinant viruses expressing LBV proteins, indicates that RABV genes can interact with the LBV M and G genes, similar to previous studies [18,28,29,58]. Finke et al. (2010) and Marston et al. (2013) showed that EBLV-1 and EBLV-2 genes can interact with RABV genes by respectively performing replacement of the M and G genes from RABV with those from EBLV-1 or EBLV-2. In addition to these studies, Genz et al. (2012) replaced the transmembrane and ectodomain of RABV G protein with those of EBLV-1 or EBLV-2. Neutralization of SPBN-LBVG, SPBN-LBVM-LBVG and SPBNGAS-LBVG-GAS with LBV antiserum confirmed that the LBV G protein

is expressed by these recombinant viruses. Failure of the same serum to neutralize SPBN shows that the VNAs were directed against the LBV G protein [59,60]. A G gene-deleted RABV (SAD Δ G) was previously shown to be unable to spread *in vivo* and *in vitro*, and also failed to produce infectious virus particles when passaged in cells that were not transfected with the plasmid expressing the G protein [61,62]. The SAD Δ G was also shown to be non-pathogenic when inoculated *i.c.* [62] and in addition, the M gene-deleted RABV had a reduced viral titer by about 500 000-fold [63]. The spread of SPBN-LBVG and SPBN-LBVM-LBVG in cells that were not transfected with the relevant exchanged genes also indicated that the recombinant viruses functionally expressed these genes. Western blotting could not be used to determine LBV M and G genes expression because of the lack of suitable antibodies directed against these proteins.

All the viruses in this study, except for SPBNGAS-GAS-GAS, were pathogenic to mice after *i.c.* inoculation (Table 3), and LBVAFR1999 and SPBN-LBVM-LBVG infection resulted in 100% mortality. The 20% mortality rate observed with SPBNGAS-LBVG-GAS compared to 0% mortality of SPBNGAS-GAS-GAS indicated that the non-pathogenic RABV G (GAS) genes were not dominant over the pathogenic phenotype of the LBV G gene. Previously, it was shown that *i.c.* inoculation of mice with a double-G gene recombinant virus (SPBNGAK-GAK) resulted in 70% mortality [48]. However, when one of the G genes was made non-pathogenic (by mutation of Lys 194 to Asn), the recombinant virus only caused 10% mortality to mice, indicating dominance of the non-pathogenic G over the pathogenic G gene [48]. This indicates that in addition to multiple G genes that result in over-expression and therefore reduced pathogenicity [25], the pathogenic domains of the G genes must be eliminated. Inoculation of the viruses into mice *i.m.* showed that only LBVAFR1999 and SPBN-LBVM-LBVG were pathogenic. In contrast, the SPBN-LBVG was not pathogenic despite expressing the G gene from a pathogenic LBV. It was previously shown that replacement of a non-pathogenic RABV G gene with that of a pathogenic RABV results in a pathogenic RABV, but with reduced pathogenicity compared to the parental strain [13,16,18]. In other studies, replacement of a non-pathogenic RABV G gene with a pathogenic RABV did not result in a pathogenic strain [16,64]. Replacement of a G gene from a non-pathogenic RABV (SN-10) with the G gene from a number of street RABV variants (pathogenic *i.m.*) resulted in a non-pathogenic RABV strain when inoculated *i.m.* to mice [16,64], as also confirmed in our study where the SPBN-LBVG was non-pathogenic after *i.m.* inoculation. Replacement of both the M and G gene of a non-pathogenic RABV with that of a pathogenic RABV was shown to result in increased pathogenicity as compared to the replacement of only the G gene, indicating the importance of the M gene in pathogenicity [12,13]. In our study, the SPBN-LBVM-LBVG was pathogenic *i.m.* while the SPBN-LBVG was non-pathogenic, which is in agreement with the previous studies indicating the role of the M gene in pathogenicity [12,13,15].

Differences in recombinant virus growth was determined in MNA cells by multi-step and single-step growth curves. SPBN and SPBN-LBVG showed the same growth rate in the multi-step growth curve, indicating that the LBV G gene is able to interact optimally with the RABV M gene during encapsidation and budding. Notably, LBVAFR1999 and SPBN-LBVM-LBVG produced the lowest viral titers during single- and multi-step growth kinetics, which is in agreement with previous findings that pathogenic RABV strains have lower replication rates than non-pathogenic strains [12,13,48]. This, thought to be one of the mechanisms by which lyssaviruses evade the host defense mechanisms, is consistent with our finding that only the LBVAFR1999 and SPBN-LBVM-LBVG were pathogenic when inoculated *i.m.* in mice. The single-step growth curve indicated no significant ($p > 0.05$) difference between SPBN, SPBNGAS-LBVG-GAS and SPBNGAS-GAS-GAS. These viruses maintained higher titers in both the single- and multi-step growth curves, indicating that insertion of multiple G genes and the LBVAFR1999 G gene did not negatively influence the growth of SPBNGAS-LBVG-GAS and SPBNGAS-GAS-GAS.

By replacing the M and G genes between laboratory-adapted SPBN and the wild-type LBVAFR1999 isolate, we have attempted to address the importance of the M and G genes in the pathogenicity of LBV. Our results emphasize again that the G protein is important in pathogenicity

of lyssaviruses, and that there is a cooperative effect of other lyssavirus genes as shown by increased pathogenicity when both the SPBN M and G genes were replaced by those of LBV.

The SPBNGAS-LBVG-GAS produced high levels of VNAs against RABV and DUVV 13 days post-primary vaccination in mice and the level was comparable to that produced by vaccination with SPBNGAS-GAS-GAS (data not shown). In previous studies, SPBNGAS-GAS-GAS produced high levels of RABV VNA and protected mice against lethal challenge with RABV, therefore suggesting that SPBNGAS-LBVG-GAS will offer the same level of protection [39,40]. In addition, SPBNGAS-LBVG-GAS also produced high levels of VNAs capable of cross-neutralizing DUVV. Although serum from RABV-vaccinated mice has already been shown to cross-neutralize DUVV [30] the VNA titers against DUVV were significantly lower than those against RABV. In our study, the DUVV VNA titers induced by SPBNGAS-LBVG-GAS were not significantly different from the RABV VNA titers ($p > 0.05$). Previous studies on cross-reactivity of RABV vaccines to other phylogroup I lyssaviruses indicated that a vaccine derived from the Pasteur virus (PV) strain protected mice from EBLV-1 challenge, while vaccines derived from Pitman-Moore (PM) and LEP-Flury (LEP) strains failed to protect mice against EBLV-1 infection [31]. However, in another study, a vaccine derived from the PM strain was shown to produce VNA that neutralize EBLV-1 and protect mice against lethal challenge [35]. This indicates that different strains from the same lyssavirus species can result in different protection levels.

Mice vaccinated with SPBNGAS-LBVG-GAS also produced VNAs against LBV and MOKV although the titers were significantly lower ($p < 0.05$) compared to RABV, with the exception of VNA titer of RABV and LBV at day 28, where no significant difference was observed. MOKV VNA titers were significantly lower ($p < 0.05$) than LBV VNA titers on day 13. Previous studies did report cross-neutralization between MOKV and LBV [30,65,66]; however, the titer was always higher when the G protein of the challenge virus was identical to the G protein of vaccine. Cross-neutralization against SHIBV was not tested in this study but it is likely that cross-neutralization will occur with other phylogroup II viruses when SPBNGAS-LBVG-GAS is used as vaccine [38]. Booster vaccination resulted in significantly higher ($p < 0.05$) VNA titer against all challenge viruses used. Mice were not challenged in this study; however, the high VNA titer produced by SPBNGAS-LBVG-GAS especially after booster vaccination indicates that this recombinant vaccine will likely protect mice against lethal challenge with RABV, LBV, MOKV and DUVV. VNA titer is regarded to be an important factor in protecting against virus challenge and can be correlated with survival, but this will have to be confirmed in challenge studies in mice in the future [67].

In future studies, the expression of LBV G should be increased by changing the gene order of the LBV G gene or by insertion of an additional G gene to determine if the level of LBV and MOKV VNA increases without booster vaccination. Although VNA titers against LBV and MOKV were lower compared to RABV and DUVV during primary vaccination, the significant increase in VNA titer after booster vaccination warrants further investigation of the efficacy of SPBNGAS-LBVG-GAS to protect against phylogroup II lyssaviruses.

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Article

Protection Against CNS-Targeted Rabies Virus Infection is Dependent upon Type-1 Immune Mechanisms Induced by Live-Attenuated Rabies Vaccines

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Abstract: Rabies remains a major public health issue worldwide, especially in developing countries where access to medical care can represent a real challenge. While there is still no cure for rabies, it is a vaccine-preventable disease with pre- and post-exposure prophylaxis regimens approved by the World Health Organization (WHO). However, many rabies-exposed individuals have limited access to vaccines and virus-neutralizing antibodies approved for post-exposure prophylaxis. Unfortunately, any delay in the administration of these reagents can have lethal consequences. This highlights the need to develop cost-effective immunological reagents with a greater window of efficacy. Live-attenuated vaccine strains of rabies virus presents a potential treatment in filling this gap. We show here that immunization with live-attenuated vaccines provide long-lasting rabies immunity, superior to the protection induced by inactivated vaccines. In the absence of an immunostimulatory adjuvant, vaccination with multiple doses of inactivated rabies virus induces a type-2 immune response. This type of immunity is highly effective at inducing neutralizing antibody but has limited efficacy in clearing the virus from central nervous system (CNS) tissues. In contrast, a single infection with live-attenuated rabies vaccine safely drives a type-1 immune response, associated with both the production of a neutralizing antibody and the clearance of wild-type rabies virus from CNS tissues. These results indicate that live-attenuated rabies strains have the potential to be more effective in post-exposure prophylaxis than conventional inactivated vaccines.

Keywords: rabies; vaccine; type-1 immunity; type-2 immunity

1. Introduction

Rabies is a central nervous system (CNS) disease, nearly always fatal for humans and most mammals, caused by host infection with the rabies virus (RABV). RABV is a single-stranded, negative sense, neurotropic RNA virus that belongs to the *Lyssavirus* genus of the *Rhabdoviridae* family [1]. Despite advances in the control of animal reservoirs and in human prophylaxis, rabies still accounts for over 60,000 human deaths worldwide, with most cases recorded in Asia and Africa [2]. Although incurable once clinical symptoms appear [3], rabies is largely preventable through mass vaccination of dogs in rabies-zoonotic regions, which aims to eliminate the virus at its source [4], or via the use of anti-rabies biologics in humans after exposure [5]. Since Pasteur's development of the first rabies vaccine in 1885, rabies prevention has evolved in two directions: pre- and post-exposure (PEP) prophylaxis. Pre-exposure prophylaxis, which involves a series of three or more intramuscular (i.m.) injections of inactivated rabies vaccine at 0, 7, and 28 days [6,7], is given only to at-risk populations

such as veterinarians, laboratory workers, and travelers to rabies endemic regions [8]. PEP, which consists of proper wound cleaning, immunization with an inactivated rabies vaccine, and injection of rabies immunoglobulins at the site of infection [8], is effective at preventing the development of the disease when administered to the patient within a short window after exposure to a suspected rabid animal [9].

Unfortunately, a large number of rabies-exposed patients fail to receive adequate PEP, primarily in resource-poor countries, largely due to the high cost or unavailability of rabies vaccine or rabies immunoglobulins [10]. The difficulties of rabies management in animal reservoirs and the inability to follow World Health Organization recommendations for PEP best practice in under-developed countries drives the need to improve the availability of safe, cost-effective rabies reagents [11]. Foremost among these advancements are both the use of human monoclonal rabies virus neutralizing antibodies in replacement of serum-based antibodies and new vaccine approaches that provide the critical, safe, and rapid induction of long-lasting immunity. The use of adjuvants in combination with inactivated RABV vaccines or infection with live-attenuated RABV vaccine strains are two strategies that may more rapidly induce rabies immunity, although it is now known that the nature of the immune response is also an important consideration. We previously reported that the outcome of RABV infection of neural tissues is dependent upon two key processes: (1) the early control of virus replication by IFN- γ -promoted innate immune mechanisms [12,13]; and (2) virus clearance from CNS tissue by the infiltration of immune effectors and the local production of virus-neutralizing antibodies (VNA) [14]. A rabies-specific immune response biased toward type-1 immunity (Th1 CD4 T cell response) is critical for both of these processes [15].

The present study is aimed at evaluating the efficacy in mice of live-attenuated RABV vaccine strains in triggering long-lasting immunity and protection against challenge with lethal wild-type RABV via routes distal from and proximal to the CNS. Several vaccine and wild-type RABV strains were used in various mice strains to take into account genetic variability in immunity. The immune response to live-attenuated RABV was compared to inactivated RABV, which more closely resembles current approved vaccines for humans [5]. We found that live-attenuated RABV strains consistently outperform inactivated vaccine strains, including a current commercial vaccine IMOVAX[®], in the induction of protection against challenge with a lethal RABV. We show that this is largely due to differences in the class of immune response elicited: type-1 by live-attenuated RABV, and type-2 by inactivated RABV.

