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Rebekah C. Kading
Centers for Disease Control and Prevention, fxk7@cdc.gov

Amy T. Gilbert
Centers for Disease Control and Prevention, Amy.T.Gilbert@aphis.usda.gov

Eric C. Mossel
Centers for Disease Control and Prevention

Mary B. Crabtree
Centers for Disease Control and Prevention

Ivan V. Kuzmin
Centers for Disease Control and Prevention

See next page for additional authors

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Authors

Rebekah C. Kading, Amy T. Gilbert, Eric C. Mossel, Mary B. Crabtree, Ivan V. Kuzmin, Michael Niezgoda, Bernard Agwanda, Wanda Markotter, M. Ryan Weil, Joel M. Montgomery, Charles E. Rupprecht, and Barry R. Miller

Short Communication

Isolation and molecular characterization of Fikirini rhabdovirus, a novel virus from a Kenyan bat

Rebekah C. Kading,[1] Amy T. Gilbert,[2]† Eric C. Mossel,[1] Mary B. Crabtree,[1] Ivan V. Kuzmin,[2]‡ Michael Niezgoda,[2] Bernard Agwanda,[3] Wanda Markotter,[4] M. Ryan Weil,[5,6] Joel M. Montgomery,[7] Charles E. Rupprecht[2]‡ and Barry R. Miller[1]

[1]Centers for Disease Control and Prevention, Division of Vector-borne Diseases, Arbovirus Diseases Branch, Fort Collins, CO, USA

[2]Centers for Disease Control and Prevention, Division of High Consequence Pathogens and Pathology, Poxvirus and Rabies Branch, Atlanta, GA, USA

[3]National Museums of Kenya, Nairobi, Kenya

[4]University of Pretoria, Department of Microbiology and Plant Pathology, Pretoria, South Africa

[5]SRA International, Atlanta, GA, USA

[6]Centers for Disease Control and Prevention, Office of Infectious Disease, National Center for Emerging and Zoonotic Infectious Diseases, Atlanta, GA, USA

[7]Centers for Disease Control and Prevention, Global Disease Detection Program, Nairobi, Kenya

Correspondence
Rebekah C. Kading
fxk7@cdc.gov

Zoonotic and vector-borne pathogens have comprised a significant component of emerging human infections in recent decades, and bats are increasingly recognized as reservoirs for many of these disease agents. To identify novel pathogens associated with bats, we screened tissues of bats collected in Kenya. Virus isolates were identified by next generation sequencing of viral nucleic acid preparations from the infected cell culture supernatant and characterized. Here we report the identification of Fikirini rhabdovirus, a novel rhabdovirus isolated from a bat, *Hipposideros vittatus*, captured along the Kenyan coast.

Since 1980, novel human pathogens have been discovered at a rate of more than three per year, and approximately 75 % of these have been viruses, with 80 % associated with non-human vertebrate reservoirs (Woolhouse & Gaunt, 2007). Bats have been recognized as reservoir hosts for highly virulent viruses, including lyssaviruses (Rupprecht *et al.*, 2011), henipaviruses (Halpin *et al.*, 1996; Chua *et al.*, 2000), severe acute respiratory syndrome (SARS)-related coronaviruses (Li *et al.*, 2005) and filoviruses (Leroy *et al.*, 2005, 2009; Towner *et al.*, 2007, 2009; Negredo *et al.*, 2011). Evolutionarily, bats have also been identified as major natural reservoirs of hepaciviruses and paramyxoviruses, two taxa which contain viruses responsible for prominent human diseases (Drexler *et al.*, 2012; Quan *et al.*, 2013). Together, these discoveries have made bats a focal target for virus discovery efforts.

