REVIEW

Rabies virus-neutralising antibodies in healthy, unvaccinated individuals: What do they mean for rabies epidemiology?

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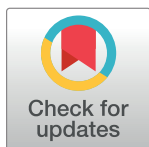
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Abstract

Rabies has been a widely feared disease for thousands of years, with records of rabid dogs as early as ancient Egyptian and Mesopotamian texts. The reputation of rabies as being inevitably fatal, together with its ability to affect all mammalian species, contributes to the fear surrounding this disease. However, the widely held view that exposure to the rabies virus is always fatal has been repeatedly challenged. Although survival following clinical infection in humans has only been recorded on a handful of occasions, a number of studies have reported detection of rabies-specific antibodies in the sera of humans, domestic animals, and wildlife that are apparently healthy and unvaccinated. These 'seropositive' individuals provide possible evidence of exposure to the rabies virus that has not led to fatal disease. However, the variability in methods of detecting these antibodies and the difficulties of interpreting serology tests have contributed to an unclear picture of their importance. In this review, we consider the evidence for rabies-specific antibodies in healthy, unvaccinated individuals as indicators of nonlethal rabies exposure and the potential implications of this for rabies epidemiology. Our findings indicate that whilst there is substantial evidence that nonlethal rabies exposure does occur, serology studies that do not use appropriate controls and cutoffs are unlikely to provide an accurate estimate of the true prevalence of nonlethal rabies exposure.

Introduction

Rabies virus (RABV) is a negative-sense RNA virus in the Rhabdoviridae family and one of 16 currently described viruses in the *Lyssavirus* genus [1]. Whilst other lyssaviruses also cause fatal disease that is indistinguishable from that caused by RABV, RABV is the greatest threat to human health. Typically transmitted in the saliva of infected hosts through bites, it is highly neurotropic and causes mortality through encephalomyelitis. Rabies kills an estimated 59,000 people annually, many of whom are children [2]. In rural Africa and Asia, where the majority of human cases occur, domestic dogs are the primary reservoir, responsible for up to 99% of rabies transmission to humans [3].

The ecology of rabies is complex, with transmission occurring between wildlife, domestic animals, and humans. However, as the host species responsible for the majority of transmission to humans, domestic dogs are the primary target for vaccination [4]. The World Health Organization (WHO) recommends vaccination coverage of at least 70% of the domestic dog population, repeated over several annual campaigns, to achieve rabies control [3]. This coverage level is supported by mathematical models of rabies dynamics [5,6,7]. However, rabies epidemiology is highly variable, with differences in viral strain, domestic dog density, and wildlife involvement between locations [8,9]. One area of this complex ecology of rabies that has long been recognised, but the significance of which remains unclear, is the occurrence of rabies virus-neutralising antibodies (RVNAs) in healthy, unvaccinated individuals.

Methodology

We conducted multiple searches using electronic databases, including Web of Science and Google Scholar. The search strategy involved using different combinations of the following words and phrases to identify relevant publications: rabies, lyssavirus, serology, antibodies, nonlethal, nonfatal, carrier, latent, recovery, survival, and 'rabies virus-neutralising antibodies'. The search covered all years and any studies in the English language. We also searched reference lists from articles identified for other relevant sources. We selected studies that were relevant under the following categories: 1) rabies serology studies in unvaccinated humans, domestic dogs, and wildlife; 2) reviews and studies on rabies serology tests; 3) studies on nonlethal rabies exposure, both experimental and under field conditions; and 4) studies on rabies mathematical modelling and surveillance with relevance to implications of naturally acquired immunity. The serology studies included in Tables 1 and 2 are not an exhaustive list but were selected as examples to cover a range of species, countries, and test methods.

Table 1. Serology surveys in unvaccinated domestic dogs yielding the estimated percentage with detectable rabies-specific antibodies in serum.

Country	Seroprevalence (95% CI)	Sample Size (# Positive)	Test Method	Cutoff	Seropositive Titre Range	Ref
Nigeria	16.1 (11.8–21.3)	254 (41)	HI	1:16	1:16–1:1,024	[114]
Nigeria	30.7 (26.5–35.1)	463 (142)	RFFIT; MNT	1:8	1:8–1:256	[11]
Ethiopia	80.0 (44.3–97.5)	10 (8)	RFFIT; ELISAs	RFFIT: 1:50 ELISA: 0.5 IU/0.2 mL	RFFIT: 1:60–1:540 ELISA: 1.2–1.8 IU/mL	[35]
Kenya	9.6 (5.7–14.9)	178 (17)	Modified RFFIT	0.5 IU/mL	NA	[115]
Namibia	30.0 (19.6–42.1)	70 (21)	LPBE	$>\log_{10}1.4$	$\log_{10}1.4$–$\log_{10}2.8$	[53]
Tanzania	LPBE: 7.4 (5.4–9.9) RFFIT: 49.4 (42.7–56.0)	567 (42) 233 (115)	RFFIT; LPBE	RFFIT: 0.5 IU/mL ELISA: $>\log_{10}1.5$	RFFIT: 0.5–2.7 IU/mL ELISA: $\log_{10}1.5$–$\log_{10}1.8$	[33]
Tunisia	LPBE: 28.8 (20.9–37.9) RFFIT: 39.0 (30.1–48.4)	118 (34) 118 (46)	RFFIT; ELISA	RFFIT: 0.5 IU/mL ELISA: 0.5 EU/ml	NA	[30]
China	13.3 (1.7–40.5)	15 (2)	RFFIT	0.5 IU/mL	NA	[94]
Kenya	28.0 (18.2–39.6)	75 (21)	RFFIT	0.05 IU/mL	0.05 to >0.56 IU/mL	[51]
Uganda	19.8 (12.5–28.9)	101 (20)	FAVN	0.24 IU/mL	NA	[116]

The 95% CI for the percentage seropositivity is shown. Sample size is shown with the number of seropositive individuals reported in brackets. Type of serology test and cutoff threshold used to define a seropositive are shown. When provided, the cutoff titre is shown converted to IU; otherwise, the dilution is shown. When reported, the range of titres in individuals defined as seropositive is shown; otherwise, this is recorded as NA. Further information on each study is provided in S1 Table in the supporting information. **Abbreviations:** CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; EU, equivalent units; FAVN, fluorescent antibody virus neutralisation; HI, haemagglutinin inhibition; IU, international units; LPBE, liquid-phase–blocking ELISA; MNT, mouse neutralisation test; NA, not available; RFFIT, rapid fluorescent focus inhibition test.

