A rabies serosurvey of domestic dogs in rural Tanzania: results of a rapid fluorescent focus inhibition test (RFFIT) and a liquid-phase blocking ELISA used in parallel

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SUMMARY

During a serosurvey of domestic dogs in Tanzania, a rapid fluorescent focus inhibition test (RFFIT) and a liquid-phase blocking ELISA (LPBE) were used to measure rabies antibodies in vaccinated and unvaccinated dogs. Post-vaccination titres measured by LPBE correlated closely with those found by RFFIT. Of 567 unvaccinated dogs tested using the LPBE, 42 (7·4%) were seropositive, with titres exceeding 32. Of this group, 233 dogs were tested using the RFFIT and 115 (49·4%) were seropositive, with titres exceeding 0·5 IU/ml. Two lines of evidence pointed to the greater specificity of the LPBE when measuring rabies antibodies induced by natural infections: (a) no seropositive dogs were detected among the 162 unvaccinated dogs from the rabies-free island of Pemba, Tanzania, when using LPBE, whereas 15/145 (10·3%) dogs of the same group were seropositive using RFFIT; (b) among Tanzanian dogs there was a close association between the location of rabies cases and location of seropositive dogs when using LPBE, but not when using RFFIT. These results suggest that LPBE may be of value in rabies seroepidemiological studies and could be developed as a reference technique for the detection of rabies antibody in domestic dogs.

INTRODUCTION

Throughout much of sub-Saharan Africa, case incidence data on canine rabies have often been fragmentary and official figures reported to the World Health Organization (WHO) provide a poor indication of both the magnitude of the rabies problem and the trends in disease incidence. In Tanzania, for example, a total of only 42 cases of dog rabies were officially reported from 1990–7 [1, 2] despite unofficial reports that the disease is a widespread problem throughout the country. With so few rabies cases reported, there has clearly been a need to develop epidemiological approaches that do not rely on case incidence data. The aim of this investigation was therefore to explore the use of serological assays for epidemiological studies of rabies in rural African domestic dog populations.

Serological surveys are widely used to investigate the dynamics of viral infections in natural pop-

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ulations. However, serosurveys have generally been considered to be of little value for the study of rabies epidemiology because, classically, antibodies are detected only in the terminal stages of infection and shortly before death. Serological tests have therefore been developed principally to measure post-vaccination antibody titres in people and animals and not to detect antibodies induced by natural infections.

The general explanation for the late appearance of antibodies in rabies infections is that the intrinsic neurotropism of the rabies virus protects it from immune surveillance and virus must multiply in the central nervous system before sufficient antigen is released to induce serum neutralizing antibody [3]. This assumption is challenged by the detection of rabies antibody in a proportion of healthy domestic dogs [4–9] and wild animals [5, 10–21] in rabies-endemic areas in various parts of the world. However, interpretation of such serological data is problematic because of the wide range of assays adopted, the variation in cut-off points and, in many cases, the lack of validation of test specificity for the species in question.

Although several rabies serological tests have been described [22], at the time of this study, only the mouse neutralization test (MNT) [23] and the rapid fluorescent focus inhibition test (RFFIT) [24] were recognized as reference techniques by the WHO. These recommendations were based on the fact that both tests measure inhibition of infectivity, a biological property usually correlated with protection. However, both tests have practical drawbacks, requiring specialized facilities, use of infective virus, several days to complete and, in the case of the MNT, large numbers of mice. A further problem with the RFFIT has been a lack of consistency between laboratories as a result of numerous modifications to the original RFFIT protocol [25, 26].

Enzyme-immunosorbent assays (ELISA) are gaining increasing popularity as a rapid and sensitive method of detecting rabies antibody. Several new techniques have been described using competitive systems [27] or enzyme-linked protein A [28] to allow sera of all species to be tested. The development of competitive assays that use monoclonal antibodies [29] also overcomes the need to produce highly purified antigen. Despite these advances, no ELISA has yet been validated as a reference technique for measuring rabies antibodies in dogs.

