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Detection and isolation of Lyssaviruses from apparently healthy unvaccinated local breed of dogs in some rural communities in Southwestern Nigeria

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Abstract

Background: Existence of rabies virus-carrying and virus-secreting but apparently healthy dogs, referred to as rabies carriers, has been reported in Nigeria. This subclinical, in-apparent and non-fatal infection of rabies or related-viruses in the carrier dogs has compounded the risk of human exposure to the infection.

Methods: This study was designed to investigate the presence of subclinical or in-apparent Lyssavirus infection in apparently healthy unvaccinated local dogs in some rural communities in southwestern Nigeria. Oral swab specimens from 89 apparently healthy unvaccinated dogs in 5 rural communities in Oyo State were examined for Lyssaviruses using tissue culture isolation test (TCIT) and direct fluorescent antibody test (dFAT).

Results: Eighteen (20.2%) of the specimens were positive for rabies or related Lyssaviruses by TCIT, 24 (26.9%) by dFAT and 27 (30.3%) overall. Among age groups, the rate of virus infection was higher among younger than older dogs. The rate of virus infection was also higher among the female than the male dogs; however, the difference was not significant. The rate of detection of virus was higher by dFAT than TCIT. The carrier state of Lyssavirus infection found in this study suggests that ecological equilibrium exists between the strains of the viruses and the dogs, hence the absence of clinical rabies in the dogs.

Conclusion: This finding is disturbing and requires urgent attention to evaluate the role of domestic dog (Canis lupus familiaris) in the epidemiology of Lyssavirus infection and its threats to humans in rural communities in Nigeria.

Keywords: *Lyssaviruses, immunofluorescence, isolation, unvaccinated dogs, rural communities, Nigeria.*

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Résumé

Contexte: L'existence des chiens apparemment en bonne santé mais transportant et sécrétant le virus de la rage, appelés transporteurs de la rage, a été signalé au Nigeria. Cette infection sub-clinique, inapparente et non fatale de la rage ou virus-apparentés chez les chiens transporteurs a aggravé le risque d'exposition humaine à l'infection. Cette étude a été conçue pour étudier la présence ou non-apparence d'infection sub-clinique du lyssavirus chez des chiens locaux non-vaccinés apparemment en bonne santé dans certaines communautés rurales du sud-ouest, Nigeria. Les spécimens de nettoyage oral de 89 chiens non-vaccinés apparemment en bonne santé dans 5 communautés rurales dans l'État d'Oyo ont été examinés pour le lyssavirus en utilisant le test d'isolement de culture tissulaire (TCIT) et le test d'immunofluorescence directe (DFAT). Dix-huit (20,2%) des échantillons étaient positifs pour la rage ou le lyssavirus liés par TCIT, 24 (26,9%) par dFAT et 27 (30,3%) globale. Parmi les groupes d'âge, le taux d'infection par le virus était plus élevé chez les chiens jeunes que les plus âgés. Le taux d'infection par le virus était aussi plus élevé chez les chiens femelles que les mâles, cependant, la différence n'a pas été significative. Le taux de détection du virus était plus élevé par dFAT que TCIT. L'état des transporteurs de l'infection du Lyssavirus trouvé dans cette étude suggère qu'un équilibre écologique existe entre les formes du virus et les chiens, d'où l'absence de la rage clinique chez les chiens. Cette constatation est inquiétante et nécessite une attention urgente pour évaluer le rôle du chien domestique (Canis lupus familiaris) dans l'épidémiologie de l'infection des lyssavirus et ses menaces à l'homme dans les communautés rurales du Nigeria.

Introduction

Rabies is a highly fatal infection of the nervous system that affects all warm-blooded animals, including humans [1, 2]. The infection is caused by rabies and the rabies-related viruses, belonging to the genus Lyssavirus in the family

Rhabdoviridae[2, 3].The viruses are usually transmitted by bite of infected animal species, particularly dogs, cats, wolves, foxes, mongooses, skunks and bats, via their infective saliva, or through licking of superficial wounds without bite[4, 5]. Accidental infection by aerosol in bat roost and the laboratory have also been reported [6].