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Table 2. Serology surveys in unvaccinated wildlife yielding the estimated percentage with detectable rabies-specific antibodies in serum.

Species	Country	Seroprevalence (95% CI)	Sample Size (# Positive)	Cutoff	Test Method	Seropositive Titre Range	Ref
Black-backed jackal (*Canis mesomelas*)	Kenya	3.6 (0.1–18.4)	28 (1)	0.5 IU/mL	RFFIT	NA	[117]
	Kenya	1.4 (0.0–7.8)	69 (1)	0.05 IU/mL	RFFIT	0.25 IU/mL	[51]
	Namibia	8.6 (3.6–17.0)	81 (7)	1:10	FAVN	NA	[74]
African wild dog (*Lycaon pictus*)	Tanzania	25.0 (5.5–57.2)	12 (3)	0.5 IU/mL	RFFIT	All 0.55 IU/mL	[54]
	Kenya	8.6 (2.9–19.0)	58 (5)	0.05 IU/mL	RFFIT	0.067 to >0.418 IU/mL	[51]
Wolf (*Canis lupus*)	US	1.1 (0.0–6.2)	88 (1)	1:10	RFFIT	NA	[118]
Ethiopian wolf (*Canis simensis*)	Ethiopia	13.3 (1.7–40.5)	15 (2)	RFFIT: 1:50 ELISA: 0.5 IU/0.2 mL	RFFIT; ELISAs	RFFIT: both 1:60 ELISA: 1.2–2.5	[35]
Arctic fox (*Vulpes lagopus*)	US	4.3 (1.2–10.8)	92 (4)	1:5	Modified SNT	1:11–1:45	[97]
Crab-eating fox (*Cerdocyon thous*)	Brazil	5.9 (1.6–14.2)	68 (4)	0.10 IU/mL	Modified SNT	0.10–0.27 IU/mL	[73]
Bush dog (*Speothos venaticus*)	Brazil	100.0 (2.5–100.0)	1 (1)	0.10 IU/mL	Modified SNT	1.6 IU/mL	[73]
Maned wolf (*Chrysocyon brachyurus*)	Brazil	14.3 (7.8–23.2)	91 (13)	0.10 IU/mL	Modified SNT	0.1–0.27 IU/mL	[73]
Spotted hyena (*Crocuta crocuta*)	Tanzania	37.0 (27.6–47.2)	100 (37)	0.5 IU/mL	RFFIT	NA	[119]
	Kenya	6.5 (2.7–13.0)	107 (7)	0.05 IU/mL	RFFIT	0.09–0.29 IU/mL	[51]
Small Indian mongoose (*Herpestes javanicus*)	Puerto Rico	39.3 (30.2–49.0)	112 (44)	0.1 IU/mL	RFFIT	0.1–50.0 IU/mL	[98]
Small Asian mongoose (*Herpestes auropunctatus*)	Grenada	18.9 (16.0–22.1)	672 (127)	1:2	Modified SNT	1:5,900	[120]
Pampas cat (*Leopardus colocolo*)	Brazil	20.0 (0.5–71.6)	5 (1)	0.10 IU/mL	Modified SNT	0.13 IU/mL	[73]
Jaguar (*Panthera once*)	Brazil	23.1 (5.0–53.8)	13 (3)	0.10 IU/mL	Modified SNT	0.10–0.13 IU/mL	[73]
Ocelot (*Leopardus pardalis*)	Brazil	20.0 (2.5–55.6)	10 (2)	0.10 IU/mL	Modified SNT	0.10–0.13 IU/mL	[73]
Puma (*Puma concolor*)	Brazil	12.5 (0.3–52.7)	8 (1)	0.10 IU/mL	Modified SNT	0.10 IU/mL	[73]
Lion (*Panthera leo*)	Zambia	40.0 (19.1–64.0)	20 (8)	0.2 IU/mL	RFFIT	0.2–1.8 IU/mL	[45]
Oncilla (*Leopardus tigrinus*)	Bolivia	100 (2.5–100.0)	1 (1)	NA	RFFIT	>70 IU/mL	[55]
Common Vampire Bat (*Desmodus rotundus*)	Argentina	Pre: 3.0 (1.9–4.6) During: 6.6 (2.2–14.7) Post: 16.8 (10.3–25.3)	Pre: 694 (21) During: 76 (5) Post: 107 (18)	1:5	MNT	NA	[22]
	Brazil	7.4 (4.2–11.8)	204 (15)	0.5 IU/mL	ELISA; RFFIT	NA	[121]
Big brown bat (*Eptesicus fuscus*)	US	9.6 (5.8–14.8)	187 (18)	1:8	Modified SNT	NA	[122]
Little brown bat (*Myotis lucifugus*)	US	2.4 (0.5–6.8)	127 (3)	1:8	Modified SNT	NA	[122]
Mexican free-tailed bat (*Tadarida brasiliensis*)	US	68.5 (65.1–71.8)	750 (514)	1:10	Modified SNT	NA	[123]
Human (*Homo sapien*)	Peru	11.1 (4.6–21.6)	63 (7)	0.1 IU/mL	RFFIT	0.1–2.8 IU/mL	[13]
	Nigeria	28.6 (23.9–33.6)	350 (100)	1:8	RFFIT	1:8–1:64	[11]
Capuchin monkey (*Cebus paella*)	Brazil	6.7 (0.2–32.0)	15 (1)	0.11 IU/mL	RFFIT	0.33 IU/mL	[57]
	Brazil	11.1 (3.1–26.1)	36 (4)	0.25 IU/mL	RFFIT	0.7–1.3 IU/mL	[58]
Black bear (*Ursus americanus*)	US	5.2 (1.4–12.8)	77 (4)	1:5	RFFIT	1:20–1:320	[124]
Opossum (*Didelphis aurita*)	Brazil	11.0 (5.8–18.4)	109 (12)	0.11 IU/mL	RFFIT	0.11–1.00 IU/mL	[61]