In this paper, we compare results of a rabies serological survey in rural domestic dog populations in Tanzania that were obtained using a RFFIT and a liquid-phase blocking ELISA (LPBE). Given the uncertainties about the interpretation of rabies seropositivity in natural infections, we adopted two approaches to assess the specificity of our results. The first was to compare titres of Tanzanian mainland dogs with those from a negative control population on the rabies-free island of Pemba, Tanzania [1]. Previous studies have addressed the specificity of RFFIT and LPBE through analysis of sera from dogs in rabies-free countries, such as UK, Mauritius and Hawaii [30, 31]. However, none of these countries provides an ideal negative control group for Tanzanian dogs because cross-reactivity to other Lyssavirus genotypes, such as Lagos Bat and Mokola virus [32], cannot be ruled out.

In the second analysis, we adopt an epidemiological approach to the assessment of test specificity, investigating the association between the location of rabies seropositive dogs and the location of rabies cases. We assume that, if non-specific or cross-reactions are the cause of rabies seropositivity in dogs, we would not expect to find a significant association between the location of seropositive dogs and rabies cases. The results are discussed with respect to the potential value of these assays for rabies seroepidemiological studies in natural populations. The significance of these findings in terms of rabies epidemiology has been discussed elsewhere [31].

MATERIALS AND METHODS

Field studies

Blood sample collection from unvaccinated dogs

Blood samples were collected from 567 domestic dogs from two Districts in north-western Tanzania; Ngorongoro District to the east of the Serengeti National Park (35°–36° E, 1° 30′–3° 7′ S), and Serengeti District to the west of the park [33]. Blood samples were collected from September–December 1993 and from August–December 1994 during systematic surveys of households within 19 villages bordering the park (Fig. 1). Details of household surveys are given elsewhere [33]. Dogs were manually restrained and muzzled while blood was collected from the cephalic vein. Blood samples were centrifuged within 24 h of collection, serum was stored for up to 5 months at between $-5\,^{\circ}\mathrm{C}$ and $-20\,^{\circ}\mathrm{C}$ and transported to laboratories on dry ice.
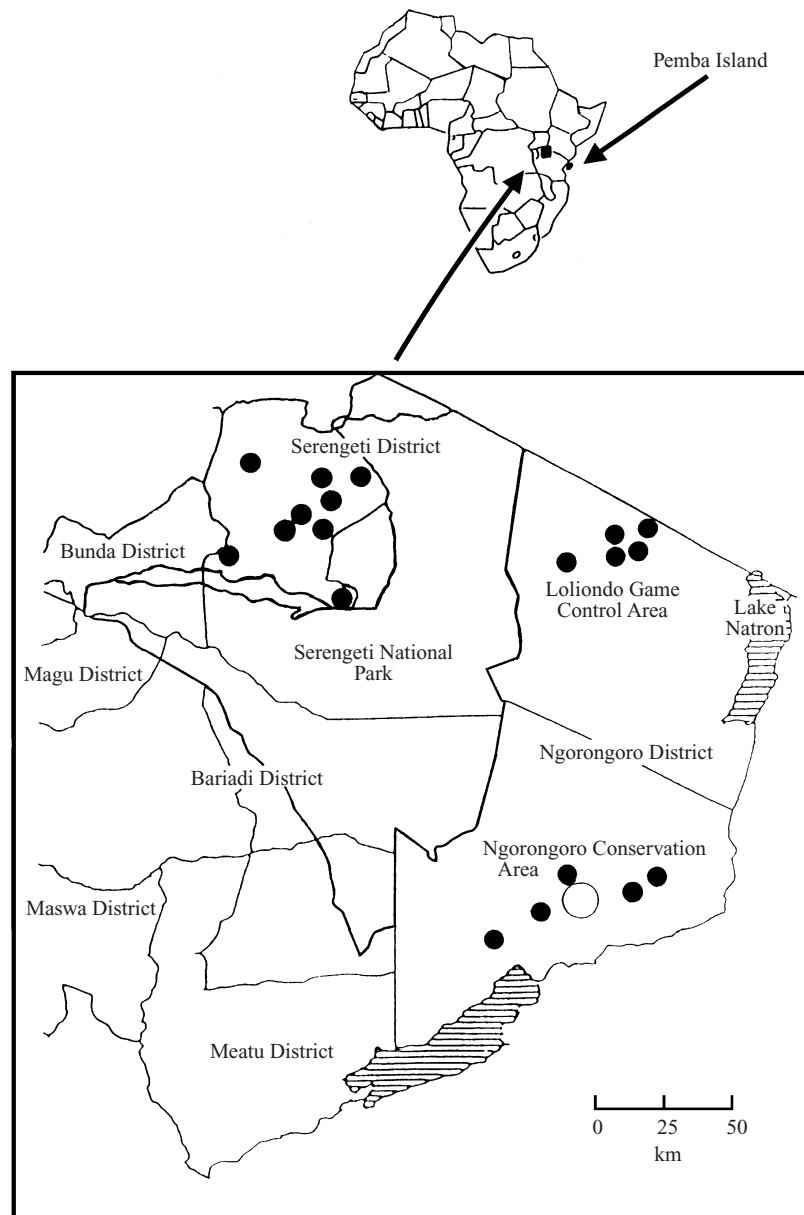
Fig. 1. Map of the study area showing location of villages (dark circles) where domestic dogs were sampled. Ngorongoro crater is shown by the large open circle.