According to WHO [7], the annual estimate of human rabies cases worldwide is 75,000; however, the true burden of the disease is largely underestimated, especially in Africa. In Nigeria, rabies constitutes a major public health problem, especially in the rural and semi-urban areas [8, 9,10], where it endangers both human and animal health [11,10]. Several cases of rabies related viruses infections in humans have also been reported in Lagos and Ibadan, Nigeria between 1958 and 1973 [12, 13]. Recently, *Aliyu et al.* [14] and Mshelbwala et al. [15] reported detection of rabies virus antigen in the brains of apparently healthy dogs in Yola and Abia state, Nigeria respectively.

Existence of rabies virus-carrying and virus-secreting but apparently healthy dogs, referred to as rabies carriers, has also been reported in Nigeria[12]. This subclinical, inapparent and non-fatal Lyssavirus infection [16, 17] may be due to adaptation of strains of rabies or related-viruses in dogs. It has also been reported, on the other hand, that dogs with clinical rabies recovered and became carriers of the viruses [18].This subclinical infection and/or recovery of dogs from clinical rabies compound the risk of human exposure to the disease [12]. This study was therefore designed to investigate the extent of subclinical Lyssavirus infection in apparently healthy unvaccinated local breeds of dogs in rural communities in Nigeria.

Materials and Methods

Study Sites

The study was carried out in five communities (Tewure, Oloya, Alaropo Nla, Awaye and Ajegunle-Igbo-Iran) in Ori-Ire Local Government area (LGA) of Oyo State, Nigeria (Figure 1) using owned local domestic dogs (Canis lupus familiaris) of varying ages and sex. The study LGA is one of the 33 in the state. The LGA is bounded in the south by Ogbomosho north and Ogbomosho south, in the southwest by Ogo Oluwa and Oyo east, in the south east by Sulurele, in the North by Olorunsogo, in the West by Atiba LGAs and in the East by Osun State.

Oral swab specimens were collected from 89 owned free-range and unvaccinated local breeds of dogs in virus transport medium (VTM) and also smeared on pre-cleaned labelled glass slides. The smears were air-dried, fixed in 98% analytical grade acetone for 15 minutes, allowed to air-dry and arranged in slide boxes. The specimens were transported to the laboratory on ice and stored at -20°C until the smears were examined by dFAT and specimens in the VTM were inoculated into tissue culture for virus isolation.

Titration of Fluorescien Isothiocyanate Conjugate

Polyclonal antinucleocapsid (conjugate N4-13) Fluorescein Isothiocyanate Conjugate (FITC), obtained from ARC-Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South Africa, was serially diluted and the working dilution determined according to manufacturer's instructions. Flurry low egg passage vaccine strain rabies virus used as control was obtained from the National Veterinary Research Institute, (NVRI) Vom, Nigeria.

Virus isolation in tissue culture

Virus isolation was carried out in tissue culture grade tubes of Vero cells (ATCC No. CCL-81) containing 1 x 10^5 cells per ml cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 2% Fetal Calf Serum (FCS) at three passage levels. To perform inoculation, EMEM initially supplemented with 10% FCS covering the fully confluent Vero cells was discarded and replaced with 1.0ml of EMEM per tube, containing 2% FCS, aseptically. The tubes were inoculated in duplicates with 200µl of each specimen per tube. Tubes inoculated were incubated at 37°C and examined daily for appearance of cytopathic effect (CPE)for 10 days using the inverted microscope. Specimens that did not show CPE after primary and first passages were subjected to blind passages until confirmed negative at the second passage. Cultures of positive specimens were harvested when there was about 75% CPE, freeze-thawed thrice and stored in aliquots of 0.5ml per vial at -80°C. Similarly, confluent 24-well plates of Vero cells were inoculated with 50µl per well of virus isolates, incubated at 37°C and examined daily for CPE. When CPE was about 50%, plates were retrieved, medium decanted and cells were carefully scrapped from the wells of the plate unto corresponding labeled glass slides. Slides were fixed in 98% analytical grade acetone and

stained with 1:700 dilution of the FITC conjugate according to standard protocol [2]: for confirmation by dFAT.