(*Continued*)

Table 2. (Continued)

Species	Country	Seroprevalence (95% CI)	Sample Size (# Positive)	Cutoff	Test Method	Seropositive Titre Range	Ref
Striped skunk (*Mephitis mephitis*)	US	9.1 (5.5–14.0)	198 (18)	0.09 IU/mL	RFFIT	0.13–2.36 IU/mL	[125]
Raccoon (*Procyon lotor*)	US	40.5 (24.8–57.9)	37 (15)	0.05 IU/mL	RFFIT	0.05 to <0.12 IU/mL	[56]
	US	MNT: 17.2 (13.1–22.0) RFFIT:25.3 (20.1–31.1)	297 (51) 253 (64)	1:2 1:5	MNT; RFFIT	1:5–1:125	[23]
Crab-eating raccoon (*Procyon cancrivorus*)	Brazil	7.7 (0.2–36.0)	13 (1)	0.10 IU/mL	Modified SNT	0.27 IU/mL	[73]
Coati (*Nasua nasua*)	Brazil	100.0 (15.8–100.0)	2 (2)	0.11 IU/mL	RFFIT	0.12–0.20 IU/mL	[57]

This is not an exhaustive list of all studies but covers a breadth of species and locations. The 95% CI for the percentage seropositivity is shown. Sample size is shown with number of seropositive individuals in brackets. The type of serology test and cutoff used are shown. A number of studies used SNTs other than the RFFIT or FAVN; for simplicity, these are referred to as modified SNTs. Where provided, the cutoff titre used to define a seropositive is shown converted to IU; otherwise, the dilution is shown. Pre, during, and post refer to time of sampling relative to a rabies outbreak in cattle in [22]. Further information on each study is included in S2 Table in the supporting information. **Abbreviations:** CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; FAVN, fluorescent antibody virus neutralisation; IU, international units; MNT, mouse neutralisation test; NA, not available; RFFIT, rapid fluorescent focus inhibition test; SNT, serum neutralisation test.

Studies of rabies-specific serum antibodies in healthy, unvaccinated individuals

Prior to the development of postexposure prophylaxis (PEP), exposure to rabies in humans was widely considered to be uniformly fatal. Although when promptly administered, PEP is highly effective in preventing clinical infection, following the onset of symptoms, the disease fatality rate is still effectively 100%. However, the inevitable fatality of untreated rabies exposure in humans has been challenged by several studies showing the presence of RVNAs in serum from apparently healthy humans who have not been vaccinated [10,11,12,13]. For example, in a study of two communities in Peru, 7 of 63 individuals tested were found to have detectable RVNAs, of which only one had any history of prior vaccination [13]. When a host is exposed to a pathogen, antibodies able to bind to the specific antigens present on the pathogen are selected for and amplified. If a host does not succumb to the exposure, this heightened response can be detected after the pathogen is cleared. Detectable levels of rabies-specific antibodies in healthy, unvaccinated individuals therefore suggest exposure to RABV that has not led to fatal disease. Whilst natural development of rabies antibodies in healthy individuals has rarely been recorded in humans, detectable RVNAs in sera have been reported in a number of studies in populations of domestic dogs and wildlife in regions where rabies is endemic (Tables 1 and 2).

Despite nonlethal rabies exposure first being reported in laboratory experiments by Pasteur in 1882 [14], whether detection of RVNAs in unvaccinated domestic dogs and wildlife is the result of nonlethal rabies exposure remains controversial. In bats, which have a long history of coevolution with lyssaviruses, it is well established that nonlethal rabies exposure regularly occurs [15]. However, whether this is true for other mammals is unclear. Interpretation of serology studies is complicated by variation in specificity of the test methods and cutoffs used and by potential cross-reactivity [16]. This has led to an unclear picture of the significance of nonlethal exposure for rabies epidemiology and control. Substantial work and discussion of this topic has previously been carried out by Bell, Carey and McLean, Fekadu, and Cleaveland and Dye [17,18,19,20].

Detection methods for rabies antibodies

Several test methods are available for detecting rabies-specific antibodies. The first developed was the mouse neutralisation test (MNT). In this method, sera at different concentrations are mixed with a constant viral dose and are then inoculated into weanling mice. The mice are then observed for development of clinical symptoms [21]. This method was used in some early wildlife serology surveys (e.g., [22,23]) but has since been replaced by in vitro methods. The most commonly used in vitro methods are virus neutralisation tests and ELISAs (enzyme-linked immunosorbent assays).

