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Shimoni bat virus, a new representative of the *Lyssavirus* genus☆

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ABSTRACT

During 2009, 616 bats representing at least 22 species were collected from 10 locations throughout Kenya. A new lyssavirus, named Shimoni bat virus (SHIBV), was isolated from the brain of a dead Commerson's leaf-nosed bat (*Hipposideros commersoni*), found in a cave in the coastal region of Kenya. Genetic distances and phylogenetic reconstructions, implemented for each gene and for the concatenated alignment of all five structural genes (N, P, M, G and L), demonstrated that SHIBV cannot be identified with any of the existing species, but rather should be considered an independent species within phylogroup II of the *Lyssavirus* genus, most similar to *Lagos bat virus* (LBV). Antigenic reaction patterns with anti-nucleocapsid monoclonal antibodies corroborated these distinctions. In addition, new data on the diversity of LBV suggests that this species may be subdivided quantitatively into three separate genotypes. However, the identity values alone are not considered sufficient criteria for demarcation of new species within LBV.
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1. Introduction

The *Lyssavirus* genus (family *Rhabdoviridae*) includes 11 recognized species. The type species, *Rabies virus* (RABV) is distributed worldwide among mammalian reservoirs—carnivores and bats. *Lagos bat virus* (LBV) circulates among pteropid bats in sub-Saharan Africa with infrequent spill-overs into other mammals (Markotter et al., 2008a). *Mokola virus* (MOKV) has been isolated in sub-Saharan Africa from shrews, domestic cats and dogs, a rodent and two humans (Nel et al., 2000; Sabeta et al., 2007). The reservoir hosts for MOKV have not yet been established. *Duvenhage virus* (DUVV) was isolated from insectivorous bats and humans who died after bat bites in sub-Saharan Africa (Markotter et al., 2008b; van Thiel et al., 2009). *European bat lyssavirus, type 1* (EBLV1) has been isolated from insectivorous bats across Europe, and the primary host species of this virus appears to be the Serotine bat (*Eptesicus serotinus*). Human cases of EBLV1 infection have been described as well (Fooks et al., 2003a; Kuzmin et al., 2006). *European bat lyssavirus, type 2* (EBLV2) was isolated primarily from insectivorous bats of the *Myotis* genus and from humans who died after bat bites in northwestern Europe (Fooks et al., 2003a,b). *Australian bat lyssavirus* (ABLV) circulates in Australia among insectivorous and pteropid bats, and has caused at least two documented cases of human rabies (Warrilow, 2005). Recently, four other lyssavirus species were ratified by the International Committee on Virus Taxonomy (ICTV Official Taxonomy: Updates since the 8th Report): *Aravan virus* (ARAV) and *Khujand virus* (KHUV), isolated from insectivorous bats of the *Myotis* genus in Central Asia (Kuzmin et al., 2003); *Irkut virus* (IRKV), isolated from an insectivorous bat, *Murina leucogaster* in eastern Siberia; and *West Caucasian bat virus* (WCBV), isolated from an insectivorous bat, *Miniopterus schreibersi* in southeastern Europe (Botvinkin et al., 2003). Seroprevalence to WCBV was also detected in *Miniopterus* spp. bats from Kenya (Kuzmin et al., 2008a), suggesting a wider geographical range than was previously believed.

Based on genetic distances, serologic cross-reactivity, and peripheral pathogenicity in a mouse model, the *Lyssavirus* genus was subdivided into two phylogroups. Phylogroup I includes RABV,

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DUVV, EBLV1, EBLV2, ABLV, ARAV, KHUV and IRKV. Phylogroup II includes LBV and MOKV. The WCBV cannot be included in any of these phylogroups, and we suggest it should be considered as a member of an independent phylogroup III (Badrane et al., 2001; Hanlon et al., 2005; Kuzmin et al., 2005).

The operational term 'genotype' has been used for lyssavirus classification since the time when molecular techniques replaced serotyping for classification purposes (Bourhy et al., 1993). Demarcation of genotypes has been based largely on genetic distances (identity values) between members of the genus, and on the bootstrap support of phylogenetic constructions (Bourhy et al., 1993; Delmas et al., 2008; Kissi et al., 1995; Kuzmin et al., 2005; Tordo et al., 1993). In addition, based on identity values, LBV was suggested to be subdivided into at least two separate genotypes (Delmas et al., 2008; Markotter et al., 2008a). However, the ICTV does not operate with viral genotypes but recognizes only viral species. Definition of a viral species is complex, and cannot be based solely on genetic distances, in the absence of other demarcation characteristics (Büchen-Osmond, 2003).

In the present paper we describe the isolation and characterization of a new, previously unrecognized lyssavirus, which should be considered a new species of the genus. We also provide additional data on LBV diversity, and discuss challenges of virus classification when different approaches are used. Furthermore, we enrich the data on the comparisons of complete lyssavirus genomes for phylogenetic purposes.