Fluorescent antibody test on the oral swab smears

Direct Fluorescent Antibody Test (dFAT) was performed on the oral swab slide smears according to standard protocol of OIE [2]. All the smears were carefully examined using CY-S-4005R I PartecCyScope® TB binocular (fluorescent microscope) with royal blue LED (455nm) excitation usingX10 eyepiece and X40 oil immersion objective magnifications. comparing the test smears with the positive and negative controls.

Data Analysis

Descriptive analysis of data generated in this study was carried out using the Statistical Package for Social Sciences (SPSS) version 20.0. Test of association between categorical variables were done by student t-test and ANOVA, where values of $P<0.05$ were considered significant. Sensitivity and specificity of TCIT were also determined as described by Akobeng [19].

Results

Titration of Fluorescein Isothiocyanate Conjugate

Varying dilutions of the FITC labeled rabies antibody (Conjugate) ranging from 1:100 to 1:1000 produced characteristic greenish yellow or apple-green fluorescence with Lyssaviruses in infected cells and oral swab smears. The highest dilution with optimum fluorescence was1:700.

Virus Isolation and Detection

In tissue culture, intracytoplasmic viral antigens were observed as minute or oval shaped granules of apple-green fluorescing inclusions scattered in the pericaryon of infected Vero cells that were stained with rabies FITC labeled conjugate [21] (Plates 1 and 2), compared to the negative control specimen (Plate 3). These fluorescing viral inclusions (Negri bodies) are diagnostic of rabies and related Lyssaviruses [2]. Plate 1 depicts fluorescence in FITC stained cell monolayer that was infected with positive control virus, plate 2 shows fluorescence in stained cells seven days post inoculation (p.i.) with virus isolate while Plate 3 portrays no fluorescence in stained uninfected cells. Intensity of fluorescence increased with days p. i. in the cultures. Plates 4 and 6 show CPE such as rounding of cells and pyknosis of nuclear contents, compared to uninfected negative control cell culture (Plate 5). Out of the 89 swab samples inoculated in Vero cells,18 (20.2%) yielded positive cultures (Tables 1 and 2).

Out of the 89 dog oral swab smears examined, 24(26.9%) were positive for rabies or related viruses by dFAT (Tables 1 and 2). The rate of infection was higher among females (30.6%) than males (22.5%), however, the difference was not significant ($P>0.05$). Although the rate of infection was slightly higher in younger dogs (Figure 2), there was no significant difference ($P>0.05$) by the age. The highest rate of infection (66.7%) was in Oloya and the lowest (18.5%) was in Alaropo Nla (Table 1).

Fifteen specimens were positive in both tests while nine and three were only positive in dFAT and TCIT respectively (Figure 4). Therefore, the overall result showed that twenty-seven specimens, representing 30.3% of the total tested were positive in the study. In relation to dFAT, the gold-standard technique for rabies diagnosis [2], the sensitivity and specificity of TCIT were found to be 63% and 95% respectively. The rate of infection was significantly ($P<0.05$) higher among female dogs in three of the five communities (Table 1). However, overall, the rates of infection in both gender was similar (Figure 3). The highest rate of isolation (66.7%) was in Oloya community and the lowest (11.1%) in Alaropo Nla community (Table 2). Across the communities, a significantly high difference in the rate of infection among age groups ($P=0.006$) was observed. Dogs that aged 1-3 years had the highest rate of virus infection (35.2%) and the lowest (18.2%) was found in those greater than 3 years old (Figure 2).