Virus neutralisation tests, which include the rapid fluorescent focus inhibition test (RFFIT) and fluorescent antibody virus neutralisation (FAVN), are the approved methods for measuring vaccination response prior to movement of animals internationally [24]. Both the FAVN and RFFIT rely on the same principle, in which the antibody concentration is measured by the ability of sera to neutralise a challenge virus, detected by the reduction in fluorescence in virus-infected cells [25]. The difference between the two tests is the method of plate-reading. Several modifications of the standard virus neutralisation tests have also been developed, for example, to allow the use of small serum volumes and pseudotype viruses [26,27].

An alternative to neutralisation tests is the use of ELISAs. In contrast to the RFFIT and FAVN, ELISAs do not measure neutralisation, but estimate the antibody concentration in a serum sample able to bind specifically to rabies antigens. This concentration does not provide a direct measure of protection against the virus because the recorded titre also includes antibodies that bind but may not contribute to defence against the virus [28]. ELISAs are faster to run than the RFFIT and do not require live RABV or cell-culture facilities. A number of different ELISAs have been developed and are available commercially, but these have been shown to vary in sensitivity and specificity [29].

Challenges to interpreting rabies serology tests

Discordance between test methods

Both rabies neutralisation tests and ELISAs have primarily been used for measuring responses to vaccination. Comparison of these tests shows that for vaccinated domestic dogs, there is a strong correlation between the titres measured [30, 31]. However, in wild carnivores, the correlation following oral vaccination has been found to be poor, with challenge experiments suggesting that the ELISA potentially provides a better measure of protection [32]. In unvaccinated individuals, both neutralisation tests and ELISAs have been used to detect antibodies arising from natural exposure. However, when results from different tests have been compared, poor agreement has been found. In a sample of 286 dogs, Bahloul and colleagues found concordance between the two tests for only 30% of the dogs defined as unvaccinated seropositives [30]. Substantial discordance was also found by Cleaveland and colleagues, with significantly higher seroprevalence detected by RFFIT than ELISA [33].

The lack of agreement between rabies serology tests in unvaccinated individuals suggests these methods differ in their specificity or sensitivity for detecting nonlethal exposures. Cleaveland and colleagues found that an RFFIT, but not an ELISA, detected false positives on a rabies-free island, suggesting that using an ELISA may be more specific for detecting nonlethal exposures [33]. This lack of specificity with the RFFIT may be a result of non-antibody neutralising factors present in samples. If serum samples are of poor quality, cytotoxicity can also occur in neutralisation tests, resulting in false positives [34]. The discrepancies observed may also be partially explained by differences in the class of antibody detected by the two tests [33,35]. Neutralisation tests detect all classes of antibodies, whereas ELISAs specifically test for

a single class of antibody [36]. Because different antibody classes are produced at different times following exposure, the two tests may differ in sensitivity depending on the time since exposure or vaccination.

Defining cutoffs

For both virus neutralisation tests and ELISAs, distinguishing between seropositive and seronegative individuals requires defining a cutoff point. The higher a cutoff is set, the lower the probability that false positives are detected—however, the higher the chance of missing evidence of exposure. Typically, for the RFFIT in domestic dogs, a titre of 0.5 international units (IU)/mL is used, which is the threshold set by WHO as confirmation of antibody generated against vaccine [37]. This titre has also been shown to be protective in some wildlife species following vaccination, with individuals with a titre of >0.5 IU/mL having a >95% probability of survival following challenge [32]. As a result, in serology studies of unvaccinated wildlife and domestic dogs, this cutoff is often used as evidence as exposure (see Tables 1 and 2). However, whilst this cutoff has been validated in response to vaccination in dogs and a small handful of wildlife species, it has not been verified for detection of exposures in unvaccinated individuals. A number of serology studies have also used lower cutoffs as evidence of exposure, often without statistical justification. Methods are available to estimate the appropriate cutoff, such as receiver operating characteristic (ROC) curve analysis [38]. However, these methods rely on having known positive and negative controls for a species, and because of the difficulties of this for most wildlife species, few serology surveys employ these [39, 40]. A further challenge of interpreting rabies serology studies is that many do not convert to IU by comparison to a standard. Without standardisation, dilutions cannot be readily compared between studies.

Cross-reactivity

A further factor that complicates interpretation of rabies serology tests is the possibility of cross-reactivity. For closely related viruses, similarity of antigens can allow antibodies generated against one virus to neutralise others. RVNAs could therefore indicate current infection or previous exposure to other lyssaviruses. However, the lyssaviruses are divided into 3 phylogroups based on serologic cross-reactivity and genetic distances within the G-protein codomain, and cross-reactivity is limited between phylogroups [41,42,43,44]. Whether RVNAs can be attributed specifically to exposure to RABV should therefore be considered within the specific context. Taking the example of lyssaviruses in sub-Saharan Africa: RABV is in phylogroup 1, whilst, with the exception of Duvenhage virus, which has only rarely been isolated, the majority of other lyssaviruses known to circulate are in phylogroup 2 or 3. By contrast, in Europe a number of other phylogroup 1 lyssaviruses circulate in bat species [1,41]. However, surveillance of lyssaviruses remains limited, and the current understanding of lyssavirus diversity and distribution may change with further study. In species that are known rabies reservoirs or that are likely to come into contact with these species, it may be justified to assume that exposure to RABV is more likely than exposure to other lyssaviruses. For example, Berentsen and colleagues found high seroprevalence of RVNAs in lions (*Panthera leo*), which, based on the species ecology, they considered to be more likely to have resulted from contact with canine rabies than lyssaviruses in bat populations [45]. When cross-reactivity is likely, serology tests using other lyssaviruses as challenge viruses could elucidate whether cross-reactivity is occurring [46]. For example, Ogunkoya and colleagues tested dog and human samples for neutralisation of RABV, Lagos virus, and Mokola virus and found no evidence of cross-reactivity [11]. In bat species, Mélade and colleagues also found no cross-reactivity in individuals seropositive for Duvenhage virus or European Bat Lyssavirus 1 (EBLV-1) and RABV, despite these

belonging to the same phylogroup [47]. However, in foxes and racoon dogs vaccinated against RABV, strong cross-reactivity with both EBLV-1 and 2 has been shown [48]. As well as cross-reactivity with lyssaviruses, in human clinical cases, the indirect fluorescent antibody test has been found to detect false rabies seropositives in patients with encephalitis caused by West Nile and Powassan flaviviruses [49]. Therefore, antibodies in unhealthy individuals may not be considered specific. However, standard rabies neutralisation tests in this study detected no false positives, suggesting this is method-specific.