2. Materials and Methods

2.1. Mice and Study Approval

C57BL/6 and Swiss Webster mice (6 to 8 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and Taconic Biosciences (Germantown, NY, USA), respectively. All animals were monitored for survival and blood samples were collected at various times post-infection for serum antibody titers. All procedures were conducted in accordance with Public Health Service Policy on Humane Care and Use of Laboratory Animals under protocols approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University (Animal Welfare Assurance Number A3085-01).

2.2. Virus and Vaccine Strains

SPBN Double GAS (GG) and SPBN Triple GAS (GGG), recombinant RABV vaccines containing two or three copies of a mutated glycoprotein, respectively, were developed as previously described [16,17]. Both the dog rabies virus 4 (DRV4) and the silver-haired bat rabies virus 17 (SHBRV-17) are highly pathogenic RABV strains of dog and bat origins. These lethal viruses were isolated from the brains of human victims and propagated in suckling mice brain [18]. The challenge virus standard F3 (CVS-F3) is an antibody escape-attenuated mutant, of dog origin, that differs from

its parental strain by a single point mutation in its glycoprotein in position 333 [19]. IMOVAX[®] is a licensed human diploid cell vaccine (Sanofi Pasteur, Swiftwater, PA, USA) prepared from the Pitman-Moore strain of RABV grown on MRC-5 human diploid cell cultures, in use in the USA since 1980 [20,21].

2.3. Virus Inactivation

IMOVAX[®] is a commercially available vaccine inactivated with beta-propiolactone (BPL), an alkylating agent reacting with nucleic acids and proteins, widely used for the inactivation and increased safety of biological reagents such as viruses [22]. In some experiments, IMOVAX[®] was reconstituted according to manufacturer's directions and stored at either 25 °C or 40 °C for three weeks prior to use. The live-attenuated vaccine strains used in this study were inactivated either by exposing the virus to UV radiation for 45 minutes or by treatment with BPL (1:100 dilution) overnight at 4 °C.

2.4. Immunization and Challenge of Mice

A variety of immunization strategies were used and are detailed in each figure legend. Briefly, mice were immunized with 1×10^5 – 1×10^8 focus-forming units (f.f.u.) of live-attenuated or inactivated (f.f.u. determined before inactivation) RABV via intramuscular (gastrocnemius, i.m.g., 50 µL), intranasal (i.n., 10 µL) or intracranial (i.c., 5 µL) routes. Mice immunized with IMOVAX[®] received 0.125–0.25 International Units (IU) of rabies antigen. Mice were challenged at 21–63 days post-immunization with 1×10^3 – 1×10^5 f.f.u. lethal RABV via one of the same routes as immunization.

2.5. Neutralizing Antibody (VNA) Titer and Serum Antibody Isotyping

Virus neutralizing antibody (VNA) titer was evaluated by the rapid fluorescent focus inhibition test as previously described [23]. The antibody isotypes were determined by ELISA using UV-inactivated Evelyn-Rokitnicki-Abelseth (ERA) virus as coating antigen and mouse-specific total IgG, IgG1, and IgG2a secondary antibodies, as described previously [24].

2.6. Statistical Analysis

Statistical significance of survival rates or IgG levels were compared with a one-way ANOVA followed by the Bonferroni multiple comparison test. Graphs were created and statistical analysis was performed using GraphPad Prism 5.0 software (La Jolla, CA, USA). Statistically significant differences between groups are denoted as follows: * $p \leq 0.01$, ** $p \leq 0.005$, and *** $p \leq 0.001$.

3. Results

3.1. Live-Attenuated RABV are Protective and Stable at Room Temperature

Current human anti-rabies vaccines are inactivated to improve both the safety for the host and the stability of the preparation. However, multiple doses are required for sufficient immunity. While live-attenuated rabies vaccines are capable of inducing a strong immune response with a single dose, the limitations most often cited for their use are safety and stability. Here we show that IMOVAX[®], a commercial BPL-inactivated vaccine, either freshly reconstituted or stored at 25 °C or 40 °C for three weeks, is fully protective (100% survival) in immunocompetent mice against an i.m. challenge with the lethal DRV4 virus (Figure 1a). The protection against DRV4 challenge was conferred by the induction of a strong rabies-specific humoral response following immunization with the IMOVAX[®] vaccine, as evidenced by high levels of circulating IgG antibodies (Figure 1b) and VNAs (Figure 1c). As expected, mice immunized with a fresh preparation of the live-attenuated GG virus resulted in 100% survival after DRV4 challenge, while only 20% of the mice survived when the animals were immunized with the vaccine stored at 40 °C (Figure 1d). However, we found that the live-attenuated GG strain stored at room temperature (25 °C) for three weeks retained sufficient activity to provide full protection against the wild-type DRV4 challenge (Figure 1d). Analysis of the peripheral humoral

response of mice infected with GG stored at 25 °C shows that rabies-specific IgGs (Figure 1e) and VNAs (Figure 1f) were both produced. While the VNA titer was lower than that elicited by fresh GG vaccine, it is likely responsible for animal survival, as administration of GG virus stored at 40 °C failed to elicit rabies-specific antibody production (Figure 1e,f) or protect against DRV4 challenge (Figure 1d).

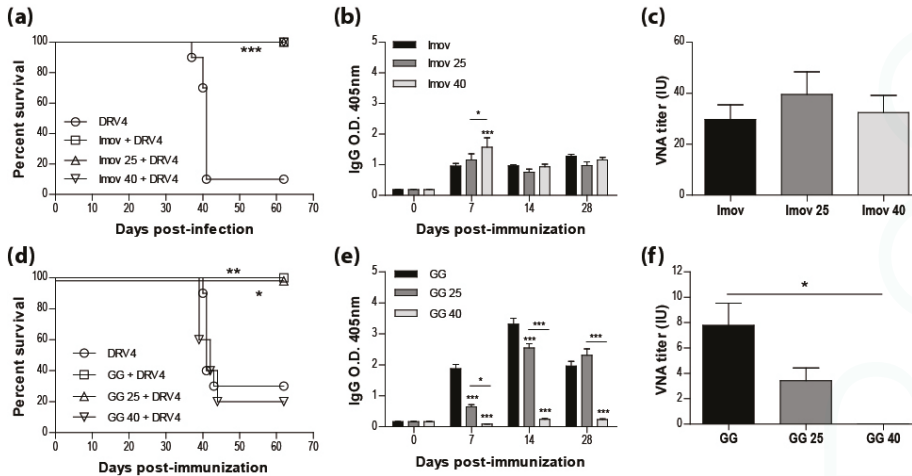


Figure 1. Live-attenuated RABV vaccines, stored at room temperature, retain sufficient infectivity to induce fully protective immunity. Swiss Webster mice were immunized with 0.25 IU of IMOVAX[®] (a) or 1×10^5 f.f.u. of GG (d) i.m.g. stored at various temperatures then challenged 30 days later with 1×10^5 f.f.u. of DRV4 i.m.g. and monitored for survival. Data are expressed as percent survival ($n = 5-10$ per group). (b,e) Virus-specific Ab response for total IgG was determined by ELISA at 7, 14 and 28 d.p.i. for all groups (dilution 1:40). (c,f) VNA titer at 28 d.p.i. was determined by the rapid fluorescence focus inhibition test, as described in Materials and Methods. Data are expressed as mean \pm SEM of international unit (IU). Statistically significant differences between groups are denoted as follows: * $p \leq 0.01$; ** $p \leq 0.005$; *** $p \leq 0.001$.

3.2. IMOVAX[®] Induces a Mixed Th1/Th2 Immune Response

In addition to the long-established requirement for VNA, we have previously reported that the bias of the immune response is critical for the clearance of RABV from CNS tissues. We speculate that the bias of the immune response elicited by vaccination is also of importance for long-term protection. As previously reported and seen here, vaccination with RABV inactivated by UV or BPL induces a response producing significantly more IgG1 than IgG2a antibodies (Figure 2a). This is characteristic of a type-2 immune response. We therefore expected the IMOVAX[®] vaccine, which is BPL-inactivated, to induce the production of IgG1 antibodies. However, both IgG1 and IgG2a RABV-specific antibodies are seen after vaccination with IMOVAX[®] (Figure 2b), indicating that the response has mixed type-1 and type-2 characteristics.

3.3. Addition of Adjuvant to Inactivated Vaccine does not Promote Protection

The mixed type-1/-2 response observed following immunization with the IMOVAX[®] vaccine raises questions about how this inactivated vaccine preparation stimulates Th1 cells. Is there a component with qualities resembling an adjuvant? These immunostimulatory reagents are extensively used for vaccination and have occasionally been reported to promote type-1 immunity. However, we show here that vaccination with UV-inactivated CVS-F3 RABV in Freund's complete adjuvant (CFA) does not protect mice from subsequent challenge with the lethal DRV4 virus (Figure 3a). The use of

CFA in the vaccine evidently did not change the type-2 bias of the response as reflected by the higher levels of RABV-specific IgG1 antibodies produced (Figure 3b).

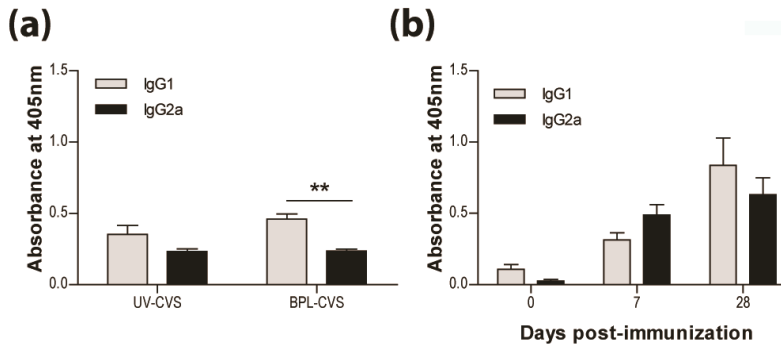


Figure 2. IMOVAX® is protective through the induction of a mixed Th1/Th2 immune response. Virus-specific Ab isotype was determined by ELISA 10 days following immunization with either 1×10^5 f.f.u. of UV- or BPL-inactivated CVS-F3 RABV (a) or 0.25 IU of IMOVAX® (b). Results are expressed as mean absorbance \pm SEM in OD (dilution 1:20). Statistically significant differences between groups ($n = 5$ –10 per group) are denoted as follows: ** $p \leq 0.005$.

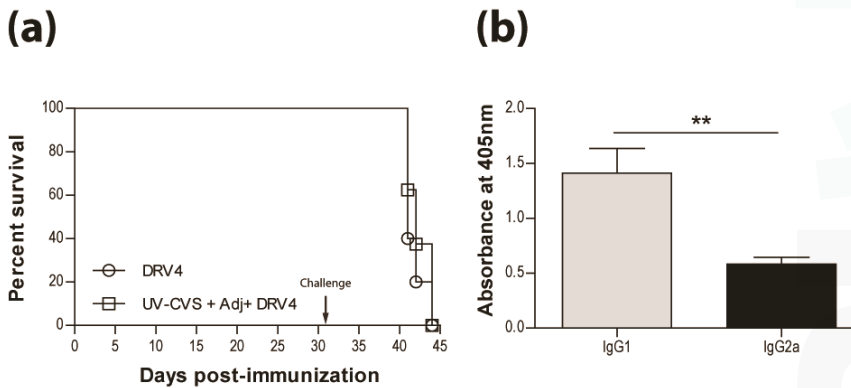


Figure 3. Adding adjuvant to UV-inactivated RABV does not induce protection. (a) C57BL/6 mice were immunized with 5×10^7 f.f.u. of UV-CVS-F3 mixed with CFA adjuvant, i.m.g. then challenged 31 days later with 1×10^5 f.f.u. of DRV4 i.n. and monitored for survival. Data are expressed as percent survival. (b) Virus-specific Ab isotyping was determined by ELISA 24 days post-immunization. Results are expressed as mean absorbance \pm SEM in OD (dilution 1:50). Statistically significant differences between groups ($n = 10$ per group) are denoted as follows: ** $p \leq 0.005$.

3.4. Vaccination Efficacy is Dictated by the Immunization Regimen and the Challenge Route

The survival of non-immunized mice infected with the SHBRV-17 RABV differs greatly according to the route of infection, with 33%, 50%, and 100% mortality, for i.m.g (Figure 4a), i.n. (Figure 4b), and i.c. (Figure 4c) routes, respectively. However, regardless of the route of infection, immunization with a single dose of the live CVS-F3 RABV conferred superior protection (90%–100% survival) against SHBRV-17 challenge 21 days later than immunization with the UV-inactivated CVS-F3 (Figure 4, top panels). The difference in protection against i.n. and i.c. immunization/challenge with SHBRV-17 persisted for at least 63 days post-immunization with survival of i.m.g., i.n., and i.c.

immunized/challenged mice respectively at 100%, 50%, and 87.5% for those vaccinated with live CVS-F3 RABV, versus 87.5%, 12.5%, and 37.5% for animals that had received UV-inactivated CVS-F3 (Figure 4, bottom panel).