2. Materials and methods

2.1. Sample collection, screening and virus isolation

During 2009, a survey of bats was conducted in Kenya for the purpose of detecting new and potential human pathogens that could emerge from bat reservoirs within the region and infect humans (Kuzmin et al., 2008a,b). In total, 616 bats representing at least 22 species were collected from 10 locations across the country (Fig. 1), including 40 sick and dead bats. The sampling and collection protocol were approved by the National Museums of Kenya and the Kenya Wildlife Service. The brain and pooled organs were collected into sterile plastic tubes. Oral swabs were placed into tubes containing Minimum Essential Medium (MEM-10, Invitrogen, Grand Island, NY). Sera were separated from blood clots by centrifugation. All samples were transported on dry ice and stored at −80 °C until used.

Bat brains were subjected to the direct fluorescent antibody (DFA) test for detection of lyssavirus antigens (Dean et al., 1996) using monoclonal (Fujirebio Diagnostics Inc., Malvern, PA) and polyclonal (Chemicon Int., Temecula, CA) fluorescein isothiocyanate-labeled anti-rabies antibodies. When a positive DFA result was documented, the brain specimen was homogenized into 10% suspension in MEM-10 and inoculated intracranially into suckling mice (Koprowski, 1996) and mouse neuroblastoma (MNA) cell culture (Webster and Casey, 1996) for virus isolation. Afterwards, three additional passages were made in MNA cells to increase virus titer and supernatant of this culture was used for intramuscular inoculation of 3-week-old mice and Syrian hamsters to assess peripheral pathogenicity of the virus.

2.2. Sequencing of viral genome

The amount of the original positive bat brain tissue was limited. Therefore, only the N, P, M and G genes of the virus were determined from the original bat brain material, whereas the L gene and the genome termini were recovered after one mouse passage. Total RNA was extracted from brain homogenates using TRIZol (Invitrogen) and subjected to RT-PCR with subsequent direct sequencing on the ABI3730 automated sequencer (Applied Biosystems, Foster City, CA). Genome termini were circularized by RNA ligation, amplified by RT-PCR, cloned and sequenced as described previously (Kuzmin et al., 2008b). Each DNA strand of a given PCR product was sequenced at least twice. The sequence assembly, alignment and consensus sequence generation, as well as DNA translation into deduced amino acids were performed in BioEdit software (Hall, 1999).

2.3. Identity calculations and phylogenetic analysis

A set of representative lyssaviruses, used in the present study, is shown in Table 1. For the phylogroup II lyssaviruses, all available sequences were included, whereas for the phylogroup I lyssaviruses, a selection was made, to cover the intrinsic diversity of representatives of each species. Multiple alignments for each viral gene, and for the alignment of concatenated coding regions of the N, P, M, G and L genes (both nucleotide and deduced amino acid sequences), were produced using the ClustalX program (Jeanmougin et al., 1998). The identity values were calculated in BioEdit. Neighbor joining (NJ) phylogenetic analysis was performed in MEGA program (Kumar et al., 2001), using p-distances, Kimura-2 parameters and maximum composite likelihood models, for 1000 bootstrap replicates. Bayesian analysis (BI) was performed using BEAST software (Drummond and Rambaut, 2007), with the general time-reversible model incorporating both invariant sites and a gamma distribution (GTR + I + G). Two simultaneous analyses, each with four Markov chains, were run for 1,000,000 generations and sampled every 1000 generations. Trees generated prior to the stabilization of likelihood scores were discarded (burning = 250). The remaining trees were used to build a 50% majority rule consensus tree. Maximum likelihood (ML) analysis was performed using the PHYLIP package (Felsenstein, 1993) for 100 bootstrap replicates. Nucleotide substitution models used transition/transversion ratios varying from 2 to 4, with empirical base frequencies, and a gamma distribution of rate variations among sites. The ML model parameters for each alignment was determined using PAUP* (Swofford, 2003).

2.4. Monoclonal antibody typing

The newly isolated virus and representatives of all described lineages of LBV and MOKV were inoculated intracranially into suckling or 3-week-old mice. When clinical signs of rabies were observed, the mice were euthanized, and brain impressions were made on 4-well teflon-coated slides (Cel-Line, Erie Scientific, Portsmouth, NH). After overnight fixation in cold acetone, the samples were subjected to typing via the indirect fluorescent antibody test, using a panel of anti-nucleocapsid monoclonal antibodies (N-MAbs) of the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA), and N-MAb 422-5 of The Wistar Institute (Philadelphia, PA, USA), as described elsewhere (Smith, 1989).

3. Results

3.1. Detection and isolation of the virus

Screening of bat brains by the DFA test revealed one positive specimen, the brain of an adult female Commerson's leaf-nosed bat (*Hipposideros commersoni*), found dead in a cave in the south-coastal Kenya (Fig. 1). The carcass of the bat was partly decomposed and therefore no tissues besides the brain were collected. This cave, along with two neighboring caves, was inhabited by at least eight bat species, including *H. commersoni*, *Miniopterus minor*,