Factors affecting sensitivity

As well as factors affecting the specificity of rabies serology tests, a number of factors can affect the sensitivity for detecting prior exposure. Repeated freezing and thawing of serum samples can cause antibodies to decay, reducing the sensitivity of serology tests [50,51]. Furthermore, the antibody level in an individual is affected by multiple factors, such as time since exposure and individual variation in immune response [16,52]. As a result, undetectable levels of antibody are not necessarily a true indicator that an individual has never been exposed because antibody levels may have decayed below the detectable threshold. For example, this has been observed in experimental rabies infection in big brown bats (*Eptesicus fuscus*) [15].

Can RVNAs be attributed to nonlethal rabies exposure?

Given the challenges of rabies serology discussed so far, it is important to consider whether rabies seropositives reported in serology surveys can be attributed specifically to nonlethal exposure to RABV. Potentially the greatest challenge is whether the test methods used are generating false positives. The majority of the domestic dog studies reporting detection of rabies-specific antibodies used an RFFIT, which, as shown by Cleaveland and colleagues, may generate false positives, with 10.3% seroprevalence found using a 0.5 IU/mL cutoff on a rabies-free island [33]. This study provides significant evidence that at least a proportion of seropositives detected by RFFIT are likely to be false positives due to either nonspecific neutralisation or cross-reactivity. However, the same study found that using an ELISA, no false positives were detected, but 7.4% of dogs in a rabies-endemic region were seropositive. The location of these dogs also correlated with known rabies cases, providing strong evidence that these are true evidence of nonlethal exposures. Other studies using ELISAs in domestic dogs have also found high seroprevalences using similar cutoffs [30,53]. Therefore, whilst false positives are likely to occur, at least a proportion of seropositives in domestic dogs appear to be true indicators of prior nonlethal exposure.

The lack of specificity of the RFFIT in domestic dogs, for which the test has been validated, raises serious concerns for its use in wildlife species, for the majority of which little or no validation of the serology tests has been conducted. As with domestic dog studies, in most of the wildlife studies identified, the RFFIT was used (Table 2). In almost no cases was a comparison to a rabies-free control population made. Exceptions were Gascoyne and colleagues and Deem and colleagues, who used zoo populations, although in both cases only for very small sample sizes [54,55]. A majority of the studies made no mention of what controls were used or used domestic dog or human controls. A number of wildlife studies also used cutoffs lower than 0.5 IU/mL, which increases the probability of detection of false positives. In a number of the studies considered, if the cutoff had been 0.5 IU/mL, no seropositives would have been detected (e.g., [56,57]). However, in other cases, the antibody titres found were well above the standard cutoff point (see Table 2). For example, Machado and colleagues detected titres of up to 1.3 IU/mL in capuchin monkeys (*Cebus apella nigritus*), Berentsen and colleagues up to 1.8 IU/mL in lions, and Deem and colleagues a titre of greater than 70 IU/mL in an oncilla (*Leopardus*

tigrinus) [45,55,58]. However, given that the serology tests have not been validated for these species, interpretation of these high titres is unclear. A further challenge for interpreting wildlife serology studies is that in a number of cases, the sample sizes used were small, leading to wide confidence intervals for the seroprevalence estimates. Verifying serology methods for the target species and achieving larger sample sizes should therefore be a priority for rabies serology surveys in wildlife.

In domestic dog studies, whilst the serology tests have been verified for this species, interpreting serology studies is complicated by the potential that seropositive individuals have been vaccinated, but this has not been recognised because of poor record keeping. Whilst this is possible in some cases, the majority of studies reported in Table 1 were conducted on owned dogs and in areas where little or no vaccination had been carried out. It is therefore presumed that the owners or researchers will know whether dogs had been previously vaccinated with relative surety. Whilst it remains possible that a proportion of individuals were vaccinated, it appears unlikely that vaccination would account for a significant proportion of the seropositives detected. In areas where oral vaccination of wildlife has been carried out, it is also possible that this could explain seropositivity. However, the wildlife studies reported were primarily carried out in areas with no wildlife vaccination or prior to distribution of vaccine (e.g., [53,59]).

Overall, rabies serology studies should be interpreted critically, and reported seroprevalences are unlikely to provide a completely accurate estimate of nonlethal exposure. However, it appears that at least a proportion of seropositives are likely to indicate true incidents of rabies exposures. Studies using rabies serology to infer exposure to the virus would benefit from greater consideration of the most suitable test method and cutoff to provide high specificity and of the use of species-specific controls.

Alternative courses of rabies infection as the cause of RVNAs

In 'classical' rabies infection, following the latent period, clinical infection is short-lived. Infected individuals display furious or paralytic symptoms, followed by death from encephalomyelitis. Typically, because of immune evasion by the virus, neutralising antibody responses are only detectable at a late stage in the course of clinical infection, by which point the infected individual is unable to effectively clear the virus [60]. For example, a study of human clinical cases found that rabies antibodies in serum were not detected until around 10 days after the onset of clinical symptoms [61]. Occurrence of rabies-specific antibodies in healthy, unvaccinated individuals must therefore be the result of an alternative course of rabies exposure. Four alternative courses of rabies infection that could lead to rabies antibody detection in healthy individuals were initially identified by Fekadu [19]. These are subclinical infection, recovery from clinical infection, a carrier state, and an extended latent period [19].