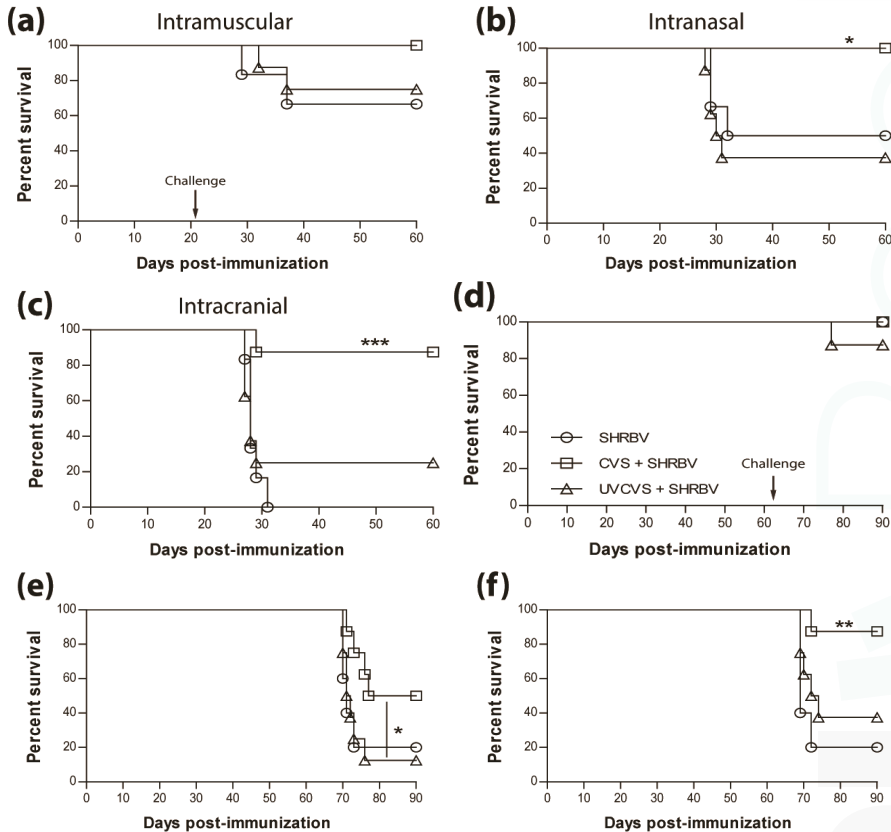


Figure 4. Single immunization with live-attenuated vaccine confer superior long-lasting protection against wild-type virus challenge. Swiss Webster mice were either mock-immunized or immunized with 1×10^5 f.f.u. of live-attenuated CVS-F3 or 1×10^8 f.f.u. of inactivated CVS-F3 (UV-CVS-F3) then challenged 21 (top row) or 63 (bottom row) days later with 1×10^4 f.f.u. of SHBRV-17 virus and monitored for survival. Data are expressed as percent survival ($n = 10$ per group). Animals were immunized either (a,d) intramuscularly in the gastrocnemius, (b,e) intranasally or (c,f) intracranially, then challenged with the corresponding route. Statistically significant differences between groups are denoted as follows: * $p \leq 0.01$; ** $p \leq 0.005$; *** $p \leq 0.001$.

3.5. Type-1 Immunity is Critical for Protection Against Wild-Type RABV Infection

The results presented above as well as in our previous publications (e.g. reference [15]) indicate that a type-1 immune response is important in dealing with wild-type RABV that reaches the CNS. This is further illustrated in Figure 5a, where immunization with live-attenuated, but not UV-inactivated, RABV efficiently protects against i.n. infection with wildtype DRV4 RABV (80% versus 20% survival). This is despite the production of levels of RABV-specific, type-2-associated IgG1 antibodies in the latter that are higher than the levels of type-1 IgG2a in mice that were vaccinated with the live-attenuated virus (Figure 5b). To determine if the prior induction of a type-2 immune

response to RABV interferes with the induction of a more protective type-1 response, mice that received UV-inactivated RABV were boosted with the virus in a live-attenuated format. Survival from an i.n. challenge with DRV4 was improved (from 20% to 50%) but did not reach the level (80%) of mice that were both primed and boosted with live-attenuated RABV (Figure 5c). A change in the bias of RABV-specific serum antibodies from IgG1>IgG2a to IgG2a>IgG1 was seen when animals immunized with UV-inactivated RABV were boosted with the live-attenuated virus (compare Figure 5b,d).

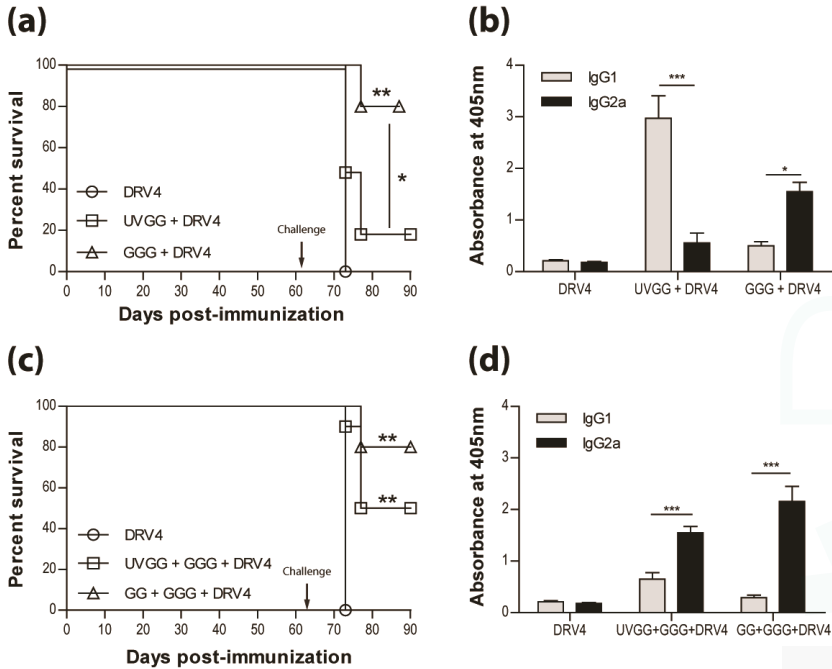


Figure 5. Live-attenuated RABV are protective through the induction of type-1 immunity. C57BL/6 mice were (a) immunized with 3 doses of 1×10^6 f.f.u. of UV-GG or 1×10^5 f.f.u. of GGG i.m.g.; (c) immunized with 1×10^6 f.f.u. of UV-GG or 1×10^7 f.f.u. of GG followed by a boost of 1×10^5 f.f.u. of GGG 28 days later. All animals were then challenged 63 days later with 1×10^5 f.f.u. of DRV4 i.n. and monitored for survival. Data are expressed as percent survival ($n = 10$ per group). (b,d) Virus-specific Ab isotyping was determined by ELISA 42 days after immunization. Results are expressed as mean absorbance \pm SEM in OD (dilution 1:20). Statistically significant differences between groups ($n = 10$ per group) are denoted as follows: * $p \leq 0.01$; ** $p \leq 0.005$; *** $p \leq 0.001$.

4. Discussion

As we previously reported, immunocompetent mice can clear attenuated RABV infection from brain tissues through the local activity of both humoral and cellular type-1 immune mechanisms [14,15,25]. Immune effector delivery across the blood-brain barrier (BBB) proves to be particularly important as this does not happen during wild-type RABV infection [24,26]. IFN- γ , a major product of type-1 immunity, is evidently important in RABV clearance from brain tissues through: (i) its induction of type-I interferons that control virus replication [13]; (ii) its contribution to the non-inflammatory changes in BBB function that promote immune cell infiltration [27]; and (iii) its role in driving the local production of VNA [14], which ultimately eliminates the virus from CNS tissues [25]. While the delivery of immune effectors across the BBB is a critical step, the type of immune

cells delivered into CNS tissues is also important. CD4⁺ Th2 cells reach CNS tissues during attenuated RABV infection but are evidently non-functional in this environment [15].

The current study reinforces the importance of type-1 immunity in rabies, not merely in the clearance of attenuated RABV but also in the prevention of wild-type RABV infection. Immunization with inactivated RABV induces a type-2 response with high levels of serum RABV-specific antibody but limited protection from an i.n. challenge dose of highly pathogenic DRV4, regardless of whether the inactivated vaccine is administered with CFA. The use of a live vaccine following initial immunization with a killed vaccine induces the production of IgG2a antibodies that are associated with type-1 immunity and improves protection against an i.n. DRV4 challenge, but not to the extent provided by the use of a live-attenuated vaccine alone. A comparison of the efficacy of immunization with live-attenuated versus inactivated RABV using the lethal SHBRV provides further insight into the differences in vaccination efficacy. SHBRV administered i.m. in the gastrocnemius is only mildly pathogenic, but lethal for around 80% of mice infected either i.n. or i.c. Infection with the live-attenuated CVS-F3 strain significantly improved survival of mice infected with SHBRV i.n. or i.c., while survival following immunization with inactivated CVS-F3 is only marginally improved in animals challenged i.c. It is also noteworthy that the superior protection against i.n. or i.c. challenge conferred by immunization with live-attenuated virus is consistent, independent of the mouse strain studied (C57BL/6 and Swiss Webster) and the RABV strains used for immunization (GG, GGG, CVS-F3) or challenge (DRV4, SHBRV-17).

Currently, rabies vaccines approved for human use are all produced with inactivated, cell culture-derived RABV. As expected, the commercial IMOVAX[®] vaccine, consisting of BPL inactivated Pitman-Moore RABV [21], protects 100% of mice against i.m. challenge with DRV4 in the gastrocnemius. While inactivated virus is expected to induce a predominant type-2 response, as shown here for UV-inactivated CVS-F3 and GG as well as beta-propiolactone-inactivated CVS-F3 viruses, IMOVAX[®] was found to induce the production of RABV-specific antibodies, reflecting a more mixed type-1 plus type-2 response. These data support the predominance of type-1 immunity protection, once triggered, over type-2 immune responses during viral infection. Previously, it has been reported that B cell hybridomas, produced from the peripheral blood mononuclear cells (PBMC) of human donors vaccinated with Rabivac[™] (Behringwerke, Marburg, FRG), another vaccine based on inactivated Pitman-Moore RABV, largely elaborate IgG1 and IgG3 rabies VNA [28], reflecting type-1 immunity in humans. This suggests the possibility that either the virus strain or some element of the manufacturing process results in products with some type-1 immunostimulatory properties which are not present in the UV or beta-propiolactone-inactivated viruses studied here. We consider that the capacity to stimulate RABV-specific type-1 immunity is an important basis for an effective rabies vaccine.

Prior work in mice has concluded that adjuvant use with inactivated vaccine enhances immune protection against i.m. challenge with pathogenic RABV [29]. While it is expected that an adjuvant would enhance the immune response, we did not find that the use of CFA with UV-inactivated RABV improved the survival of mice challenged with DRV4 i.n. This suggests that the immune response elicited by inactivated RABV is unable to prevent virus spread in the CNS. It has also been found that rabies immunoglobulin must be administered in conjunction with inactivated vaccine to achieve 100% survival post-exposure [30]. Our finding that live-attenuated RABV vaccines are more effective in the induction of protection against i.n. and i.c. challenge with wild-type RABV provides an explanation as to why GGG [16] and other highly attenuated RABV such as ERAg333 [31] are effective in post-exposure regimens in the absence of added rabies VNA [16]. Infection with GGG would rapidly induce the IFN- γ -dependent immune mechanisms capable of preventing wild-type virus replication and trigger the processes that promote immune effector activity in CNS tissues. Thus, treatment with live-attenuated GGG can contain the virus until sufficient antibody production is elicited to mediate virus clearance. As supported by the current data and prior reports of the failure of late stage PEP [9], administration of an inactivated vaccine, and VNA is likely to only be effective if wild-type RABV has not yet reached CNS tissues. Based on the current data, a similar consideration holds for an

immunized individual that is exposed to wild-type RABV by a means that introduces the virus in close proximity to the CNS. Pre-existing type-1 rabies immunity would be expected to be protective, but not a type-2 response.