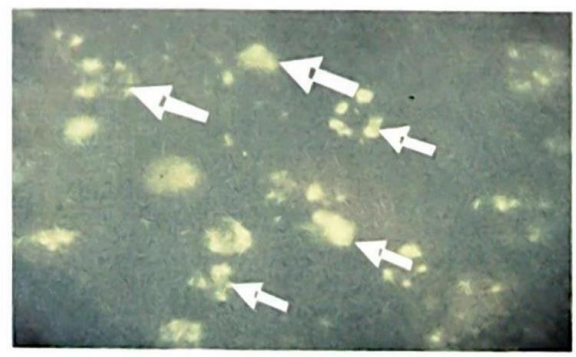

Plate 1: FITC Stained Positive Control Showing fluorescence (arrows)

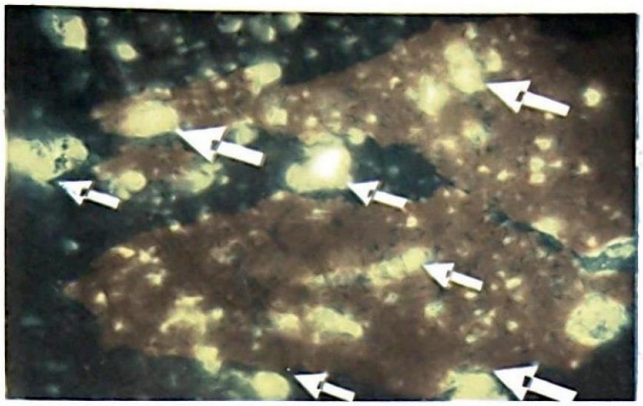

Plate 2: FITC stained virus infected Vero cells that showed advanced CPE (7 days post infection -PI) with Virus isolate showing Fluorescence (Arrows)

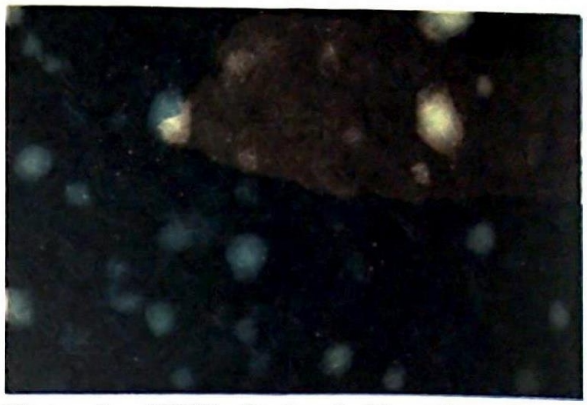

Plate 3: FITC Stained Negative Control (Showing no fluorescence)