Subclinical infection

Subclinical infection refers to the clearance of the virus before the onset of recognisable clinical symptoms. As shown by the effectiveness of vaccination, rabies antigens are highly immunogenic [60]. In classical rabies infection, immune evasion by the virus in the central nervous system (CNS) results in a failure of the host to develop an effective antibody response [62]. However, if clearance of the virus can be achieved before entry into the CNS, clinical infection can be avoided, leading to a subclinical or 'aborted' infection with mild or no symptoms. A number of studies have observed subclinical infections following experimental exposure with RABV, including in dogs [63,64] and mice [65,66]. In bats, the occurrence of subclinical rabies infection is particularly prevalent [67]. Several studies have demonstrated survival of bats following challenge with RABV without development of symptoms and in some cases with

subsequent development of RVNAs [15,67,68,69,70]. Lyssaviruses have coevolved with bat species, which, though susceptible to lyssaviruses, have considerably lower fatality rates when exposed compared to other mammals [71,72]. In other mammals, in cases in which true rabies-specific antibodies are detected in healthy, unvaccinated individuals under field conditions, subclinical exposure also appears to be the most parsimonious explanation. Seropositive individuals have been shown to have no evidence of symptoms, and later development of clinical infection has not been observed [33,73,74]. In the situation of recovery or an extended latent period, evidence of previous clinical symptoms or lower survivorship due to later development of rabies would be expected.

The probability of developing clinical rabies infection, relative to subclinical, will vary depending on the host species and viral stain. In vampire bats, Blackwood and colleagues estimated the probability of developing fatal infection following exposure to be 0.1 [75]. By contrast, in a study of domestic dogs, the proportion of exposed dogs that developed clinical infection was considerably higher at 0.49 (95% confidence interval: 0.45–0.52) [76]. In humans who did not receive postexposure treatment after a probable rabies exposure, Changalucha and colleagues found that 0.165 developed rabies [77]. This probability was dependent on the site of exposure, with the highest risk from bites to the head [77]. Of these exposures not leading to clinical infection, it may be that the reported contacts were not with infectious individuals or that contact failed to lead to viral establishment, with the host remaining susceptible. However, a proportion of these exposures may be cleared by the immune system and lead to development of immunity. Although further study is needed, subclinical infection appears to be the most likely alternative course of rabies infection that could lead to RVNA development under natural conditions.

Recovery

Recovery here means clearance of the virus following the onset of recognisable clinical symptoms, in contrast to subclinical infection, in which clearance occurs before these symptoms appear. Recovery from clinical infection in domestic dogs was first reported by Pasteur in 1882, who noted that dogs can 'sicken and recover' from rabies [14]. Recovery in humans has also been reported on a handful of occasions; however, in most cases only when vaccine had been administered before onset of clinical symptoms [78]. When recovery has occurred, the majority of cases were left with permanent neurologic sequelae and severe cognitive disability [79]. In experimental infection of animals, recovery has been recorded in a number of species, including mice [17,66,80], dogs [81], a ferret [82], and rabbits [83]. As with human cases, the majority of these individuals had lasting sequelae following recovery.

Symptomatic rabies infection occurs after the entry of the virus of the CNS. Therefore, recovery depends on clearance of the virus from the CNS. However, once the virus is in the CNS, it is protected from the humoral immune response unless antibody is able to cross the blood–brain barrier (BBB). In cases in which recovery has been recorded, permeability of the BBB and the presence of antibody in the cerebrospinal fluid (CSF) have been shown to be key correlates of recovery [63,84,85,86]. As a result, in cases in which antibody is detected in the CSF of healthy individuals as well as the serum, this provides a key indication of recovery rather than subclinical infection. Because parenteral vaccination only leads to development of serum antibody, CSF antibody can also rule out that vaccination has occurred [87].

Whilst recovery can occur, in most cases, development of CSF antibody occurs too late to allow clearance [60]. Reports of recovery under natural conditions are scarce, despite the distinctive clinical symptoms associated with rabies that are likely to increase the probability of detecting cases of recovery relative to subclinical infection. For example, in a study of 957

naturally infected dogs, none survived more than 10 days after admission into quarantine [88]. In the case of wildlife, survival with clinical symptoms may be even less likely as because of the debilitating nature of the disease, they would be highly vulnerable to predation or starvation. Therefore, given the scarcity of recorded cases of natural rabies recovery and the permanent disability associated with clinical rabies, it is unlikely to be a common course following rabies exposure under field conditions. However, testing for antibodies in the CSF of individuals with seropositive sera could allow for distinction between subclinical infection and recovery.

