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Evidence of Lagos Bat Virus Circulation among Nigerian Fruit Bats

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ABSTRACT: During lyssavirus surveillance, 350 brains from four species of fruit bats and one species of insectivorous bat were collected from seven locations in Northern Nigeria during May to October, 2006. Lyssavirus antigen was not detected in the brains, and isolation attempts in mice were unsuccessful. However, serologic tests demonstrated the presence of lyssavirus-neutralizing antibodies in bat sera. Of 140 sera tested, 27 (19%) neutralized Lagos bat virus, and two of these additionally neutralized Mokola virus. The positive samples originated from the straw-colored fruit bat (*Eidolon helvum*) and the Gambian epaulet bat (*Epomophorus gambianus*). No neutralizing activity was detected against other lyssaviruses including rabies, Duvenhage, and West Caucasian bat viruses.

Key words: *Eidolon helvum*, *Epomophorus gambianus*, fruit bats, Lagos bat virus, Nigeria, serology.

Bats are recognized as important reservoirs of many emerging infectious agents, including lyssaviruses (order *Mononegavirales*, family *Rhabdoviridae*). The classic rabies bat virus (RABV) circulates in bats in the Americas. Five other lyssavirus species, including Lagos bat virus (LBV), Duvenhage virus (DUVV), European bat lyssaviruses 1 and 2, and Australian bat lyssavirus have been reported in other parts of the world (WHO, 2005). More recently, four new putative lyssavirus species (Aravan, Kudjand, Irkut, and West Caucasian bat viruses) have been identified in bats (Kuzmin and Rupprecht, 2007).

To date, four lyssavirus species have been documented in Africa. Of these, RABV occurs worldwide, but LBV, Mokola virus (MOKV), and DUVV have not been naturally encountered outside of Africa (WHO, 2005). Nigeria is particularly important for lyssavirus surveillance because the initial isolations of LBV and MOKV occurred in that country. Historically, LBV was isolated over four decades ago from the brains of the straw-colored fruit bat, *Eidelon helvum*, collected on Lagos island in 1956 (Boulger and Porterfield, 1958), and MOKV was isolated from the viscera of shrews (*Crocidura* sp.) in 1968 in Mokola province (Shope et al., 1970). A study carried out in Ibadan (western Nigeria) in 1990 suggested the presence of RABV-neutralizing antibodies in the sera of fruit bats (Aghomo et al., 1990). Human consumption of bats is practiced in many parts of Nigeria, and the public health implication of this practice remains to be assessed. Surveillance is particularly important for nonrabies lyssaviruses because commercially available rabies biologics do not provide reliable protection against LBV, MOKV, and West Causcasian bat virus (WCBV) (Hanlon et al., 2005). In this study, we performed active surveillance for lyssaviruses among bats in the northern part of Nigeria in order to provide the baseline information necessary to begin assessment of the epidemiology of lyssaviruses in this part of the world.

A total of 350 bats were captured between May and October, 2006 in small settlements around Zaria (11°08′45″N and 7°32′20″–7°25″E), Kaduna State of northern Nigeria (Karfi, Mil Goma, Tsibiri, Tsibirin Iya, Biye, and Bijimi). Additional samples were collected from Mubi (10°16′8″N and 13°16′14″E), northeastern Nigeria (Fig. 1). Bats were collected from existing colonies with the consent of the community leaders where the colonies

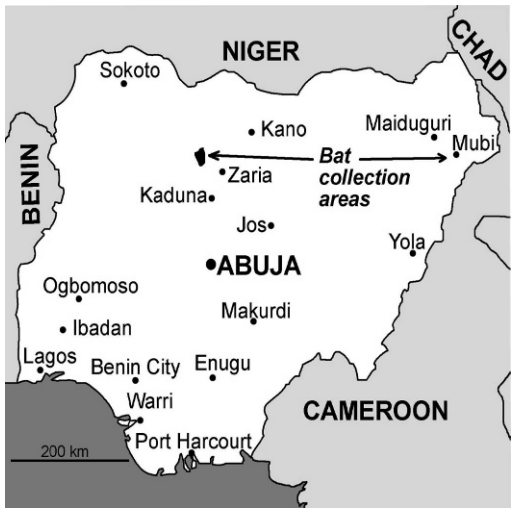

FIGURE 1. Locations of bat collection sites in Nigeria.

were located. Most bats were trapped using hand nets. Blood samples were collected from the jugular veins in animals intended for consumption, or by cardiac puncture following inhalational anesthesia (using chloroform) from insectivorous bats. Sera were separated by centrifugation after allowing the blood to clot. Brain specimens were collected and stored individually in either glass bottles or in 50% buffered glycerol saline at −20 C. The age and sex of the bats was assessed based on body size of the bats and observation of the reproductive organs, respectively. Representative carcasses were stored in 10% formalin for species identification. Sera could not be collected from several bats that died before processing.