Survivorship studies

In 1994, 76/193 (39·3%) households visited in 1993 were re-visited to determine survival of seropositive and seronegative dogs sampled previously. If a dog had died, information was collected from the owner on the date of death, clinical history and whether rabies was a suspected cause of death. Although results relied on owners identifying rabies as a cause of death, the disease is well-recognized in rural Tanzania. In this study area, villagers were able to detect rabies in domestic dogs with a diagnostic specificity of 83·3% and sensitivity of 86·5%, based on an analysis of 139 cases where dogs had died and samples had been sent for laboratory diagnosis.

Post-vaccination samples

From September–December 1993, 240 dogs from 6 villages were vaccinated against rabies, by a single subcutaneous injection of 1 ml Rabisin (Rhône-Poulenc, Nairobi). Ninety-one vaccinated dogs were re-bled between 28–31 days after vaccination to assess post-vaccinal titres; 62 vaccinated dogs were also re-bled 1 year after vaccination.

Rabies case incidence data

Records of reported rabies cases for the three study areas were obtained from veterinary offices in Mugumu (Serengeti District), Loliondo and Ngorongoro (Ngorongoro District). Suspected rabies cases were reported by village extension officers stationed in each village. Wherever possible, samples from suspect rabies cases were examined for rabies antigen using immunofluorescence diagnostic tests [34] carried out on brain stem samples collected in WHO collection kits containing 50% glycerol-saline with 0·01% methiolate as preservative [35]. Tests were carried out at the WHO Collaborating Centre for Zoonoses, Centre National d'Études Vétérinaires et Alimentaires (CNEVA), France.

Negative control population

From November–December 1996, 166 serum samples were collected from domestic dogs on Pemba by sampling every household within four rural villages, Fundo Island, Kizambani, Mchanga Mdogo, Gombani, and one urban location Bahrein/Masipa. Samples were obtained and treated as described above.

Serological tests

Liquid phase blocking ELISA

A total of 729 serum samples were analysed with the liquid phase blocking ELISA (LPBE) at the Onderstepoort Institute for Exotic Diseases, South Africa, using a technique adapted for the measurement of antibodies to foot-and-mouth disease virus [36, 37]. Details of the protocol and methods of calculating serum titres have been described fully elsewhere [27]. In this study, the protocol differed from that described previously only in the use of CVS strain of rabies virus as test antigen in place of Flury HEP virus. Sera were initially screened at dilutions 1:16–1:128. Any serum showing a titre > 1:16 was re-titrated at least once using dilutions from 1:16–1:1024 and the geometric mean titre adopted as the value. Titres were expressed as the logarithm$_{10}$ of the reciprocal dilution (referred to as log dilution). Previous studies have demonstrated specificity of the LPBE at a threshold > log dilution 1·5 [27, 31] and we adopt this cut-off point to define seropositivity in this analysis.