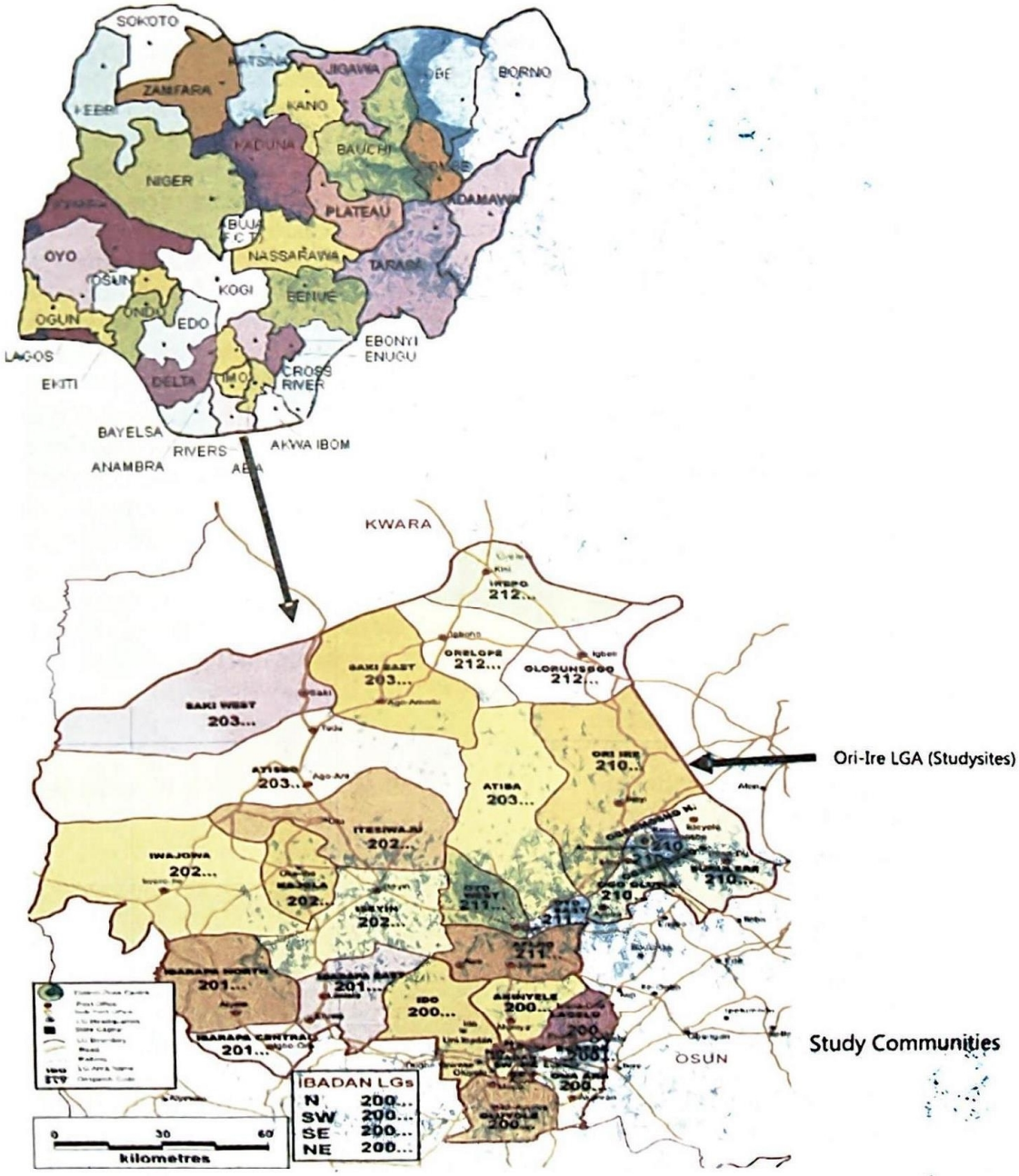

Fig. 1: Map of Nigeria showing Oyo State and Ori-Ire Local Government Area (studysites) (source: Official website of the Oyo State Government-The Pacesetter State, Nigeria). Available from http://www.oyostate.gov.ng/

Plate 4: Culture of positive control virus showing CPE in Vero cells, 5days PI (Arrows)

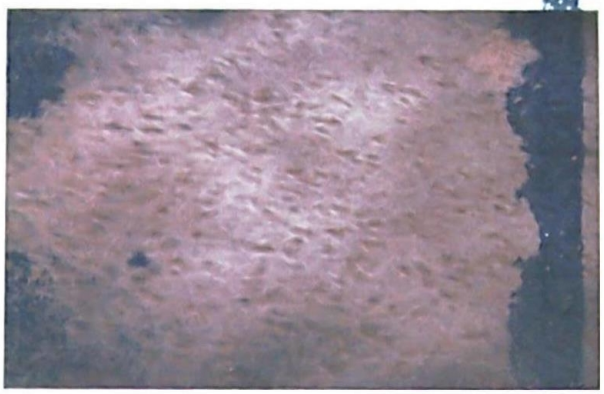

Plate 5: Uninfected cell culture showing no CPE

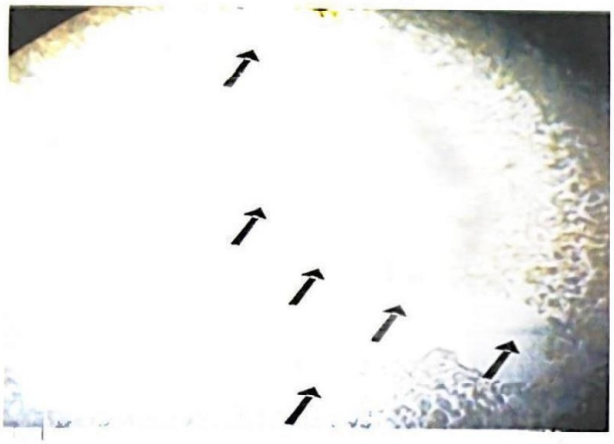

Plate 6: Virus culture showing advanced CPE (arrows) in Vero cell culture (7days PI)