In total, bats of five species were collected, including *Eidolon helvum, Epomophorus gambianus* and *Micropteropus pusillus* (lesser epaulet bat). The fourth species of fruit bat could not be identified. One specimen of the microchiroptera bat, *Rhinolophus alcyone*, was collected (Table 1). Fifty-three percent of the bats were females, and adults composed 93% of the bats in this study.

All brain samples were tested for the presence of lyssavirus antigens by the direct fluorescent antibody (DFA) test, as described (Dean et al., 1996). Touch-brain impressions were made on 4-well, teflon-coated slides. The slides were fixed in cold acetone, air dried, and stained with four different flourescein isothiocyanate conjugates, including anti-rabies monoclonal (Light Diagnostics™, Southampton, UK and Fujirebio Diagnostics, Inc., Malvern, Pennsylvania, USA) and polyclonal (Light Diagnostics) antibodies. RABV-infected and normal mouse brains were used to prepare positive- and negative-control slides.

A mouse inoculation test (MIT) was used to attempt virus isolation. Ten-percent suspensions of bat brains prepared in Eagle's minimum essential medium (MEM-10, Invitrogen, San Diego, California, USA) were inoculated intracranially into 3-wk-old mice, as described (Koprowski, 1996). Brain impressions from mice which died following inoculation were tested by DFA for the presence of lyssavirus antigens and subjected to a second intracerebral passage following filtration of the brain suspension through a 0.22-μm filter.

Of the 350 bats collected, 140 serum samples were available. A modified rapid fluorescent focus inhibition test (RFFIT) was used for testing of sera for neutralizing antibodies. The reaction was performed using 4-well (6 mm) glass slides (Cel-Line, Erie Scientific, Portsmouth, New Hampshire, USA). Heat inactivated serum samples were initially tested in 1:10 and 1:25 dilutions. Approximately 14 μl MEM-10 was added to the 1st and 3rd well of 4-well Teflon-coated slides, and 7.5 μl of the same medium was dispensed into the 2nd and 4th well. Approximately 3.6 μl of two separate test sera were added to the 1st and 3rd wells, respectively, and the contents of each well were properly mixed using the pipette tip. About 5.0 μl of the mixture was then transferred to the corresponding 2nd and 4th wells and mixed properly.

For RFFIT, LBV (isolate from Nigeria, 1956), MOKV (isolate from South Africa,

TABLE 1. Demographic, virologic, and serologic data on bats captured during surveillance for lyssaviruses in northern Nigeria.

Location and species	Sex (males/females)	Age (adults/nonadults)	Brains tested by DFA and MIT[a,b]	Serum tested by RFFIT (LBV-positive/MOKV-positive/Tested)[c]
Mubi				
Eidolon helvum	33/52	85/0	85	6/0/42
Biye				
Eidolon helvum	7/4	10/1	11	4/0/9
Tsibirin Iya				
Micropteropus pusillus	28/27	53/2	55	0/0/21
Tsibiri				
Eidolon helvum	14/30	44/0	44	10/0/39
Epomophorus gambianus	3/9	7/5	12	3/0/12
Karfi				
Eidolon helvum	40/28	54/14	68	4/2[d]/15
Unidentified fruit bat	4/13	17/0	17	NA[e]
Mil Goma				
Rhinolophus alcyone	27/16	42/1	43	0/0/2
Bijimi				
Eidolon helvum	8/7	15/0	15	NA
Total	164/186	327/23	350	27/2/140

[a] All brains were negative for detection of lyssavirus antigens and evidence of virus isolation; DFA = direct fluorescent antibody; MIT = mouse inoculation test.

[b] Total number of each species per site.

[c] All samples tested negative against rabies virus, Duvenhage virus, and West Causcasian bat virus; RFFIT = rapid fluorescent focus inhibition test; MOKV = Mokola virus.

[d] Both MOKV-neutralizing samples also neutralized Lagos bat virus (LBV).

[e] NA = not available.