Rapid Fluorescent Focus Inhibition Test (RFFIT)

A modification of the original RFFIT technique [24] was used to measure neutralizing antibodies to rabies virus in 378 serum samples. This technique was developed at CNEVA, Nancy, using an adaptation for use of microtitre plates, based on the techniques of Zalan and colleagues [38] and Perrin and colleagues [39]. The protocol is described in detail elsewhere [30]. The titre of the serum was determine as the dilution at which 50% of fluorescence was inhibited (D_{50}) according to the Spearman–Karber method [40]. The antibody titre in International Units (IUs)/ml was determined by comparison with the WHO standard immunoglobulin. Titres were also expressed as the logarithm$_{10}$ of the reciprocal D_{50} (referred to as log dilution) for comparison with LPBE titres. In this study, we use the international reference threshold of 0·5 IU/ml (equivalent to log dilution 1·5) to define seropositivity.

Data analysis

Frequency distributions of Serengeti and negative control populations were compared by grouping values into four categories in contingency tables and analysed using a χ^2 test. Associations between the location of seropositive dogs and the location of rabies cases were investigated by calculating odds ratios (OR) expressed with 95% confidence limits using Epi-Info 5.0 [41].

RESULTS

Sample sizes

The number of serum samples tested with each serological test is shown in Table 1. Eleven dogs were sampled in both 1993 and 1994, and sera analysed using the LPBE. Of these dogs, only one sample was included for each individual to avoid pseudo-replication. There was no significant difference in the distribution of titres that included only the first samples and the distribution of titres that included only the second samples ($\chi^2_{(3)} = 0·04$, $P > 0·05$). For consistency, results including the first sample only are used in subsequent analyses.

Frequency distributions

The frequency distributions of LPBE values are shown for Serengeti and Pemba dogs in Figure 2a. The distribution of titres of Pemba dogs differed significantly from the distribution of Serengeti dogs ($\chi^2_{(3)} = 12·7$, $P < 0·01$). None of 162 Pemba dogs was seropositive. In the Serengeti, 21/286 (7·34%) unvaccinated dogs were seropositive in 1993 and 21/281 (7·47%) seropositive in 1994.

Table 1. *The number of serum samples analysed by each of the serological tests*

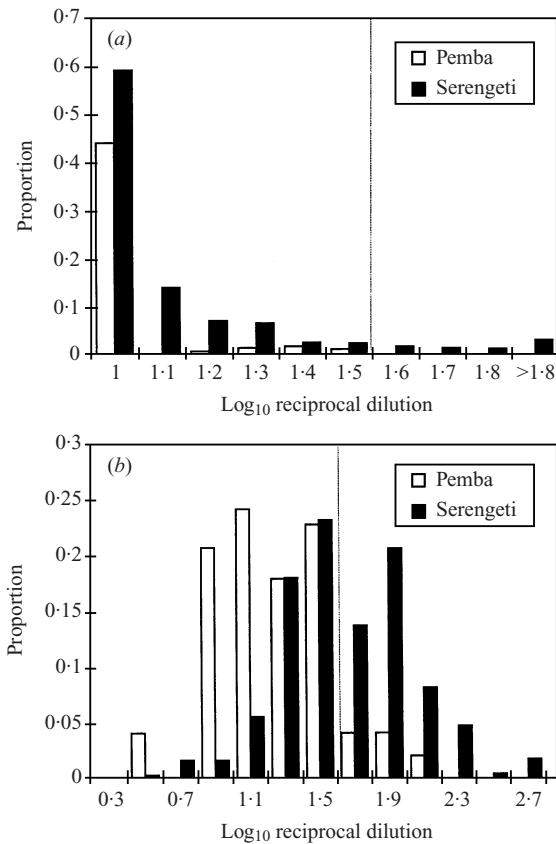| Origin of samples	LPBE (Onderstepoort)	RFFIT (CNEVA, Nancy)	Samples analysed on both LPBE and RFFIT
Serengeti, 1993	286	134	126
Serengeti, 1994	281	99	90
Pemba, 1996	162	145	141

Fig. 2. Distribution of rabies antibody in unvaccinated domestic dogs from Pemba and Serengeti measured by (*a*) liquid phase blocking ELISA (LPBE) and (*b*) rapid fluorescent focus inhibition test (RFFIT).