Carrier state

In the carrier state, the infected host sheds virus across an extended period whilst remaining apparently clinically healthy. The occurrence of the carrier state for rabies is highly controversial. The first suggestion of its occurrence was in vampire bats (*Desmodus rotundus*) [89]; however, later experimental studies testing for carriers in this species found little evidence, with excretion in the saliva only detected for a short period after clinical infection [90,91]. Potentially, a greater cause for concern is if domestic dogs are able to act as carriers, in which case bites from apparently healthy individuals could still cause human deaths. A carrier state has occasionally been reported in domestic dogs; for example, in one experimentally infected dog, RABV was isolated from saliva up to 305 days following recovery from clinical infection until death during whelping [92]. In Nigeria, Aghomo and Rupprecht isolated RABV from the saliva of 4 healthy, unvaccinated dogs brought for routine veterinary examination [93]. The strains isolated were found to be pathogenic in mice, but not in puppies, which was taken as evidence for a host-adapted strain with lower pathogenicity. A further study of 153 dogs in China found rabies antigen in the saliva of 15 dogs. However, observation of these dogs for 6 months showed detection of antigen was inconsistent, and no viral RNA was detected, suggesting the ELISA may have provided unreliable results [94]. Two further studies screening dogs in Argentina and India found no evidence for a carrier state [95,96]. Therefore, clear evidence for a carrier state in domestic dogs is very limited. In wildlife, evidence for a carrier state is also scarce. Of the wildlife serology studies considered, a number tested for virus present in the brain or saliva of seropositive animals with no evidence found [51,97,98]. The implications of a carrier state for rabies epidemiology are worth consideration because even infrequent occurrence of carriers could significantly influence rabies dynamics through the exposure of large numbers of other individuals [20]. However, evidence for a carrier state is very scarce, and it is highly unlikely to account for any significant number of healthy, unvaccinated rabies-seropositive individuals.

Extended latent period

The latent or incubation period is the interval between initial exposure to a pathogen and the onset of clinical symptoms. For rabies, the latent period varies considerably. Although in humans, this period lasts between 20 to 90 days on average [79], latent periods over a year have been reported [99]. In domestic dogs, Hampson and colleagues found an average incubation period of 22.3 days, but periods over 200 days were also recorded [76]. RVNAs might therefore indicate active infection that has not yet reached the clinical stage. However, in typical rabies infection, because of immune evasion by the virus, antibodies are not detectable during the latent period; therefore, the occurrence of an extended latent period is unlikely to explain the phenomenon of unvaccinated seropositive individuals [60]. Furthermore, there is no evidence that healthy seropositive individuals later develop clinical rabies, as would be expected if they were harbouring latent infection. For example, in Tanzania no significant difference in mortality was found between seropositive and seronegative domestic dogs, and

none of the 32 dogs defined as seropositive by the RFFIT were reported to have developed clinical rabies [33]. In the wildlife studies reported, in many cases individuals were released without follow-up, making it impossible to rule out that they were incubating rabies. However, in studies that did follow up, all seropositive individuals remained healthy. It is therefore unlikely that any significant proportion of seropositive individuals detected are in the latent period.

Implications of nonlethal infection

Whilst it remains unclear what proportion of seropositive unvaccinated individuals can be truly attributed to nonlethal rabies exposure, there is significant evidence that subclinical rabies infection does occur. This observation has potential implications for rabies epidemiology, surveillance, and control.

Immunity to reinfection

The population-level consequences of nonlethal rabies infection depend on whether the immune response generated results in protection against future infection. The titres of RVNAs detected vary widely within populations and even in longitudinally sampled individuals. However, these titres often exceed those regarded as protective following vaccination in domestic animals. In the case of domestic dogs, challenge experiments suggest a titre of 0.2 IU/mL is sufficient for protection [100], whilst in vaccinated wildlife, a titre of >0.5 IU/mL was found to be protective in 95% of cases [32]. Even individuals with low titres because of waning antibody levels following exposure may remain protected because of the ability to develop antibodies more rapidly following secondary exposure. Furthermore, although cross-reactivity with other lyssaviruses could mean that not all RVNAs are true indicators of rabies exposure, they may still provide protection against RABV exposure [101].

Directly testing for protection would require challenging seropositive wildlife or domestic dogs with RABV. Whilst experiments of this kind may be possible, they evidently present major ethical and practical challenges. An alternative strategy to gauge the immune response of seropositive individuals to rabies exposure is to look at their response to vaccination. Initial exposure to an antigen, either through vaccination or infection, 'primes' the immune system; therefore, on subsequent exposure, the immune reaction is faster and stronger [102]. This heightened response is known as an anamnestic response and allows for rapid clearance of the pathogen before onset of clinical infection. Testing for this anamnestic response can provide an indication of how seropositive individuals would react to challenge. However, vaccination differs significantly from natural exposure because the antigen is highly purified and immunogenic. Therefore, a response to vaccination does not equal protection to exposure to a wild-type strain. In Haiti, measurement of RVNA before and after vaccination showed a greater increase following vaccination in dogs that were seropositive prevaccination than seronegative matched controls [103], suggesting an anamnestic response occurred in seropositive individuals. However, only 10 seropositive dogs were studied. Bahloul and colleagues also found that dogs in field conditions showed higher antibody titres postvaccination than those kept under experimental conditions, potentially due to previous subclinical infection [30]. In cattle, Gilbert and colleagues also found that prevaccination RVNA was marginally associated with a stronger postvaccination response [104]. By contrast, a study of dogs in Thailand found no evidence of an anamnestic response, with seropositive results detected by an RFFIT suggested to be false positives [105]. These studies all compared the response at between 13 days to 6 months postvaccination. Because even fully naïve individuals will have generated a response to vaccination by these time points, distinguishing between an anamnestic and naïve response is challenging. Further experiments of this kind are therefore recommended to help clarify

whether seropositive individuals show an anamnestic response, as expected if they result from nonlethal exposure.

Incorporating nonlethal infection into models of rabies dynamics

Estimates of vaccination coverage required to control rabies are based on mathematical models of the disease. Classically, rabies is modelled using SEI (susceptible, exposed, infectious) or SEIV (susceptible, exposed, infectious, vaccinated) compartmental models, with no recovered class (e.g., [106,107]). In some cases such as fox rabies, in which susceptibility is very high with little evidence of postinfection immunity, this assumption of 100% fatality may be appropriate [108]. However, in species in which high seroprevalences have been detected in a number of studies, such as bat species, domestic dogs, and mongoose species, this assumption may be invalid (see Table 2). Consideration of naturally acquired immunity through inclusion of a recovered class (susceptible, exposed, infectious, recovered [SEIR]) in models may therefore provide more accurate model estimates. However, further study is required to accurately inform parametrisation of immunity in rabies models; for example, on the proportion of individuals developing immunity and the duration of immunity. This could have implications for both our understanding of rabies dynamics and the design of control strategies.