Little is known about the public health burden, prevalence and distribution of African bat lyssaviruses (Markotter *et al.*, 2008). For example, in Kenya, van Thiel *et al.* (2009) reported a fatal human case of Duvenhage virus following exposure to a bat. In response to this knowledge gap, enhanced surveillance for lyssaviruses and other zoonotic pathogens among bats in Kenya has uncovered multiple viruses including the novel Shimoni bat virus (family *Rhabdoviridae*: genus *Lyssavirus*) from *Hipposideros vittatus* (formerly known as *H. commersoni*) (Kuzmin *et al.*, 2010a) and Lagos bat virus (*Rhabdoviridae*: *Lyssavirus*) from *Eidolon helvum* and *Rousettus aegyptiacus* (Kuzmin *et al.*, 2008a), as well as serological evidence for West Caucasian bat virus (*Rhabdoviridae*: *Lyssavirus*) in *Miniopterus* spp. (Kuzmin *et al.*, 2008b). In addition, viruses from numerous families have been isolated and/or detected from a diversity of bat species, such as Marburg virus (*Filoviridae*: *Marburgvirus*) from *R. aegyptiacus* (Kuzmin *et al.*, 2010b), a rotavirus (*Reoviridae*: *Rotavirus*) from *E. helvum*

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†Present address: United States Department of Agriculture, National Wildlife Research Center, Fort Collins, CO, USA.

‡Present address: Global Alliance for Rabies Control, Manhattan, KS, USA.

The GenBank/EMBL/DDBJ accession number for the genome sequence of Fikirini rhabdovirus is KC676792.

Two supplementary tables are available with the online version of this paper.

(Esona *et al.*, 2010), 41 coronaviruses (*Coronaviridae*: *Alphacoronavirus*, *Betacoronavirus*) from 14 genera of bats (Tong *et al.*, 2009; Tao *et al.* 2012), 11 distinct lineages of polyomaviruses (*Polyomaviridae*: *Polyomavirus*) (Tao *et al.* 2013) and a variety of hepaciviruses (Quan *et al.*, 2013). Historically, Mount Elgon bat virus (*Rhabdoviridae*: Oita group) was isolated from *Rhinolophus hildebrandtii eloquens* (Metselaar *et al.*, 1969). Given this information, the specific aim of this study was to screen tissues of bats captured in Kenya for the presence of additional novel pathogens.

Bats were captured throughout Kenya during August–September 2011 (Fig. 1). Bat capture and tissue sampling techniques were performed as described by Kuzmin *et al.* (2008a) under animal protocol 2096FRAMULX-A3, approved by the Institutional Animal Care and Use Committee of the US Centers for Disease Control and Prevention (Atlanta, GA, USA) and local authorities (permit KWS/5001). All bats were killed humanely to harvest tissues for virus isolations. In total, tissues were analysed from 447 bats, including 31 *Coleura afra*, 8 *E. helvum*, 78 *H. vittatus*, 153 *Miniopterus minor*, 80 *Miniopterus* spp., 2 *Nycteris* spp., 12 *Otomops martiensseni*, 9 *Rhinolophus landeri*, 7 *Rhinolophus* spp., 56 *R. aegyptiacus*, 1 *Taphozous* spp. and 12 *Triaenops afer* (formerly known as *T. persicus*). Liver and/or spleen specimens were homogenized for virus isolation.