While our data support, in principle, the use of live-attenuated RABV vaccines as alternatives to the inactivated preparations currently used for pre- and post-exposure rabies prophylaxis, there are several considerations for its use. Cost-effectiveness is likely to be excellent as considerably less virus and only a single dose is required to induce strong immunity. However, inactivated RABV are quite stable at different temperatures and live viruses are less so, unless prepared by a vaporization method [32]. Nevertheless, as found in this study, our live-attenuated RABV strain can retain sufficient infectivity to induce fully protective immunity after three weeks at 25 °C without additional processing. In addition to efficacy, safety is the most important prerequisite for the use of a live attenuated virus in vaccination. In this regard, the GGG variant is a good candidate. This vaccine strain was reverse engineered [33] to increase the amount of the immunogenic glycoprotein expressed during infection [34]. GGG has proven nonpathogenic even for developmentally immunocompromised baby mice [16], and does not spread extensively into nervous system tissue [35]. Nevertheless, GGG infection induces immune mechanisms that can clear wildtype RABV from CNS tissues [36] and can be used to establish long-term protection against a wildtype RABV exposure targeting the CNS. Historically, vaccines such as the measles, mumps and rubella (MMR), based on live-attenuated viruses, have proven to be most effective with the benefits far outweighing potential risks. The safety of live-attenuated RABV vaccines in animal models has been well established. For example, GG has been found to be safe and effective in a target species [37]. The transition of live-attenuated RABV vaccines from animals to humans may be driven by the understanding that therapeutic intervention after the virus has entered peripheral nerves requires a type-1 immune response.

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Article

In Vivo Efficacy of a Cocktail of Human Monoclonal Antibodies (CL184) Against Diverse North American Bat Rabies Virus Variants

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Abstract: Following rabies virus (RABV) exposure, a combination of thorough wound washing, multiple-dose vaccine administration and the local infiltration of rabies immune globulin (RIG) are essential components of modern post-exposure prophylaxis (PEP). Although modern cell-culture-based rabies vaccines are increasingly used in many countries, RIG is much less available. The prohibitive cost of polyclonal serum RIG products has prompted a search for alternatives and design of anti-RABV monoclonal antibodies (MAbs) that can be manufactured on a large scale with a consistent potency and lower production costs. Robust *in vitro* neutralization activity has been demonstrated for the CL184 MAb cocktail, a 1:1 protein mixture of two human anti-RABV MAbs (CR57/CR4098), against a large panel of RABV isolates. In this study, we used a hamster model to evaluate the efficacy of experimental PEP against a lethal challenge. Various doses of CL184 and commercial rabies vaccine were assessed for the ability to protect against lethal infection with representatives of four distinct bat RABV lineages of public health relevance: silver-haired bat (Ln RABV); western canyon bat (Ph RABV); big brown bat (Ef-w1 RABV) and Mexican free-tailed bat RABV (Tb RABV). 42–100% of animals survived bat RABV infection when CL184 (in combination with the vaccine) was administered. A dose-response relationship was observed with decreasing doses of CL184 resulting in increasing mortality. Importantly, CL184 was highly effective in neutralizing and clearing Ph RABV *in vivo*, even though CR4098 does not neutralize this virus *in vitro*. By comparison, 19–95% survivorship was observed if human RIG (20 IU/kg) and vaccine were used following challenge with different bat viruses. Based on our results, CL184 represents an efficacious alternative for RIG. Both large-scale and lower cost production could ensure better availability and affordability of this critical life-saving biologic in rabies enzootic countries and as such, significantly contribute to the reduction of human rabies deaths globally.

Keywords: bat viral diseases; monoclonal antibody; immune globulin; lyssavirus; post-exposure prophylaxis; rabies; vaccine; zoonosis

1. Introduction

Rabies is an acute progressive encephalitis caused by lyssaviruses. Despite significant progress in our understanding of rabies pathobiology and epidemiology, and major advancements in the

development of safe and effective biologics for disease prevention, this neglected zoonosis causes approximately 60,000 human deaths annually [1,2]. Although dogs are the major global reservoir for rabies virus (RABV), bats are responsible for the majority of human rabies fatalities in the Americas, Australia and Western and Central Europe. Regardless of the source of viral exposure, human rabies is preventable with proper wound care, prompt administration of modern vaccine and rabies immune globulin (RIG) [3,4]. Over the past several decades, post-exposure prophylaxis (PEP) schedules have evolved, encompassing fewer doses of both intramuscular (i.m.) as well as dose-sparing intradermal (i.d.) routes for administration of inactivated vaccine in as few as four doses. However, in the absence of licensed, commercially available, live-attenuated rabies vaccines, administration of RIG remains a critical component of PEP when inactivated vaccines are used [3,5].

Current commercially available human and equine RIGs (HRIG, ERIG) are produced via pooling of human or equine plasma from immunized donors. Such production processes are associated with significant costs as well as with a possibility for transmission of potential bloodborne pathogens. Low-scale manufacturing, coupled with prohibitive cost, renders these immune globulins virtually unavailable for a majority of the population at risk in rabies-zoonotic countries such as Asia and Africa, where the demand is the highest. New approaches, such as the use of hybridoma and humanization technologies, as well as use of single chain and VHH single domain antibodies, allow for cell culture or microbial expression systems production of monoclonal antibodies (MAbs), a promising alternative to polyclonal RIG with reduced risks for transmission of pathogens and large-scale production for a reduced cost. An inherent disadvantage of any MAb, however, is the specificity/affinity to a single binding epitope on a viral protein and consequently a diminished breadth of neutralizing activity for certain virus variants with amino acid substitutions that prevent MAb binding [5,6].

The concept of using a cocktail of at least two MAbs, which target distinct, non-overlapping epitopes and that do not compete for binding to the RABV glycoprotein, as a potential alternative to RIG in PEP, has been widely accepted by the scientific community and also endorsed by WHO [3,7–10]. CL184 is a cocktail of two human MAbs (CR57 and CR4098), produced in human PER.C6[®] cells. CL184 meets the criteria of binding to different epitopes (CR57 to epitope I, CR4098 to IIIa) and does not engender competition for the binding to RABV glycoprotein [8,11]. Previously, CR57, CR4098 and CL184 were evaluated in vitro against a panel of 26 distinct RABV isolates of public health and veterinary significance [12]. Although CR57 alone did not neutralize a south central skunk RABV and big brown bat RABV (*Eptesicus fuscus* western lineage 1, Ef-w1); and CR4098 alone did not neutralize mongoose RABV from South Africa, big brown bat RABV (*Eptesicus fuscus* eastern lineage 1, Ef-e1) or western canyon bat (*Parastrellus hesperus* from Arizona) [8], it was shown that the combination of these two MAbs, CL184, did provide neutralization of all 26 tested RABV isolates [12], as well as neutralization of an additional panel of 18 RABV isolates (reported in this manuscript, Table 1). Furthermore, it was shown retrospectively that the lack of neutralization was related to epitope mutations introduced during cell culture amplification of the primary RABV isolates in the case of the south-central skunk RABV and the western canyon bat (Ph) RABV from Arizona (previously unpublished results). During one in vivo experiment, CL184, in combination with vaccine, protected hamsters against a lethal challenge with canine RABV, when administered 24 h after exposure, which was comparable with the results obtained for HRIG. In addition, CL184 was similar to HRIG in demonstrating a lack of interaction with vaccine [12]. These results suggested that CL184 could be an efficacious alternative to RIG as a part of rabies PEP.

Table 1. Breadth of in vitro neutralization of HRIG, CL184 and its components against selected RABV isolates not covered by previous publications [12].

Lyssaviruses	HRIG *	CR57	CR4098	CL184
Cow/dog, Sri Lanka	+	+	+	+
Dog, China 2005	+	+	+	+
Dog, China (RV342)	+	+	+	+
Dog, India (I 148)	NT	NT	NT	+
Dog, India (I 151)	+	+	+	+
Dog, India (I 155)	+	+	+	NT
Dog, Philippines	+	+	+	+
Dog, Philippines (231/002)	+	+	+	NT
Dog, Tunisia	+	+	+	+
Human/dog, UK ex India	NT	NT	NT	+
Human/wolf, Russia Siberia (RVHN)	+	+	+	+
Mongoose, South Africa	+	+	-	+
Raccoon dog, Russia/Far East	+	+	+	+
Skunk, south central (SK4384)	+	+	+	+
Bat, <i>Lasiurus borealis</i> , TN (tn132)	NT	NT	NT	+
Bat, <i>Lasiurus borealis</i> , TN (tn269)	NT	NT	NT	+
Bat, <i>Lasiurus borealis</i> , VA (VA399)	NT	NT	NT	+
Bat, <i>Lasiurus cinereus</i> , TN	NT	NT	NT	+

* Imogam (Sanofi Pasteur); + indicates neutralization; NT—not tested.

Given the public health importance and the diversity of bat RABV present in the Americas, as well as the frequency and distribution of isolates with mutations in the MAb binding epitopes [13], the objective of this study was to evaluate the efficacy of CL184 against selected distinct bat RABV variants from North America (including those having a critical mutation in the MAb-binding site on the viral glycoprotein). The work was done using an animal model to compare vaccine protection using standard PEP (that included HRIG and commercial rabies vaccines) against those using CL184 in substitution for HRIG.

2. Materials and Methods

2.1. Animals and Viruses

Two-month-old female Syrian hamsters (*Mesocricetus auratus*), weighing approximately 100–120 g, were obtained from commercial suppliers and held for acclimation for 3–7 days upon arrival before use. Four different RABV isolates, representatives of distinct bat-associated RABV lineages (Figure 1), were used as a challenge in PEP experiments. An *Eptesicus fuscus* Ef-w1 RABV (A09-2400L), $10^{6.1}$ 50% mouse intracerebral lethal doses (MICLD₅₀)/50 μ L, was isolated from the salivary glands of a naturally infected gray fox (*Urocyon cinereoargenteus*) in Arizona. A *Parastrellus hesperus* RABV (Ph 3860 RABV, A07-0449), 10^4 MICLD₅₀/50 μ L, was isolated from the salivary glands of a naturally infected western canyon bat from Arizona. A *Lasionycteris noctivivans* RABV (WA Ln RABV, A04-0723 and A12-6377), 10^6 MICLD₅₀/50 μ L, was isolated from the salivary glands of a naturally infected silver-haired bat from Washington. A *Tadarida brasiliensis* RABV (Tb RABV, A14-3368 and TX3368), approximately 10^5 TCID₅₀/100 μ L, was isolated from the brain of a naturally infected Mexican free-tailed bat from Texas. All the original isolates were amplified in cell culture or following i.c. challenge in mice. The titer of viruses was determined in mouse neuroblastoma cell culture and expressed in the 50% tissue culture infectious doses (TCID₅₀) or focus forming units (FFU) as well as via titration in mice (MICLD₅₀) and relative pathogenicity was determined in naive Syrian hamsters prior to experimental prophylaxis. Only RABV isolates that produced at least 75% mortality in this model were selected for further experiments with a sample size determined accordingly. All animal handling and experimental procedures were undertaken in compliance with CDC Institutional Animal Care and Use Committee guidelines (protocols #1593FRAHAMC and 2266FRAHAMC).

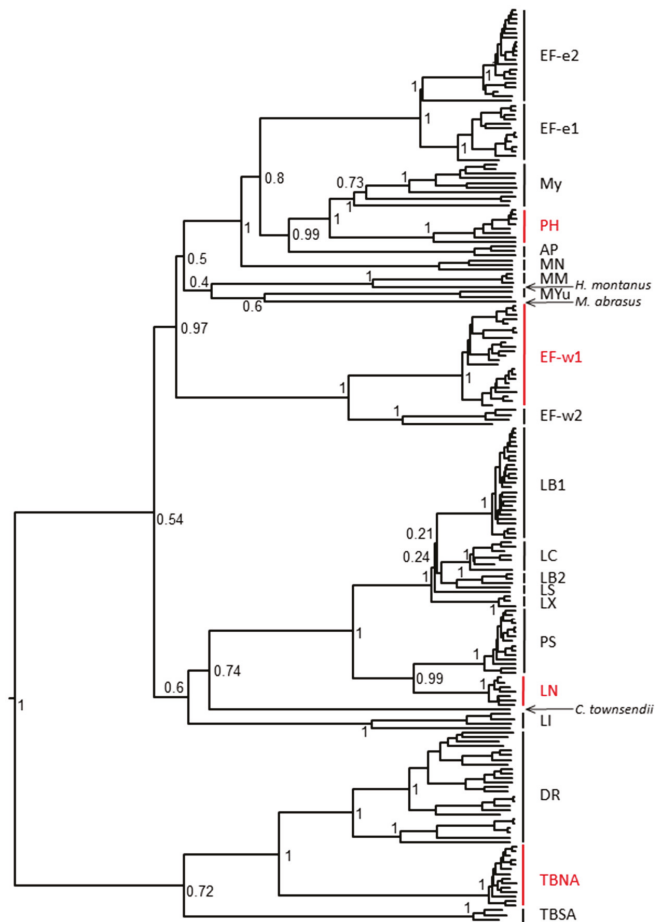


Figure 1. Phylogenetic relationship of rabies virus isolates used in this study with other representatives of bat RABV lineages. RABV used in this study are highlighted in red (PH—*Parastrellus hesperus*, Ef-w1—*E. fuscus* western lineage 1, LN—*Lasionycteris noctivagans*, TBNA—*Tadarida brasiliensis* North America).