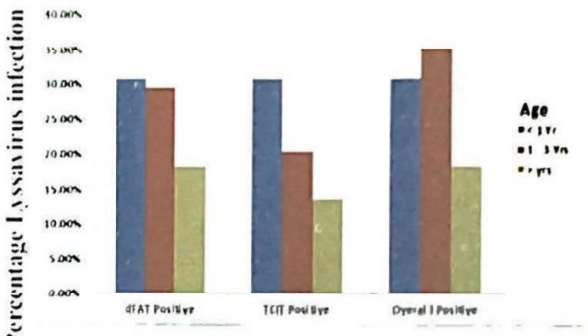

Figure 2: Rates of virus infection between genders of dogs sampled in Ibadan

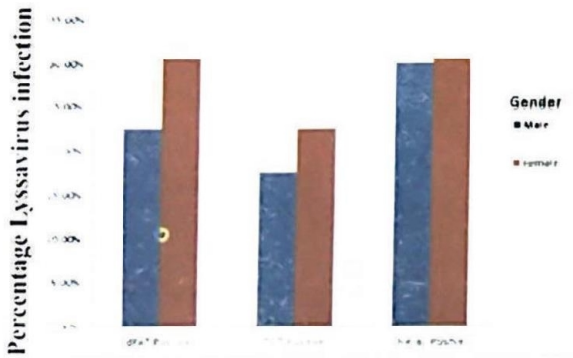

Fig. 3: Rates of virus infection among age groups of dogs sampled in Ibadan

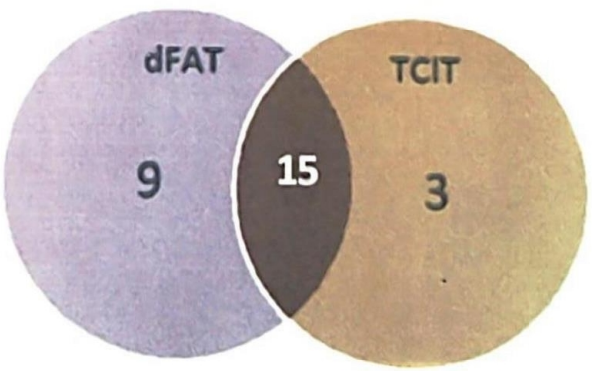

Fig. 4: Numerical relationship between dogs that tested positive for Lyssavirus infection by dFAT and TCIT in some rural communities of southwestern Nigeria

Table 1: Distribution of dogs tested for Lyssavirus infection by dFAT and TCIT in some rural communities of south-western Nigeria according to gender

	Tewure			Olova			Alaropo-Nla			Awave			Ajagunle-Igbo-Igran			Total tested	Total +ve		Overall Total +ve (%)
N tested	dFAT N(%) +ve	TCIT N(%) +ve	N tested	dFAT N(%) +ve	TCIT N(%) +ve	N tested	dFAT N(%) +ve	TCIT N(%) +ve	N tested	dFAT N(%) +ve	TCIT N(%) +ve	N tested	dFAT N(%) +ve	TCIT N(%) +ve			dFAT (%)	TCIT (%)	
9	4(44.4)	3(33.3)	3	1(33.3)	1(33.3)	12	2(16.7)	2(16.7)	10	1(10.0)	0 (0.0)	6	1(16.7)	1 (16.7)	40	9 (22.5)	7 (17.5)	12(30.0)	
17	6(35.3)	4(23.5)	3	3(100)	3(100)	15	3(20.0)	1(6.7)	4	1(25.0)	0 (0.0)	10	2(20.0)	3 (30.0)	49	15 (30.6)	11 (22.4)	15(30.6)	
26	10(38.5)	7(26.9)	6	4(66.7)	4(66.7)	27	5(18.5)	3(11.1)	14	2(14.3)	0 (0.0)	16	3(18.7)	4 (25.0)	89	24 (26.9)	18 (20.2)	27(30.3)	