Role of nonlethal rabies in persistence

The high host mortality rate associated with RABV, together with its infection of slowly reproducing mammalian hosts, has led to questions as to how the virus persists within populations. Whilst the high cross-species transmissibility of the virus means it is not limited to a single host population, strains of RABV are closely associated with and adapted to specific host species [109]. If RVNAs are indicative of frequent nonlethal exposure, this could suggest that rabies occurs in a more typical host–parasite relationship with lower mortality than is often portrayed [18]. For example, in vampire bats, the occurrence of immunizing nonlethal infections, together with between-colony dispersal, was found to be necessary for the maintenance of rabies within populations [75, 110]. Although immune hosts do not transmit virus, they are still able to reproduce, therefore replenishing the susceptible population and providing the virus with new hosts. Lower virulence may therefore benefit viral persistence in smaller populations or when cross-species transmission is less frequent. If the virus is able to adapt to facilitate a carrier state in which it can be transmitted without causing symptoms, this could also favour viral persistence. Cleaveland and Dye found that in a model of rabies in dog populations, persistence was much more likely if seropositive individuals are carriers [20]. When cycles of rabies infection occur, the presence of carriers makes extinction much less likely in the 'troughs' between peaks of infection. As a result, if a carrier state does occur, it could be an important mechanism in long-term maintenance in a system.

Rabies surveillance

Canine vaccination has been shown to be effective in reducing the rabies public health burden, and progress is being made towards the goal of eradicating human rabies transmitted by dogs by 2030 [111]. Oral vaccination has also been demonstrated to be a successful strategy for control of independent cycles of rabies in wildlife. However, in not all cases is it evident which species are maintaining rabies [112]. Surveillance for rabies is therefore of key importance for control efforts, both to monitor the progress of eradication in domestic dogs and to inform the focus of control efforts in wildlife. Serology provides a method of assessing pathogen exposure without the need to fatally sample or sample during active infection. However, detection of false positives remains a significant challenge, and identifying the appropriate test and cutoff

to provide high specificity is central to the use of rabies serology in surveillance [113]. Interpreting disease prevalence from serology data is also limited by current understanding of rabies serological responses. Factors such as the case fatality ratio, the probability of seroconversion, and the duration of the antibody response have implications for how serology studies are interpreted [52]. Further study is required to clarify these factors, as well as the test methods most appropriate for identifying true nonlethal exposures.

Conclusions

The occurrence of nonlethal rabies infections has been recognised since Pasteur; however, the view of rabies exposure as inevitably fatal is still widespread. A key line of evidence against this view is the detection of RVNAs in healthy, unvaccinated individuals. Whilst experimental studies support that subclinical rabies infection with subsequent immunity does occur, estimating the prevalence of this under natural conditions is complicated by the challenges of interpreting serology methods. To fully understand the role of nonlethal rabies, the following questions require further study: 1) what test methods and cutoffs are most appropriate for specific detection of rabies antibodies in naturally exposed individuals; 2) what proportion of RVNAs in healthy, unvaccinated individuals are true indicators of subclinical rabies exposure; and 3) are naturally exposed rabies-seropositive individuals immune to reinfection? Answering these questions could help to clarify rabies ecology and challenge the still widely held view of rabies exposure as inevitably fatal. This could lead to improved models for understanding rabies dynamics and designing surveillance methods.

Key learning points

- Rabies-specific antibodies in the sera of healthy, unvaccinated individuals have been reported in a number of studies of wildlife, domestic dogs, and humans. These antibodies provide potential evidence for nonlethal rabies exposure.

- The specificity and sensitivity of serology tests for detecting rabies exposures depends on the test method and cutoff titre chosen, which varies between studies. Together with the possibility for cross-reactivity with other lyssaviruses, this poses a significant challenge to estimating the true prevalence of nonlethal rabies exposure.

- In cases in which nonlethal rabies exposure occurs, subclinical infection, in which the virus is cleared before the onset of recognisable clinical symptoms, is the most likely alternative course of infection.

- Improved estimates of rabies seroprevalence in wildlife and domestic dogs could potentially be used to inform rabies disease models and surveillance methods.

Key papers

1. Bell JF. Latency and abortive rabies. In: Baer GM, editor. The natural history of rabies. New York, New York: Academic Press; 1975. pp. 331–354.

2. Carey AB, McLean RG. The ecology of rabies: evidence of co-adaptation. J Appl Ecol. 1983:20(3):777–800.

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5. Gilbert AT, Petersen BW, Recuenco S, Niezgoda M, Gómez J, Laguna-Torres VA, et al. Evidence of rabies virus exposure among humans in the Peruvian Amazon. Am J Trop Med Hyg. 2012;87(2):206–215.

Supporting information

S1 Table. Additional information on serology studies in unvaccinated domestic dogs. Row number of study relative to Table 1 is shown. Information is provided on whether each study followed seropositive individuals to see if they developed rabies, evidence for previous vaccination, notes on the test method used, and any additional information of use for interpreting the study results.
(PDF)

S2 Table. Additional information on serology studies in unvaccinated wildlife. Row number of study relative to Table 2 is shown. Additional information of use for interpreting the reported seroprevalence is shown. This includes whether low cutoffs were used, variation in test methods, and whether follow-up was carried out to check for development of symptoms.
(PDF)

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