2.2. Biologics

A volume of 50 µL of the commercial inactivated human diploid cell vaccine (HDCV), Imovax® (Sanofi Pasteur, Lyon, France) or purified chicken embryo cell vaccine (PCECV), RabAvert® (Novartis Vaccines, Marburg, Germany; for Tb groups), with a minimum potency of 2.5 IU/mL was administered via the intramuscular (i.m.) route, according to the Essen (on days 0, 3, 7, 14 and 28) or modified Essen (on days 0, 3, 7 and 14) regimen. In addition, 50 µL (20 IU/kg) of HRIG (Imogam® Rabies-HT (Sanofi Pasteur, 150 IU/mL)) or 6, 12, 16, 18 or 24 µg/kg of CL184 (mixture of CR57 and CR4098 in 1:1 protein ratio) was administered i.m. into the site of virus inoculation at day 0.

In all of our experiments, the amount of MAbs administered was expressed in µg/kg as opposed to IU/kg standardly indicated for polyclonal RIG products. HRIG is a polyclonal product consisting of many non-specific proteins with a very small fraction of rabies-specific antibodies, and hence correlation between protein concentration (µg/kg) and rabies virus specific neutralization (IU/kg) could not easily be established. In contrast, cell cultures producing only one anti-rabies MAb combined

with protein purification techniques result in highly purified MAb devoid of other contaminants. Hence, such antibodies can be accurately quantified and thus dosed on basis of $\mu\text{g}/\text{kg}$, thereby excluding dosing variability as a result of inconsistency in potency measurements.

2.3. Experimental Design

The calculations of group sample sizes for each individual RABV isolate were based on statistical analysis and taking into account the mortality of naïve (non-treated) hamsters during preliminary experiments. To achieve the statistical power required to demonstrate the potential added benefit of tested biologics, we selected a cutoff in mortality of $\geq 75\%$. If mortality was 100% in RABV-challenged hamsters (via a titration experiment), a group size of 12 animals was considered adequate and selected for consequent experiments. If, however, the mortality of naïve animals was $<100\%$ but $>75\%$, a group size of 21 animals was selected as adequate for comparative non-inferiority non-clinical experiments.

2.4. Model Validation/Post-Exposure Prophylaxis (PEP) Initiation Determination

Prior to the evaluation of PEP efficacy, determination of the PEP initiation window was conducted. Approximately two-month-old female Syrian hamsters ($n = 12$ or 21) were assigned randomly to experimental groups and infected into the left gastrocnemius muscle with an expected lethal dose of RABV (Ef-w1 RABV, Ph 3860, WA Ln, Tb RABV, based upon prior observations; unpublished data). Thereafter, PEP was initiated 2, 6 or 24 h following the challenge. On days 0 (set as the day of PEP initiation), 3, 7, 14, $+/-$ 28 the animals received a dose of rabies vaccine (HDCV for Ef-w1, Ph and WA Ln; and PCECV for Tb) applied into the right gastrocnemius muscle. In addition, HRIG at a dose of 20 IU/kg was administered at the initiation of PEP into the same i.m. location as virus challenge. For comparison, besides a control (PBS only) group, a vaccine-only group was included with the same time windows of PEP initiation. Monitoring was the same as below for efficacy experiments.

2.5. Evaluation of the Efficacy of HRIG/Vaccine versus CL184/Vaccine during PEP

Female Syrian hamsters ($n = 12$ or 21) were assigned randomly to experimental groups and infected in the left gastrocnemius muscle with a lethal dose of RABV. Timeline for challenge and initiation of PEP, as well as viral dose used, were selected based on the model validation experiments and prior experimental data regarding particular virus pathobiology in hamster model. The PEP was initiated 24 h (Ln, Ph and Ef-w1 RABV) or 2 h post infection (p.i.) (Tb RABV). On days 0, 3, 7, 14, and 28 the animals received a dose of rabies vaccine. In addition, 50 μL of HRIG at 20 IU/kg or 50 μL of different doses of CL184 at (6, 12 or 16 $\mu\text{g}/\text{kg}$ for Ln, Ph and Ef-w1 RABV or 12, 18 and 24 $\mu\text{g}/\text{kg}$ for Tb RABV), were administered i.m. at the site of virus inoculation on day 0. The animals were followed for 45 days and their clinical signs were monitored. All animals developing any specific signs of rabies were euthanized immediately according to an IACUC approved clinical score. Brains were removed at necropsy and subjected to detection of rabies virus antigens by the direct fluorescent antibody (DFA) test, as described below. Similarly, all animals surviving at the end of the experimental period were euthanized and their brains examined for the presence of RABV antigens.

2.6. Laboratory Methods

2.6.1. Direct Fluorescent Antibody (DFA) Test

The RABV antigens were detected in brain samples using the DFA test [14] with a fluorescein-isothiocyanate (FITC)-conjugated anti-RABV MAb (Fujirebio Diagnostics, Inc., Malvern, PA, USA).

2.6.2. Rapid Fluorescent Focus Inhibition Test (RFFIT)

The rapid fluorescent focus inhibition test (RFFIT) was performed according to a standard, previously described protocol [15].

2.6.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR), Hemi-Nested RT-PCR (hnRT-PCR) and Sequencing

To confirm identity of RABV in central nervous system (CNS) tissue of euthanized animals with the initial inoculum and to identify any potential selection of escape mutations, total RNA was extracted from the CNS tissue samples using TRIZol reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's recommendations. The RT-PCR was performed as described elsewhere [16]. The RT-PCR products were purified and subjected to direct sequencing on an ABI 3730 DNA Sequencer (Applied Biosystems, Carlsbad, CA, USA). The complete and partial nucleotide G gene sequences were assembled and converted into amino acid sequences using the Bio Edit program, v.7 (Ibis Biosciences, Carlsbad, CA, USA) [17]. Amino acid sequences of the aligned MAb binding epitopes were compared across the dataset.

2.7. Statistical Analysis

Kaplan–Meier survival curves were calculated in the Statistical Analysis Software (SAS, version 9.2. SAS Institute Inc., Cary, NC, USA). The log-rank test was used to test differences between group survival distributions. The null hypothesis of identical survival functions was rejected at $p < 0.05$. GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA, USA) was used to create survival curve graphs.

3. Results

3.1. Neutralization of Selected RABV Isolates *In Vitro*

An additional set of 18 RABV isolates of public health importance from Africa, Asia and Americas were tested for neutralization to complement the initial panel of 26 RABV isolates [12]. Twelve of the isolates were tested with CR57, CR4098, and HRIG (Imogam), and all isolates were neutralized with the exception of one, South Africa mongoose RABV, which was not neutralized by CR4098 (Table 1). This RABV isolate contains a N336D mutation in its glycoprotein which explains the observed lack of neutralization (data not shown). Further, 16 of 18 RABV isolates were tested and were shown to be efficiently neutralized by CL184 (Table 1).

3.2. Model Validation/PEP Initiation Window

In the experiments dedicated to the determination of PEP initiation window, survivorship of hamsters in control (placebo) groups was 8.3% for the Ln RABV, 0% for the Ph RABV, and 16.7% for the Ef-w1 RABV challenge. In contrast, in the vaccine-only group with PEP initiated 6 h p.i. the survivorship was 16.7%, 4.8%, 8.3%, whereas with PEP initiated 24 h p.i. it was 25%, 19%, 16.7% for these viruses, respectively (Table 2, Figure 2).

When HRIG + HDCV were administered 6 h p.i., 83.3%, 57% and 75% of experimental animals survived in the Ln RABV, Ph RABV, Ef-w1 RABV groups, respectively. Similarly, 83.3%, 66.7% and 91.7% of animals survived challenge with the Ln RABV, Ph RABV and Ef-w1 RABV, respectively, when HRIG and vaccine were administered 24 h p.i.

In the experiment with the Tb RABV, 0% survivorship was observed in the control as well as in the vaccine-only and in the HRIG + PCECV groups when biologics were administered 2 h p.i. (Table 2, Figure 2).

Table 2. Validation of animal model and postexposure prophylaxis (PEP) initiation (*p*-values based on log-rank Mantel-Cox test, comparing CL184 with standard PEP regimen 20IU/kg HRIG/vaccine).

Group	Survival after 45 days observation							
	Ln (%)	<i>p</i> -Value *	Ph (%)	<i>p</i> -Value	Ef-w1 (%)	<i>p</i> -Value	Tb (%) ‡	<i>p</i> -Value
Control (PBS only)	8.3	-	0	-	16.7	-	0	-
Vaccine only, 6 h p.i.	16.7	0.9984	4.8	0.5224	8.3	0.0023	0	0.8619
Vaccine only, 24 h p.i.	25	0.5174	19	0.6200	16.7	0.3391	NA	
HRIG/vaccine, 6 h p.i.	83.3	0.0002 0.0007	57	<0.0001 <0.0001	75	0.0005 0.0002	0	0.3901 0.3161
HRIG/vaccine, 24 h p.i.	83.3	<0.0001 0.0023	66.7	<0.0001 0.0002	91.7	<0.0001 0.0001	NA	-

* *p*-Value for vaccine-only group is comparison to control; HRIG/vaccine groups, first *p*-value is comparison to control group and second is comparison to vaccine-only group. (Ph—*Parastrellus hesperus*, Ef-w1—*Eptesicus fuscus* western lineage 1, Ln—*Lasionycteris noctivagans*, Tb—*Tadarida brasiliensis* North America). ‡ Groups challenged with Tb RABV were administered PEP at 2 h p.i. with PCECV vaccine.

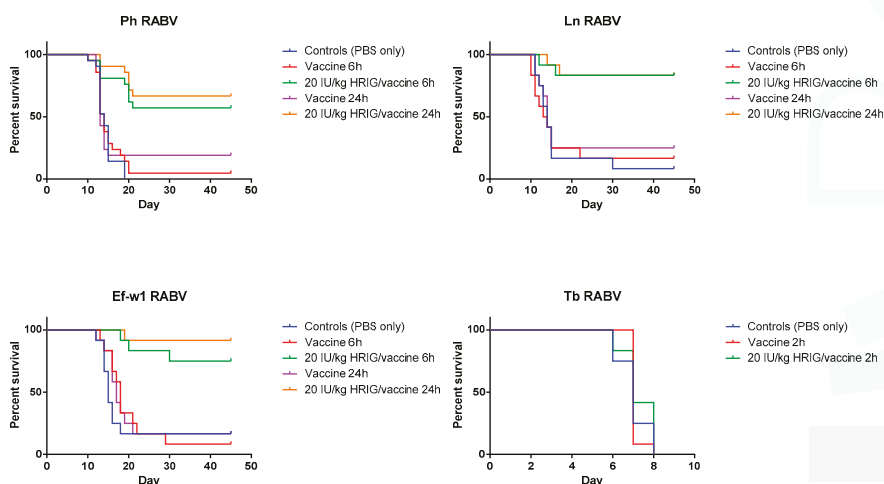


Figure 2. Validation of model and PEP initiation—Kaplan-Meier survival curves for Syrian hamsters after infection with bat rabies viruses. Hamsters (*n* = 12 or 21 per group) infected with the indicated RABV isolate 2, 6, or 24 h prior to intervention, received PEP consisting of either vaccine only (HDCV or PCECV) or vaccine in combination with 20 IU/kg HRIG.

3.3. Evaluation of the Efficacy of HRIG/Vaccine versus CL184/Vaccine during PEP

In the experimental evaluation of the efficacy of biologics when administered 24 h p.i., Ln RABV, HRIG (20 IU/kg) + rabies vaccine (HDCV) resulted in 58% survival, whereas survival of animals in groups treated with 6 µg/kg, 12 µg/kg, or 16 µg/kg of CL184, 42%, 50% and 67% survived, respectively (Table 3, Figure 3). In a mock-control group and in the vaccine-only group, 11% and 25% of experimental animals, respectively, survived the challenge.

In the experiment where a Ph RABV isolate was used, administration of HRIG (20 IU/kg) + HDCV resulted in 19% survival whereas survival in groups treated with 6 µg/kg, 12 µg/kg and 16 µg/kg of CL184 was 57%, 48% and 57%, respectively (Table 4, Figure 3). In contrast, survivorship of 17% and 0% was observed in the mock-control and in the vaccine-only group, respectively, following Ph RABV challenge.

Table 3. Evaluation of the efficacy of CL184/vaccine during PEP (*p*-values based on log-rank Mantel-Cox test, comparing CL184 with standard PEP regimen 20 IU/kg HRIG/vaccine).