Table 2: Distribution of dogs tested for Lyssavirus infection by dFAT and TCIT in some rural communities of south-western Nigeria according to age group

Age (Years)	Tewure			Oloya			Alaropo-Nla			Awaye			Ajagunle-Igbo-Igran			Total tested	Total +ve		Overall Total +ve (%)
	N tested	dFAT N(%) +ve	TCIT N(%) +ve	Ntested	dFAT N(%) +ve	TCIT N(%) +ve	N tested	dFAT N(%) +ve	TCIT N(%) +ve	N tested	dFAT N(%) +ve	TCIT N(%) +ve	N tested	dFAT N(%) +ve	TCIT N(%) +ve		dFAT (%)	TCIT (%)	
< 1	3	2(66.7)	1(33.3)	1	0(0.0)	0(0.0)	3	0(0.0)	1(33.3)	0	0(0.0)	0(0.0)	6	2(33.3)	2(33.3)	13	4(30.8)	4(30.8)	4(30.8)
1-3	13	5(38.5)	3(23.1)	5	4(80.0)	4(80.0)	16	5(31.2	2(12.5)	12	1(8.3)	0(0.0)	8	1(12.5)	2(25.0)	54	16(29.6)	11(20.4)	19(35.2)
>3	10	3(30.0)	3(30.0)	0	0(0.0)	0(0.0)	8	0(0.0)	0(0.0)	2	1(0.0)	0(0.0)	2	0(0.0)	0(0.0)	22	4(18.2)	3(13.6)	4(18.2)
Total	26	10(38.5)	7(26.9)	6	4(66.7)	4(66.7)	27	5(18.5)	3(11.1)	14	2(14.3)	0(0.0)	16	3(18.7)	4(25.0)	89	24(26.9)	18(20.2)	27(30.3)

Discussion

High rate of Lyssavirus infection was detected in dogs in the rural communities by dFAT (26.9%) and virus isolation in tissue culture (20.2%). In a survey of apparently healthy dogs in Thailand, it was found that only 0.03% of the subjects were carriers of rabies virus [20]. The rate of virus infection in this study is obviously higher than the rate found in Thailand due, probably, to large population turnover and poor vaccination coverage of dogs in Nigeria [12, 21, 22, 23] generally, and lack of vaccination of the dogs in the communities. Though owned, the dogs in this study freely roamed about within communities giving rise to easy access of uninfected, but susceptible dogs to Lyssavirus carrier dogs, making the rate of spread of the viruses to be fast and high compared to the situation in the Thailand study. Consequently, a good knowledge of local dog ecology (dog numbers, turnover rates, reasons for ownership, owners' management of their dogs and other diseases of dogs) is crucial for effective control of rabies [24]. From an ecological point of view however, it was reported that feral dogs do not appear to be an important factor in the epidemiology of rabies in KwaZulu-Natal [25].

In two previous independent but related studies, evidence of rabies virus and non-rabies Lyssavirus infection in apparently healthy dogs in some parts of Nigeria was reported[26, 27]. Furthermore, a protocol used for the intravitam detection of rabies virus genomic RNA in saliva and skin biopsy samples from 28 patients suspected of having rabies[28], allowed detection of rabies virus in 9 of the patients[2, 3,29, 30].

Evidence of Lyssavirus infection was established by the presence of characteristic apple-green fluorescing Negri bodies in rabies FITC labeled conjugate stained infected Vero cells and oral swab smears using a fluorescent microscope. Lyssaviruses were detected by immunofluorescence in the dogs across all the communities while there was no isolation by TCIT in one. These could be as a result of the higher sensitivity of dFAT compared to TCIT. The dogs mingled freely within communities, having different forms of direct contacts such as bite, licking of each other's wounds and/or mucous membranes, especially bitch to puppies and sexual contact. Most of these forms of contacts are means by which Lyssaviruses are transmitted from infected to susceptible animals [3,12, 22,31].