The frequency distributions of RFFIT titres from unvaccinated dogs is shown in Figure 2*b*. The distribution of Pemba dogs was significantly different from Serengeti dogs ($\chi^2_{(3)} = 56{\cdot}8$, $P < 0{\cdot}001$). 15/145 (10·34%) dogs from Pemba were seropositive. In 1993, 71/134 (53·0%) Serengeti dogs were seropositive and in 1994, 44/99 (44·4%) Serengeti dogs were seropositive.

Survivorship

Six of 14 (42·9%) dogs that were defined as seropositive by the LPBE were alive 1 year later. There was no significant difference in survival between
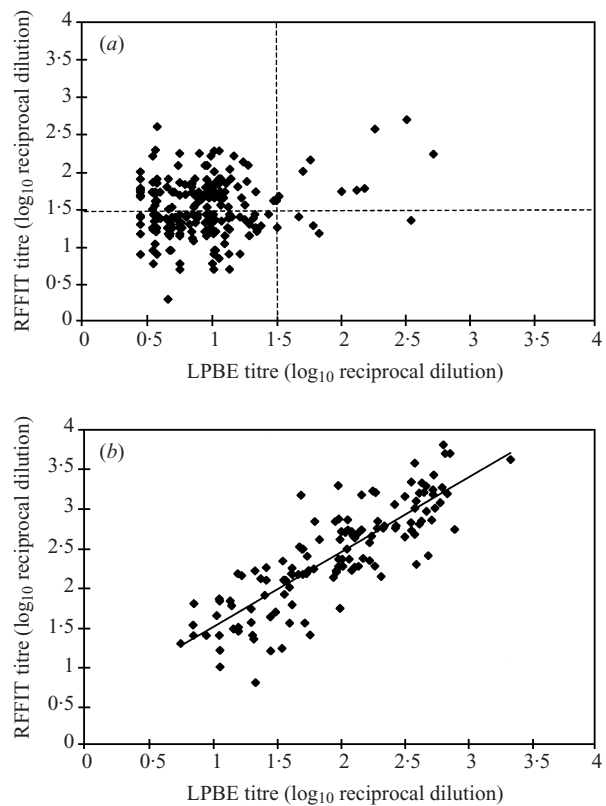

Fig. 3. Correlation between LPBE and RFFIT titres in (*a*) unvaccinated Serengeti domestic dogs and (*b*) vaccinated Serengeti domestic dogs.

seropositive and seronegative dogs ($\chi^2_{(1)} = 1{\cdot}28$, $P > 0{\cdot}05$, $n = 135$). No seropositive dogs were reported to have died with clinical signs consistent with rabies.

Of 32 dogs defined as seropositive by the RFFIT, 19 (59·4%) were alive 1 year later. Survival of seropositive and seronegative dogs was very similar ($\chi^2_{(1)} = 0$, $P > 0{\cdot}05$). No seropositive individuals were reported to have died with clinical signs consistent with rabies.

Correlation analysis

There was only a weak correlation between LPBE and RFFIT titres in unvaccinated Serengeti dogs ($r = 0{\cdot}18$, $n = 216$, $P < 0{\cdot}05$) (Fig. 3*a*). There was a much closer agreement between LPBE and RFFIT titres in

vaccinated dogs ($r = 0.84$, $n = 131$, $P < 0.001$) (Fig. 3*b*).

Geographical associations

Dogs defined as seropositive on the LPBE were significantly more likely to be found in villages where rabies had been confirmed during the past 12 months than in villages where it had not; OR = 5·16 (1·45–18·05), $P < 0.01$, $n = 216$. In contrast, there was no significant association between the location of RFFIT seropositives and confirmed rabies cases; OR = 1·26 (0·53–2·97), $P > 0.05$, $n = 216$.