Groups	Survival after 45 Days Observation							
	Ln (%)	<i>p</i> -Value *	Ph (%)	<i>p</i> -Value	Ef-w1 (%)	<i>p</i> -Value	Tb † (%)	<i>p</i> -Value
Control (PBS only)	11.1	-	16.7	-	33.3	-	0	-
Vaccine only	25	0.3630	0	0.0034	38.1	0.8464	8.3	0.6668
20 IU/kg HRIG/vaccine	58.3	0.0430	19	0.8705	95.2	<0.0001	66.7	0.0007
24 µg/kg CL184/vaccine	NA	0.1477	NA	0.0003	NA	<0.0001	NA	0.0006
18 µg/kg CL184/vaccine	NA	-	NA	-	NA	-	100	0.0319
16 µg/kg CL184/vaccine	NA	-	NA	-	NA	-	83.3	0.3959
16 µg/kg CL184/ vaccine	66.7	0.5951	57.1	0.0177	100	0.3173	NA	-
12 µg/kg CL184/ vaccine	50	0.8931	47.6	0.0699	95.2	0.9862	66.7	0.9483
6 µg/kg CL184/ vaccine	41.7	0.5257	57.1	0.0062	85.7	0.2847	NA	-

* *p*-Value for vaccine-only group is comparison to control; HRIG/vaccine groups, first *p*-value is comparison to control group and second is comparison to vaccine-only group; CL184/vaccine groups is comparison to HRIG/vaccine group. (Ph—*Parastrellus hesperus*, Ef-w1—*Eptesicus fuscus* western lineage 1, Ln—*Lasionycteris noctivagans*, Tb—*Tadarida brasiliensis* North America). † PCECV vaccine was used for Tb group while HDCV for Ef-w1, Ph and WA Ln.

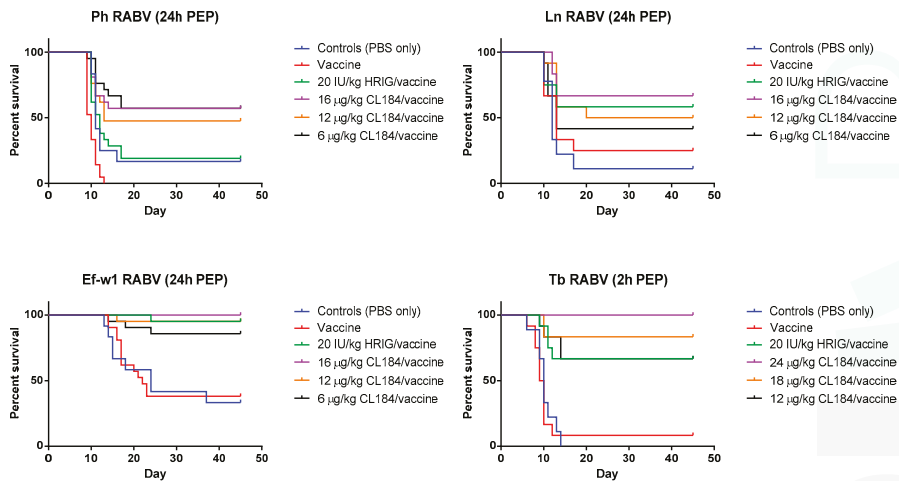


Figure 3. Evaluation of the efficacy of CL184/vaccine during PEP—Kaplan-Meier survival curves for Syrian hamsters after infection with bat rabies viruses. Hamsters (*n* = 12 or 21 per group) infected with a RABV isolate 2 or 24 h prior to intervention received PEP as outlined in Materials and Methods. Hamsters received 20 IU/kg HRIG or CL184 at a dosage of 6, 12 or 16 µg/kg for the Ln, Ph and Ef-w1 RABV or CL184 at a dosage of 12, 18 and 24 µg/kg for the Tb RABV.

Table 4. Bat RABV isolates used in the animal studies.

RABV Isolate	CR57 Epitope (226–231)	CR4098 Epitope (330–338)	CR57 Neutralization	CR4098 Neutralization
Bat, <i>Lasionycteris noctivagans</i>	KLCGVP	KSVRTWNEV	Yes	Yes
Bat, <i>Parastrellus hesperus</i>	KLCGVP	KSVRTWNET *	Yes	No
Bat, <i>Eptesicus fuscus</i> w1 lineage §	KLCGVP	KSIRTWNEI †	Yes	Yes
Bat, <i>Tadarida brasiliensis</i>	KLCGVS	KSVRTWNEI	Yes	Yes

* Ph 3860 RABV isolate used in our study has I338T mutation in CR4098 epitope precluding its neutralization by that particular MAb. We used this mutation (resulting from cell culture passage) as a model to test CL184 in vivo against virus which is not neutralized in vitro by one MAb from the cocktail. † Although some naturally-occurring *Eptesicus fuscus* Ef isolates have N336D mutation in the antigenic site III precluding neutralization of CR4098, we have not had that particular isolate available for in vivo experiments. Our isolate with N336 was neutralized by both CR57 and CR4098. § Isolated from gray fox, *Urocyon cinereoargenteus*.

Combination of HRIG + HDCV resulted in 95% survivorship when administered 24 h p.i. with the Ef-w1 RABV variant, whereas administration of 6 µg/kg, 12 µg/kg and 16 µg/kg of CL184, in combination with HDCV, resulted in 86%, 95% and 100% survivorship, respectively (Figure 3). A survivorship of 33% and 38% was observed in the mock-control and in the vaccine-only groups, respectively, for this virus (Table 3, Figure 3).

When PEP was initiated 2 h p.i. with Tb RABV, 67% of animals survived in the HRIG + PCECV group, whereas administration of 12 µg/kg, 16 µg/kg and 24 µg/kg of CL184, in combination with PCECV resulted in 67%, 83% and 100% survivorship, respectively (Figure 3). A survivorship of 0% and 8% was observed in the mock-control and the vaccine-only group.

3.4. Sequence Analyses of the Original Inoculum and Virus Detected in CNS of Experimental Animals

When G nucleotide sequences of the original Ph 3860 isolate and its cell culture passages were compared to each other, it was confirmed that the first cell culture passage contained a mix of two variants, I338 and T338 (within the CR4098 binding epitope) and that consensus sequences of viral populations from further cell culture passages demonstrated solely the T338 variant. Virus recovered from the infected hamsters (following experimental challenge) resulted in a detection of either I338, or T338, or both phenotypes irrespective of biologics used in PEP and in the mock-control groups. All other output viruses matched the input virus demonstrating CL184 did not select for escape mutations. Phylogenetic relationship of Ph 3860 as well as other bat RABV isolates used in this study to other relevant bat RABV viruses is depicted in Figure 1 and relevant epitopes for virus variants used in this experiment are shown in Table 4.

4. Discussion and Conclusions

The scarcity of conventional RIG prompted research and development of alternatives. Multiple MAbs and their combinations have been evaluated *in vitro* and *in vivo* during the past decade as potential replacements for RIG (e.g., SO57 [7]; CR57, CR4098, [8,9,12]; RAB1 [18]; E559.9.14, 1112-1, 62-71-3, M727-5-1, and M777-16-3 [19]; RVC20-RVC58 [3,10,20]).

A common denominator for all individual MAbs is their limited breadth of neutralization, inevitably resulting in the inability of one MAb to neutralize the entire spectrum of RABV variants. However, as previous *in vivo* experiments of [8,11] have demonstrated, this can be compensated by a combination of two MAbs, which bind to non-overlapping epitopes.

Our study has shown that in cases of severe exposures to bat RABV (i.e., high virus doses delivered intramuscularly), administration of either HRIG or CL184, is critical for rapid peripheral neutralization and clearance of rabies virus. In both mock-control (placebo) and inactivated rabies vaccine-only groups, the mortality of 62–100% was observed (Tables 3 and 4). In contrast, CL184, when administered in a dose ≥ 6 µg/kg in combination with vaccine, provided a significant benefit compared to vaccine alone.

In addition, the efficacy of CL184 plus vaccine in a dose ≥ 12 µg/kg was not inferior when compared to PEP consisting of HRIG (20 IU/kg) and rabies vaccine (HDCV), with 50–67% of experimental animals surviving a Ln RABV challenge, 48–57% surviving Ph RABV challenge, 95–100% surviving Ef-w1 challenge, and 67–100% surviving a Tb RABV challenge (Table 4, Figure 3).

Importantly, our experiments have shown that CL184 is efficacious against challenge with Ph RABV, which was not neutralized *in vivo* by the MAb CR4098, a component of the CL184 cocktail, given the mutation I338T (Tables 1, 2 and 4; Figure 3). Of note, this mutation was introduced during cell culture amplification of the primary Ph RABV isolate as indicated by G gene sequencing of a series of virus stocks. Epitope mutations might result either from adaptation of the primary RABV isolates to cell culture as shown in the case of south central skunk RABV (Rupprecht, Marissen, personal communication). Alternatively, both sequence variants might be present in the original field isolate in different proportions. Although initially described as a result of selection of CVS rabies strain mutants following culture with neutralizing anti-glycoprotein antibodies [21], our study demonstrated that the

I338T mutation affecting neutralization could also occur as a result of amplification in cell culture or in the laboratory rodent model without antibody-mediated selection pressure. Of note, 338T did not appear in consensus sequences of 10 natural Ph RABV isolates (data not shown). The Ph 3860 virus with the predominant I338T substitution served as a good model to assess the in vivo efficacy of CL184 given that it was not neutralized in vitro by one of the cocktail MABs.

Natural occurrence of RABV with mutation(s) in the MAB binding epitope is, however, critical for the assessment of the adequacy of a particular MAB or cocktail of MABs to be used as PEP in a particular geographic area. Sequencing of epitopes has been shown to be a reliable predictor of MAB neutralization capacity in vitro and in vivo. As previously described [13], the binding epitope for CR57 is relatively conserved with only one isolate/sequence exception (frequency 0.1%; 1/1042, Chinese dog, SE Asia-2), the K226M substitution, shown to preclude binding (data not shown). However, this reported mutation is more likely to be a sequence error rather than representing a true natural isolate, as in more than 175 Chinese RABV isolates no critical mutation in the CR57 epitope was observed (data not shown). Although the frequency of substitutions precluding neutralization of MAB CR4098 in antigenic site III is higher (N336D, 63/1042, 6%, including besides others big brown bat (*Eptesicus fuscus*) RABV from North America), our study showed that combining CR57 and CR4098 in a cocktail can effectively neutralize virus in vivo even if one of those MABs does not neutralize it in vitro. Of note, CR4098 was still shown to bind to RABV glycoprotein harboring an N336D mutation [8] which could facilitate viral clearance in vivo. In addition, in neutralization experiments using a natural big brown bat (*Eptesicus fuscus*) RABV isolate harboring a N336D mutation, complete neutralization by CR4098 at 15 µg/mL was observed (data not shown). Overall, these findings emphasize the importance of WHO recommendations requiring inclusion of at least two MABs [3] with non-overlapping epitopes in biologics for PEP as well as a need for continuous surveillance for natural occurrence of RABV isolates with mutations which may preclude MAB binding.

Although effective concentration of immune globulin in the circulation of individual experimental animals was not measured during the observation period, mortality and survivorship data demonstrated a dose effect, with lower Mab doses resulting in higher mortality.

Virulence of different RABV variants influences the efficacy of PEP. As demonstrated in this study, viruses which are more pathogenic in a particular model (e.g., Tb RABV) and possibly spread more rapidly towards the CNS, require an earlier initiation of PEP (2 hours p.i.) as compared to other viruses, for which PEP initiated 6 or 24 h p.i. still seemed to provide an adequate prophylactic effect within the hamster model (Table 4). Although pathogenesis is dependent on route, viral dose, host species and proximity the exposure site to the CNS, further studies may elucidate differences in the kinetics of peripheral neuronal entry and axonal spread of various RABVs.

In this study, the efficacy of CL184, when administered in a dose ≥ 12 µg/kg in combination with a commercial inactivated rabies vaccine, was not inferior to PEP consisting of HRIG and the same vaccine. As such, CL184 presents a promising, non-inferior alternative for RIG during rabies PEP. Large scale and lower cost production of MABs could ensure availability and affordability of this critical life-saving biologic in rabies enzootic countries and would significantly contribute to the reduction of human rabies deaths globally.

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Review

The Imperative of Palliation in the Management of Rabies Encephalomyelitis

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Abstract: The aim of this review is to guide clinicians in the practical management of patients suffering from rabies encephalomyelitis. This condition is eminently preventable by modern post-exposure vaccination, but is virtually always fatal in unvaccinated people. In the absence of any proven effective antiviral or other treatment, palliative care is an imperative to minimise suffering. Suspicion of rabies encephalomyelitis depends on recognising the classic symptomatology and eliciting a history of exposure to a possibly rabid mammal. Potentially treatable differential diagnoses must be eliminated, notably other infective encephalopathies. Laboratory confirmation of suspected rabies is not usually possible in many endemic areas, but is essential for public health surveillance. In a disease as agonising and terrifying as rabies encephalomyelitis, alleviation of distressing symptoms is the primary concern and overriding responsibility of medical staff. Calm, quiet conditions should be created, allowing relatives to communicate with the dying patient in safety and privacy. Palliative management must address thirst and dehydration, fever, anxiety, fear, restlessness, agitation, seizures, hypersecretion, and pain. As the infection progresses, coma and respiratory, cardiovascular, neurological, endocrine, or gastrointestinal complications will eventually ensue. When the facilities exist, the possibility of intensive care may arise, but although some patients may survive, they will be left with severe neurological sequelae. Recovery from rabies is extremely rare, and heroic measures with intensive care should be considered only in patients who have been previously vaccinated, develop rabies antibody within the first week of illness, or were infected by an American bat rabies virus. However, in most cases, clinicians must have the courage to offer compassionate palliation whenever the diagnosis of rabies encephalomyelitis is inescapable.