Comparison of the rate of infection in the communities shows that, Oloya, the smallest among the communities, had the highest rate of infection (66.7%) by immunofluorescence and tissue culture assays (Tables 1and 2). This relatively high value may be because Oloya is a small community compared to the rest communities. In such a small community, dogs from different households had closer interactions than the ones in larger communities; hence higher chances of spread of the viruses among the dogs. The low infection rates of 18.5% and 11.1% observed by dFAT and TCIT respectively in a large community such as Alaropo Nla was probably due to little interaction amongst the dogs, hence the detection of Lyssaviruses in relatively fewer dogs. Also, higher prevalence of rabies or related-viruses observed in females in most communities is not surprising as this group is reared for longer period for breeding. The significantly high Infection rate observed amongst younger than older dogs could be so because the younger ones were at their prime of hunting and mating activities hence, the higher risk of infection.

Information from the dog owners in the communities indicated that their dogs, mainly used to hunt for wild animals (locally described as bush meat) have never been vaccinated against rabies. It is therefore reasonable to postulate that many of these dogs could have been bitten at various times by rodents, such as ground squirrels and shrews, suspected to be vectors of Lyssaviruses in Nigeria [32, 33]. The dogs were also at risk of bites by bats, wild cats and other species of wild animals known to be important maintenance hosts for Lyssaviruses[12]. Unknown sylvatic reservoir hosts of Lyssaviruses in the wild may be transmitting the viruses to the domestic dog, which in turn transmits same to humans and domestic animals in Africa [34]. This could therefore explain in part, why rabies continues to constitute major public health problem in many parts of Nigeria, especially the rural and semi-urban areas, where epizootics occur from time to time [30, 35, 36, 37, 38].

Despite the high prevalence of rabies or related virus infection in these dog populations, there were no reports of apparent clinical rabies in the communities during the period of this study. This could imply that, either that the dogs possessed protective level of antibodies against the viruses, or though protective, the level of antibody is not high enough to have neutralized the viruses, giving room for existence of ecological balance between circulating strains of the viruses and dogs in the study communities [12]. The common practice in these communities was that, any animal that showed a sign of strange or deranged behaviour was killed immediately. This could as well be responsible for the absence of clinical rabies cases at the time of this study. It is also possible that existence of quasi species, due to high level of intrinsic heterogeneity [39, 40] or variation due to mutation in several genes [41] of the viruses during adaptation in various host systems in nature could have resulted to avirulent strains of the Lyssaviruses in the communities, leading to the observed subclinical infection in this study.

Dog rabies has often erroneously been referred to as "urban" rabies but the disease is clearly more of a rural problem [24, 25] in Nigeria. It is almost exclusively endemic in developing countries where control measures are difficult to apply and education of the public is complicated by competing priorities. It

is therefore highly recommended that veterinary and public health authorities develop a working policy for effective national surveillance for rabies and intensive dog annual vaccination campaigns especially in rural communities where residents, who often do not have access to good health care facilities, are at risk. High-risk occupational groups, such as veterinary staff, wildlife workers, surveillance staff, animal welfare officers, laboratory personnel working with rabies or related viruses, as well as all members of medical teams that handle rabies cases should receive pre-exposure prophylaxis at recommended intervals [5] and use appropriate personal protective equipment (PPE) when they need to.

In conclusion, this study has shown and reiterates the significance of reservoir status of domestic dog in the epidemiology of rabies and related viruses and their threats to human health in Nigeria. This finding, in addition to previous reports on rabies in some urban and rural areas of Nigeria [23, 24, 32, 33,40, 41], necessitates the need for evaluation of the role of unvaccinated dogs in the epidemiology of rabies in Nigeria. Further studies will focus on serological and molecular characterization of the virus isolates obtained in this study.

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