DISCUSSION

The results of this study demonstrate that a proportion of unvaccinated domestic dogs from a rabies-endemic area of Africa had detectable levels of rabies antibodies when measured by a serum neutralization test (RFFIT) and by a liquid-phase blocking ELISA (LPBE). No seropositive dogs were reported to have died of suspect rabies and a proportion remained alive for at least a year. Our findings thus suggest that seropositive dogs were unlikely to be incubating rabies, but that seropositivity was consistent with aborted infection [42]. Although this outcome differs from that classically described for rabies, much of our understanding about the pathogenesis of rabies is derived from experimental, not natural, infections. In natural populations, the potential variation in infection routes, infective dose and virus characteristics may lead to a broader spectrum of outcomes than occurs in most experimental situations.

In this study, uncertainties arose in the interpretation of seropositivity because the two serological tests identified different dogs as seropositive. A key issue was therefore to investigate the relative specificity of the LPBE and the RFFIT to assess whether seropositivity reflected genuine exposure to rabies virus. Results of both analyses suggested that the LPBE was the more specific test when detecting rabies antibody induced by natural infection. Thus, fewer Pemba (rabies-negative) dogs were defined as seropositive ('false positives') when using the LPBE (0%) than when using the RFFIT (10·3%). Furthermore, the location of LPBE seropositive dogs was significantly associated with villages where rabies had been confirmed, whereas there was no significant association between the location of RFFIT seropositive dogs and rabies cases.

The close correlation between the location of LPBE seropositive dogs and rabies cases demonstrates the potential value of this technique for detecting rabies infection in unvaccinated populations. We have previously shown that rabies antibodies induced by natural infection are short-lived [31], which raises the possibility that seropositivity may also provide a useful comparative measure of the incidence of infection. The application of rabies serology may be of particular value in areas where incidence data are difficult to obtain, as occurs throughout much of rural Africa. Even in areas where active surveillance measures have significantly improved rabies detection rates [43], the high costs involved may favour rabies serology as a more cost-effective alternative for monitoring epidemiological trends. Still required, however, are more detailed longitudinal data comparing trends in both disease incidence and rabies seropositivity.

For wildlife populations, in which only a small fraction of rabies cases are ever detected, sero-epidemiological approaches clearly also have potential value. However, the conclusions of our study regarding the specificity of rabies seropositivity in natural infections applies only to domestic dogs and cannot be freely extrapolated to other species. Even where serological tests have been validated for wildlife populations, seroprevalence data may be difficult to interpret. It is likely that seropositivity in wildlife populations varies both with host species and virus strain. In highly susceptible hosts, such as red foxes and jackals, only very low seroprevalences have been recorded [10, 11, 13, 44], presumably because most infected animals die of the disease. However, virus strain also plays a role; in experimental infections, for example, red foxes infected with domestic dog isolates develop an immune response more often than when infected with red fox isolates [45].

We cannot explain why RFFIT and LPBE tests produce similar results when measuring antibody induced by a known dose of immunogenic antigen (rabies vaccine), but showed a very poor correlation when measuring antibody resulting from possible natural exposure. This is not just a matter of titre range, since unvaccinated dogs with high titres on one test had low titres on the other, whereas even low post-vaccinal titres correlated more closely. These results may simply reflect variability in the test systems, but biological differences in anti-N (detected by the LPBE) or anti-G responses (detected by the RFFIT) in non-lethal infections could also give rise to

discrepancies in titre. To our knowledge, however, there are no data comparing the kinetics of anti-G and anti-N antibody in infection.

In summary, the results of this study demonstrate the existence of rabies seropositivity in a proportion of healthy, unvaccinated domestic dogs in rural Africa and support the view that the liquid phase blocking ELISA is more specific than the RFFIT for detection of rabies antibody in unvaccinated dogs. ELISAs are intrinsically more easily standardized among laboratories than neutralization tests and allow simple and more rapid large-scale processing of sera. These practical advantages, coupled with the relatively high specificity demonstrated in this study, should encourage the development and validation of ELISAs for use in rabies epidemiological studies and as a reference technique for the detection of rabies antibody in domestic dogs.

ACKNOWLEDGEMENT

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