Keywords: rabies; encephalomyelitis; palliation; diagnosis; treatment

1. Introduction

The primary aim of this review is to re-emphasise the humanitarian role of palliative treatment in the compassionate care of patients with rabies encephalomyelitis, who have no chance of recovery. We provide practical details of this management and summarise the clinical and laboratory evidence leading to the fateful diagnosis of rabies encephalomyelitis.

Rabies infection following bites by dogs and other terrestrial mammals is eminently preventable by full modern post-exposure treatment, but if this opportunity has been missed and the virus has infected the nervous system, the condition is almost always fatal in unvaccinated people. Two children bitten by bats in the USA, only one of whom had received rabies post-exposure vaccination, have recovered from rabies encephalomyelitis to live independent lives [1,2]. Another person, who was

bitten by a dog in Turkey and was incompletely vaccinated, also recovered [3]. However, most rabies patients given expert intensive care have either died in the acute phase or have survived for varying periods of time, but with incapacitating neurological impairment. [4,5]. In the absence of any proven effective antiviral or other treatment, how should clinicians manage patients with this appalling disease, particularly in poorer countries, where dog rabies is endemic?

The practical management of patients in this dire situation has been neglected, while thousands of patients continue to die each year after terrible suffering. For example, in India, 12,700 symptomatically identifiable furious rabies deaths occur annually, 41% in children under the age of 15 years [6]. However, patients dying of paralytic or dumb rabies are much more difficult to diagnose clinically and therefore to enumerate. No practical advice about the clinical management of rabies patients is available in dog rabies-endemic areas, where palliative care is the best and only option. As a result, clinicians presented with the occasional rabies patient have been forced to improvise. They feel unable to cope with the unpredictable, sometimes agitated, behaviour typical of 'furious' rabies encephalomyelitis. As a result, patients have been abandoned in isolated rooms and bound to their beds. The hydrophobic spasms of furious rabies are associated with feelings of terror and great agitation, and some patients may become aggressive [7]. Relatives needing an opportunity to ask questions and comfort the victim are also neglected, and patients may be sent home to families who are unable to deal with their distressing symptoms and are frightened of being infected themselves.

2. Clinical Recognition of Rabies Encephalomyelitis

The incubation period may be highly variable, but is usually 20–60 days after the bite exposure. Itching or other paraesthesiae at the site of the healed bite wound are common prodromal symptoms. However, many patients present with non-specific fever, headache, myalgia, fatigue, sore throat, gastrointestinal symptoms, irritability, anxiety, insomnia, or even hallucinations and may be referred to a wide variety of specialists. Correct diagnosis depends on taking a full history that may reveal the possibility of exposure to a potentially rabid mammal.

Usually within a week, the disease progresses to either furious or paralytic rabies encephalomyelitis [8]. Furious rabies is the commoner and more easily recognisable form. Hydrophobic spasms are pathognomonic and may be the only physical sign in the early stages. Growing thirst forces patients to ask for water, but attempts to drink, the sound of a tap running, or the mere mention of water, a draught of air (aerophobia), touching the palate, bright lights or loud noises may provoke powerful, jerky contractions of the diaphragm and accessory muscles of inspiration, sometimes with gripping retrosternal pain suggesting oesophageal spasms. There is no evidence of the laryngeal spasms and upper airway obstruction implied in some accounts [9]. At the end of the attack, the patient may convulse and become contorted into a position of transient opisthotonos. Patients describe a feeling of terror that they are unable to explain, associated with the spasms. Many die of respiratory or cardiac arrest during these episodes [10]. Phases of extreme excitement, aggression, anxiety, or hallucinations are interspersed with lucid intervals, during which patients may fully comprehend their appalling predicament. Other features include fever, meningism, cranial nerve lesions, autonomic nervous system overactivity (labile temperature, blood pressure, pulse rate, sweating, hypersalivation, lacrimation), cardiac tachyarrhythmias, myocarditis, dramatic polyuria due to diabetes insipidus, haematemesis from Mallory-Weiss oesophageal tears, and hypersexuality. Even with intensive care, patients soon become comatose and may die within a few days or survive for many weeks or months.

The more insidious paralytic or 'dumb' form of the disease is far less distinctive and consequently less often recognised [11–13]. It is characteristic of vampire bat-transmitted rabies and some cases of failed postexposure vaccination. Prodromal symptoms are followed by paraesthesiae, fever, and flaccid paralysis, starting in the bitten limb and then ascending with fasciculations [14], sensory symptoms, sphincter dysfunction, and terminal bulbar and respiratory paralysis. Hydrophobic spasms and

excitation are usually absent. Even without supportive care, these patients may survive as long as 30 days [11].

If rabies is suspected, every effort should be made to eliminate potentially-treatable differential diagnoses, including encephalopathies caused by other viruses, bacteria including rickettsia, fungi, parasites, drugs, toxins, and psychotic states [15]. This point is exemplified by the case of an agitated teenage girl admitted to hospital with a diagnosis of acute psychosis. She then became drowsy and developed seizures and phobic spasms. Rabies encephalitis was diagnosed, but when she was referred to another hospital in coma, investigations revealed *P. falciparum*. After treatment for cerebral malaria, she recovered fully [16]. Conversely, however, among 133 children dying of central nervous system infections in Malawi, 10.5% had unsuspected rabies encephalitis confirmed at post-mortem. Three (11.5%) of 26 of the fatal cases originally diagnosed as cerebral malaria were in fact due to rabies [17].

3. Confirmation of Suspected Rabies

This is important to stop the search for a potentially-treatable condition, to decide on a clinical management plan, to warn others possibly infected by the same animal but unvaccinated, and to offer pre-exposure prophylaxis to relatives and caregivers. Positive diagnoses also contribute to public health surveillance, and guide prevention. However, in most countries with enzootic dog rabies, the diagnosis of human rabies is rarely feasible. Animal testing laboratories are scarce and usually found only in major cities. With advances in techniques, the diagnosis could be made at a remote laboratory, or possibly on a post-mortem needle necropsy by a rapid test on-site [18–21]. Clinicians must be made aware of the possibilities and provide adequate specimens for testing.

3.1. *Intravital Confirmation of Human Rabies Encephalitis (Table 1)*

The diagnosis of rabies can be made by isolation of virus, identification of antigen or, in unvaccinated patients, antibody detection. First, it is important to find out from the local rabies reference laboratory which tests they can perform, which samples to take, whether to store them in a fridge or frozen, and how to package and send them. Isolation of the virus in tissue culture or by inoculation of suckling mice confirms the diagnosis with high specificity but lower sensitivity than other methods. It is also less technically demanding, uses cheaper reagents, and is the method of choice.

Positive results are most likely during the first week of illness, from saliva, throat, trachea or eye swabs, brain biopsy samples, and CSF. Viraemia has not been detected.

Rabies diagnosis is most commonly made by a variety of reverse transcription polymerase chain reaction (RT-PCR) tests on saliva, skin biopsy or CSF, but this is possible only in specialist laboratories [22]. Remote testing can be very successful. A reverse-transcription hemi-nested polymerase chain reaction (RT-hnPCR) gave excellent results in Cambodia, Madagascar, Senegal, and France when performed on a skin biopsy specimen taken any time after the onset of symptoms, or from at least three successive saliva samples from each patient [23].

A direct immunofluorescent antibody (IFA) test rapidly confirms antigen in frozen sections of skin biopsies taken from a hairy area, usually the nape of the neck [19,24–26]. False positives have not been reported. Unfortunately, this test is rarely performed. The corneal smear IFA test is very insensitive, and the results are unreliable [19,26].

In unvaccinated patients, detection of rabies neutralising antibody is diagnostic. This might appear in the second week of illness, but patients often die before generating any antibody response. In vaccinated people, very high and rising levels of antibody in the serum, and especially in the CSF, suggest the diagnosis [1,2].

The two validated serological methods, the fluorescent antibody neutralization test (FAVN) and the rapid fluorescent focus inhibition test (RFFIT), are technically demanding. Commercial ELISA kits are simpler but do not always correlate with the standard tests. Direct IF antibody methods are unreliable at low levels as cross-reactions occur with other viruses [27].

Table 1. Samples and methods recommended for rabies diagnosis in patients

Sample	Purpose	Method
Full thickness skin punch biopsy, including hair follicles [‡]	Antigen detection	IFA test on frozen vertical section [†] RT-PCR
Saliva [★] or throat swab Tears CSF	Virus isolation and Antigen detection	Tissue culture Mouse inoculation test RT-PCR
Serum	Neutralising antibody test	Presence of antibody is diagnostic in unvaccinated patients Take sample on admission to save for comparison 7 days later
CSF	Neutralising antibody test	Test in parallel with serum
Brain post-mortem: Needle necropsy [¶] or Autopsy sample brain stem & cerebellum	Virus isolation and Antigen detection	Tissue culture Mouse inoculation test IFA test on impression smear [°] RT-PCR

[‡] Highest positivity rate; [†] rabies antigen seen in nerve twiglets around the base of hair follicles by immunofluorescence (IFA) test; [★] easiest sample for antigen detection, repeat daily at least x 3, until a diagnosis is confirmed; [¶] necropsies are taken with a long biopsy needle via the medial canthus of the eye through the superior orbital fissure; via the nose through the ethmoid bone; through the foramen magnum or open fontanelles in children; [°] alternative: a direct rapid immunohistochemical test with biotinylated monoclonal antibodies and light microscopy.

3.2. Postmortem Diagnosis

All the methods mentioned above may be used to confirm the diagnosis postmortem, especially if the clinical illness was very short. Brain samples offer the best chance of successful postmortem diagnosis. These can be obtained without a full necropsy examination (Table 1). Other encephalitides or cerebral malaria may also be diagnosed from brain tissue.

In any part of the world, but especially where there are cultural or religious objections to delayed burial or a full autopsy that includes craniotomy, post-mortem needle necropsy is a most useful technique for obtaining brain samples from anterior or posterior fossae and brain stem [28] (Table 1). The usual diagnostic technique is the direct IFA test as used on animals [29]. A local veterinary laboratory may be helpful if the test is done routinely. If no IF microscope is available, the alternative is a direct rapid immunohistochemical test (dRIT) using biotinylated monoclonal antibodies and light microscopy.

Rabies rapid antigen immunodiagnostic test (RIDT) lateral flow kits are being developed for animal testing in the field [20] and could also be used with human brain samples.

4. Care of Patients with Confirmed Rabies Encephalomyelitis

In a disease as agonising and terrifying as rabies encephalomyelitis, alleviation of distressing symptoms is the primary concern and overriding responsibility of medical staff. However, in many clinics and hospitals across rabies endemic areas of the world, patients suspected of having rabies are deemed to be untreatable. They are either sent home with their relatives without advice or drugs or are isolated and sometimes abandoned in a remote part of the health facility and denied any medical attention. These practices ignore the fundamental precept that a doctor's responsibility is to relieve suffering even if there is no expectation of cure.

4.1. Accommodation

To avoid provoking hydrophobic or aerophobic spasms, calm, quiet conditions should be created, ideally in a dimly-lit, draught-free, single-bedded room. Restraining the patient in bed can be attempted initially with loose and comfortable ties and cot-sides, and ultimately by adequate sedation. Relatives must be able to communicate with the dying patient with dignity, in safety and privacy, according to their cultural and religious traditions. Other visitors should be restricted, including hospital staff not directly involved in management. However, frequent monitoring is needed so that patients can be given adequate supportive treatment.

4.2. Protection of Staff, Relatives, and Other Patients

Although there is no virologically documented instance of person-to-person rabies transmission, patients' saliva and secretions may contain the virus, and so they should be isolated, and their relatives and medical attendants protected with gloves, masks, and gowns, reassured, and offered pre-exposure vaccination. Objects, such as utensils, contaminated with patients' saliva or other secretions should be thoroughly washed with soap, or detergent.

4.3. Thirst/Dehydration

Hydrophobic patients cannot tolerate drinking, while those with paralytic rabies often cannot swallow. As a result, these patients may become dehydrated and desperately thirsty. Some may be able to eat fruit such as bananas and suck citrus fruits to combat thirst, and their lips and tongue may be moistened with a damp sponge or flannel. Treatment demands a secure intravenous (iv) line, ideally a catheter rather than a needle. The iv site should be immobilised by splinting. Isotonic 5% glucose, 0.9% saline, or Hartmann's (Ringer's lactate) solution (of sodium chloride, sodium lactate, potassium chloride, and calcium chloride) can be used as appropriate. Other possible routes for parenteral rehydration, depending upon available skills and equipment, include intraperitoneal, intraosseous, subcutaneous (sc), or intrarectal (proctoclysis).