1998), DUVV (isolate from South Africa, 1970), RABV (strain CVS-11), and WCBV (isolate from Russia, 2002) were used. About 50% fluorescing foci doses (FFD_{50}) of each virus was used in the RFFITs, as confirmed by a titration on a control slide with each reaction run. The slides were incubated in a humidity chamber at 37 C for about 90 min. Thereafter, 25 µl of mouse neuroblastoma cells were added (concentration approximately 2×10^6 cells/ml), and the slides were incubated at 37 C for 20 hr for RABV and MOKV, or for 44 hr for LBV and DUVV. Slides were then fixed in cold acetone for 30 min, air dried, and subjected to the DFA test as described above. At microscopy, 10 separate fields were counted for each well. For all positive or inconclusive RFFIT results, additional titration of the sera was conducted in dilutions of 1:10, 1:25, 1:625, and 1:15,625. Each positive result was confirmed at least twice. The titers were calculated by the Reed and Muench method (Reed and Muench, 1938) and expressed as log dilution of the test serum. Only the samples that had a 50% endpoint neutralizing titer greater than 1 $\log_{10}$ (e.g., less than 5 fields contained infected cells at serum dilution 1:10) were considered as positive.

None of the 350 bat brains tested by DFA were positive for lyssavirus antigens. During the MIT, several specimens caused mortality of mice. However, no lyssavirus antigen was detected in the

mouse brains. Subsequent passage following filtration caused no mortalities, suggesting bacterial contamination of field samples.

Most of the serum samples (105/140 [75%]) originated from *E. helvum*. Of the remainder, *M. pussilus* constituted 15%, whereas *E. gambianus* and *R. alcyone* comprised 9% and 1% of the samples, respectively (Table 1). Twenty-seven samples (19%) had neutralizing activity against LBV, two of which also neutralized MOKV, both at a dilution of 1:625. None of the sera neutralized DUVV, RABV, or WCBV. Sera of *E. helvum* originated from four locations and made up 89% (24/27) of the sera that neutralized LBV.

Between colonies, antibody prevalence of *E. helvum* ranged from 14% to 44% (mean 28±12%); this is a migratory species that may travel hundreds of kilometers during certain seasons. Based on this observation, and the fact that the study areas around Zaria were separated from each other by only a few kilometers, there is a possibility that the bats moved from one roost to another during the period of the study. *Epomophorus gambianus* made up the remaining 11% (3/27) of the sera positive for LBV. Antibody prevalence in this species was 25%. Overall, the bat species was not statistically associated with seropositivity (OR=0.89, 95% CI 0.20–4.53). Both samples that demonstrated neutralizing activity against MOKV came from *E. helvum* in Karfi. Other studies in Africa record about 38% virus neutralizing activity to LBV among fruit bats in Ghana and Kenya (Hayman et al., 2008: Kuzmin et al., 2008).

Previously, LBV had been isolated from *E. helvum*, (Boulger and Porterfield, 1958; Kuzmin et al., 2008), *M. pusillus*, (Sureau et al., 1980), *Rousettus aegyptiacus* (Aubert, 1999), and *Epomophorus wahlbergi*, (Markotter et al., 2006). We obtained serologic evidence of LBV circulation in *E. helvum* and *E. gambianus*; however, viral infection was not detected, based on our limited dataset. As was documented for RABV, infection prevalence in gregarious colonial bat species is usually significantly less than 1%, whereas seroprevalence may be as high as 70% (Constantine, 1967; Steece and Altenbach, 1989). This association may be attributed to a lengthy co-evolution period between bats and lyssaviruses. Due to limited susceptibility (Warrilow et al., 2003), a majority of exposures may lead to the development of immunity, caused by peripheral virus activity and abortive clearance rather than by central nervous system infection (Shankar et al., 2004). All bats collected in our study were apparently healthy. The absence of reports of human infection with LBV may be attributable to poor surveillance in Nigeria. Since the first isolation of LBV in 1956, this is the second surveillance attempt of bats.

Mokola virus has been isolated from terrestial mammals in Africa (Swanepoel, 2004), but never from bats. Serologic cross-reactivity between LBV and MOKV has been demonstrated previously (Shope et al., 1970; Hanlon et al., 2005). Therefore, based on our cumulative serologic results, we have no substantive reason to consider the possibility of MOKV circulation in bats.

Bats are abundant and segregate into large colonies, often in close proximity to human dwellings. There are frequent interactions between humans and fruit bats in certain parts of Nigeria, where bats are hunted either for food or religious rituals. Additional surveillance is needed for a better understanding of the epidemiologic situation, circulation patterns, and public health significance of lyssaviruses and other emerging pathogens in Nigeria and other African countries.

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