4.4. Fever

Since physical methods such as tepid sponging and fanning are intolerable to most patients with furious rabies, antipyretic drugs are necessary to control fever. Aspirin, ibuprofen or diclofenac, and paracetamol (acetaminophen) can be given by non-oral routes, such as iv, im, or intrarectal (Table 2). Many patients have evidence of a generalised inflammatory response (e.g. peripheral neutrophil leucocytosis) as a cause for fever [7,8]. However, when the fever is central (neurogenic) in origin, antipyretics may be ineffective. Drugs that have proved effective in individual cases of central hyperthermia include baclofen, bromocriptine, amantadine, dantrolene, and propranolol, but these are unlikely to be widely available in developing countries [30].

4.5. Anxiety, Fear, Restlessness, Agitation, Seizures—Use of Sedatives and Tranquillisers

Benzodiazepines are drugs of choice as they are usually available and are on the World Health Organization's list of essential medicine [31]. They are used widely in daily clinical practice in most places and can be administered by various routes (Table 2). Diazepam can be given in the same doses intramuscularly (im) (which may be painful), intravenously (iv), or intrarectally. Although this is a fatal condition, it is important to avoid depressing respiration by giving the diazepam too rapidly by iv injection. Diazepam will alleviate the patient's suffering, while giving the family time to adjust and consider the possibility of taking them home to die, if that is their personal or cultural preference [32]. This can be achieved by giving a slow injection (0.5 ml/min, which is 2.5 mg/min) of diazepam. The same doses of diazepam given intrarectally may be useful if the family wish to take the patient home.

Midazolam, an alternative benzodiazepine, has a much shorter half-life. When given im, sc, or iv, it should be 'titrated' against the patient's clinical condition, which must be assessed frequently. Small doses are injected im at frequent intervals or by continuous iv infusion using an electric syringe.

A study of 45 patients in Manila identified their principal distressing symptoms and signs as hydrophobia, aggression, hyperexcitability, aerophobia, disorientation, hypervigilance, difficulty swallowing, hallucination, hypersalivation, anxiety/fear, seizure activity, and restlessness/agitation [33]. Anxiety/fear and restlessness/agitation were relieved by giving either haloperidol alone or diazepam alone, while a combination of the two was also effective against aggression/disorientation/hallucinations. Hypersalivation, hydrophobia and aerophobia were not affected by these treatments. Adult dosages were: haloperidol 5 mg sc or im every hour for at least three doses or until the patient became calm, then haloperidol 5 mg sc or im every 4 or 6 hourly and as needed; and diazepam 20 mg im every 2 h. Haloperidol can also be given by sc injection or infusion and diazepam intrarectally and by iv injection, but only under strict supervision. A patient with rabies encephalomyelitis in Wisconsin, USA, developed neuroleptic malignant syndrome that was attributed to haloperidol [34], but this is too rare a complication of treatment with drugs like haloperidol and chlorpromazine to contraindicate their use in likely-fatal disease. If none of these drugs is available, other possibilities include lorazepam (im, slow iv) or barbiturates (slow iv).

Table 2. Drugs for the palliative management of patients with confirmed or strongly suspected rabies encephalomyelitis that are included in the WHO Model List of Essential Medicines 20th List (March 2017) and WHO Model List of Essential Medicines for Children 6th List (March 2017). <http://www.who.int/medicines/publications/essentialmedicines/en/> Recommended doses are taken from <https://www.bnf.org/products/bnf-online/> and <https://bnfc.nice.org.uk/>.

Indication	Drug	Route of administration	Dose: adult	Dose: paediatric
Fever	paracetamol	iv infusion over 15 minutes	1 g every 4–6 h, maximum 4 g/24 h	125–500 mg every 4–6 h
		intrarectal	1 g every 4–6 h, maximum 4 g/24 h	
	ibuprofen	intrarectal	300–400 mg 6–8 hrly	
	aspirin	intrarectal	450–900 mg 4 hrly, maximum 3.6 g/day	
Anxiety, agitation, seizures	diazepam	iv (slow! caution!)	10 mg in 3–5 minutes, repeated 1–4 hrly	0.1–0.3 mg/kg in 3–5 min, repeated 1–4 hrly to provide 2.4–12 mg/kg/24 h
		im (painful!) intrarectal	20 mg 2 hrly 10 mg 1–4 hrly	0.1–0.3 mg/kg 1–4 hrly 0.1–0.3 mg/kg 1–4 hrly
	lorazepam	im or slow iv injection into large vein (slow! caution!)	25–50 microg/kg 6 hrly	25–50 microg/kg 6 hrly
	midazolam	im	0.08–0.2 mg/kg repeated 1–4 hrly	0.07–0.1 mg/kg repeated 1–4 hrly
iv or sc injection sc infusion intrarectal		2.5 mg hrly 10–30 mg over 24 h by pump	300–500 microg 1–4 hrly	
Anxiety, agitation	haloperidol	im or sc injection	5 mg hourly until calm, then 4 or 6 hrly and when necessary	age 1 month–12 y: 25–85 microg/kg/24 h
		iv or sc infusion	5–15 mg/24 h	12–18 y: 1.5–5 mg/24 h
	chlorpromazine	deep im intrarectal	25–50 mg/6–8 hrly 100 mg/6–8 hrly	500 microg/kg 6–8 hrly
Hypersecretion	hyoscine (scopolamine) hydrobromide	sc or iv injection	400 microg 4 hrly	10 microg/kg 4–8 hrly
		sc infusion	1.2–2 mg/24 h	40–60 microg/kg/24 h
Pain	morphine	slow iv, sc or im intrarectal	10 mg 4 hrly 15–30 mg 4 hrly	100 microg/kg
	fentanyl	transdermal patch	12–25 microg/h every 72 h	12 microg/h every 72 h

4.6. Hypersecretion (Salivation, Lacrimation, Sweating)

This may be reduced by anti-muscarinic anticholinergic drugs, such as hyoscine (scopolamine) hydrobromide, that block parasympathetic secretory activity.

4.7. Pain

There is a role for opioids and other powerful analgesics, when they are available, to relieve pain and suffering in rabies victims. Morphine can be given iv, sc, im, or intrarectally, and fentanyl transdermally by patch, which may be especially valuable for terminal management of patients after they have returned home.

As the infection progresses, coma and respiratory, cardiovascular, neurological, endocrine, or gastrointestinal complications will eventually ensue. When the diagnosis is clear, palliative care is the only compassionate strategy for treating previously unvaccinated patients infected by dogs or other terrestrial mammals.

At this stage, neuronal infection will be widespread. Animal experiments show that, even if cells are cleared of the virus, abnormal gene expression remains. Alterations in some neuronal functions would be permanent [35].

5. The Likelihood of Recovery Will Vary According to the Origin of Infecting Virus and Whether the Patient Has Been Vaccinated

In the Americas, all bat rabies viruses are rabies genotype/species 1, but are genetically distinct from the classic rabies viruses of terrestrial mammals in the same group [36]. Recovery from encephalomyelitis has been seen in two patients infected by these American insectivorous bat strains that may be less pathogenic in man. Rabies-related lyssaviruses, which infect bats in the rest of the world, show clinical signs similar to classical rabies viruses. Rabies viruses from dogs and other terrestrial mammals have been rapidly fatal in unvaccinated patients.

In patients who have had some rabies vaccine before the onset of symptoms, the progression of the disease can be more gradual. The surprising case of a man bitten by a dog in Turkey and incompletely vaccinated, and who developed rabies, is reported to have shown signs of spontaneous recovery with decreasing hydrophobia, beginning on his second day in hospital. Rabies antibody was present when first tested within a week of the onset of symptoms. He had had supportive treatment but no specialised intensive care and returned to normal life as a shepherd [3].

This case suggests that keeping the patient in hospital with simple clinical management does not prevent recovery if the patient's immunity is able to control the infection. In contrast, ITU treatment of patients who were vaccinated before the onset of symptoms can prolong the illness, but without any signs of spontaneous improvement, and leave them with severe persistent neurological deficits.

6. A Dilemma Arises when Expert ITU Facilities are Available

The decision whether or not to give intensive care relies on confirming the diagnosis in patients at an early stage before they have developed signs of overt clinical rabies, and rapidly testing the neutralising antibody level. If there is a history of an insectivorous bat bite in the Americas, ITU therapy may be considered. However, the diagnosis of rabies might be made only at a terminal stage or post-mortem, because the patients were unaware of any exposure, especially contact with a bat.

Analysis of clinical data leads to the conclusion [37,38] that, if rabies is diagnosed in a previously-vaccinated patient infected by a dog, the relatives must be made aware of the fact that, although intensive care can prolong life, the patient is most unlikely ever to regain full consciousness and will be left with multiple disabilities [39]. It is therefore wise to decide against intensive care for unvaccinated patients infected by a terrestrial mammal in routine clinical practice. This also applies to previously-vaccinated patients with clear signs of rabies who fail to show a rapid steep increase

in neutralising antibody levels and early signs of improvement. Progression of the disease despite intensive care indicates that palliation rather than intervention is the better course of action.

Recovery from rabies is extremely rare and associated with the presence of neutralising antibody in the blood and also in the CSF.

ITU therapy could therefore be considered if patients:

1. have been vaccinated previously;
2. develop rabies antibody within the first week of illness;
3. were infected by an American bat rabies virus (see above), especially if they present early.

Alleviating symptoms and signs of respiratory, cardiac, and other complications demands expert ITU management. The aim is to maintain homeostasis until the endogenous antibody and probably cellular immunity generated by the patient inhibits viral replication and clears virus from neurological tissue by unknown mechanisms [1,2].

If the diagnosis were initially in doubt and intensive care has already been started, the pressure to continue may be difficult to resist. However, clinicians should be confident in recognising that the disease is beyond recovery, stop aggressive interventions, and begin palliative care to avoid the patient's and their relatives' suffering further.

Striving to keep patients alive without a reasonable prospect of restoring them to a good quality of life would be considered unethical.

7. Antiviral Therapy?

At present, there is no specific antiviral agent or other therapy to kill or neutralise rabies virus in the brain. The 'Milwaukee' protocol gave rise to much hope but has proved no more effective than expert ITU therapy alone [38]. Several antiviral or immunotherapeutic methods have been suggested or tried [40,41]. One of these, favipiravir, a purine analogue, was effective against other ssRNA viruses. It showed some suppression of street rabies virus replication *in vitro*, but this effect may vary with different neuroblastoma cell lines. When used in mice as oral post-exposure prophylaxis an hour after inoculation as an alternative to rabies immunoglobulin, it reduced mortality and the titre of virus in the brain when the highest dose of 300 mg/kg/day was used. If favipiravir was delayed, starting four days post-inoculation or at the onset of symptoms, there was no effect on mouse mortality [42]. A similar study of favipiravir given to mice soon after inoculation showed no effect on mortality [43].

In the future, however, more effective treatment of rabies encephalitis may become possible. A promising method is injection of an attenuated rabies virus intrathecally, or perhaps iv injection of neutralising antibody, if the blood brain barrier can be bypassed by intraventricular administration [41], or breached pharmacologically [44,45]. Such apparently drastic therapy would be ethical for some sufferers of this fatal infection, but should be undertaken only when there is evidence of effectiveness in animals, and in an ethical and specialised research setting.

Hospitalization often provides little palliative care in developing countries. To correct this deficiency, clear practical guidelines based on recent relevant literature should be made available on national and international websites. The WHO and the World Medical Association are in a good position to promote this compassionate policy.

8. Prophylaxis

Rabies vaccine prophylaxis has been 100% successful if pre-exposure immunisation is followed by post-exposure booster vaccination.

Although there are no confirmed cases of rabies transmitted to caregivers, hospital staff and the patients' relatives will be reassured by the protection afforded by vaccination. Giving rabies vaccine intradermally (id) at multiple sites induces rapid seroconversion, which has been known for decades [46]. To complete a pre-exposure course, a second dose im or id should be given at least seven days later [47].

Giving pre-exposure vaccination to children living in dog rabies-endemic areas would be ideal, but is unaffordable. Pre-exposure vaccination is recommended for anyone, especially children living in dog rabies-endemic areas, if they have access to and can afford it. Vials of vaccine can be shared economically between groups, e.g. families, if injected *id* [47]. A new syringe and needle must be used for each person. For the vaccinee, this is an insurance against having to pay for full post-exposure vaccination and RIG if exposed for the rest of their lives. If exposure does occur in subsequent years, only wound washing and *im* or *id* booster post-exposure vaccination, requiring one or two clinic visits, are needed [47]. Intradermal inoculation of rabies vaccine is not accepted by some authorities, in which case the national protocols should be followed.

9. Conclusion

Rabies encephalomyelitis inflicts on its sufferers one of the most agonising deaths imaginable [48]. Unfortunately, the inescapably fatal outcome has discouraged medical staff from active management of patients with rabies encephalomyelitis, distracting them from the imperative of humane palliative care. Consideration of the terminal care of rabies patients has been long delayed and deserves formal attention to minimize the suffering of those dying of this terrible disease. Clinicians must have the courage to offer compassionate palliation whenever the diagnosis is evident.

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