Sections of tissue (approximately 0.5–1 cm^3) were mechanically homogenized in a 2.0 ml snap cap tube containing 1.5 ml BA1 medium (Hanks M-199 salts, 0.05M Tris pH 7.6, 1 % BSA, 0.35 g sodium bicarbonate l^{-1}, 100 U streptomycin ml^{-1}, 1 µg Fungizone ml^{-1} Fungizone) and one or two 5 mm stainless steel beads in a mixer mill (Qiagen) at 25 cycles s^{-1} for 4 min. Homogenates were clarified by centrifugation at approximately 12 800 g for 8 min at 4 °C and stored at −80 °C. One hundred microlitres of homogenized tissue supernatant was inoculated directly onto Vero cell monolayers, with one sample per well on a six-well plate for virus isolation by
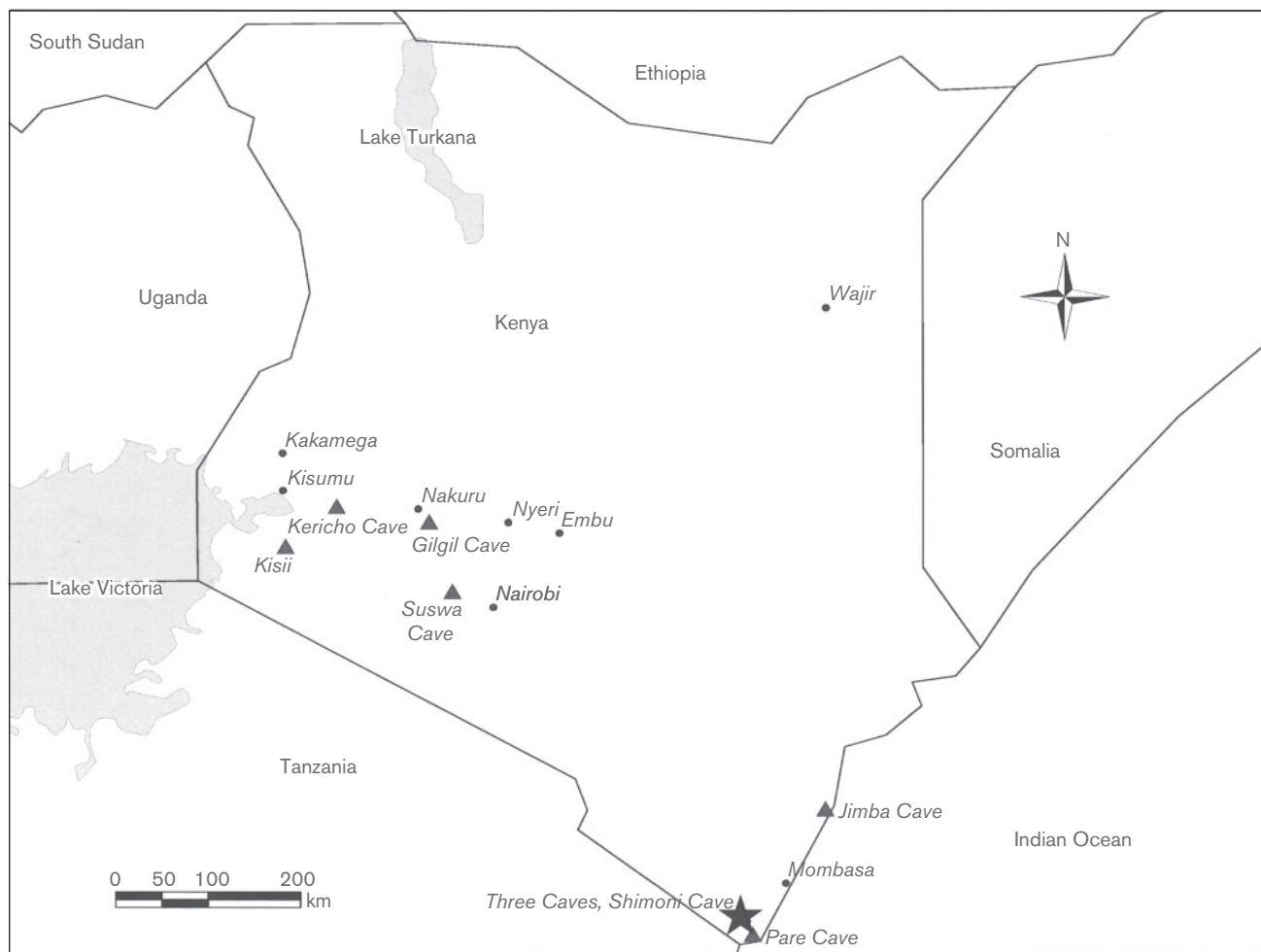
Fig. 1. Map of bat collection sites in Kenya, August–September 2011. The collection sites are shown with triangles and the site where Fikirini rhabdovirus was isolated is denoted by a star.

double-overlay plaque assay (Miller *et al.* 1989). A second overlay containing neutral red was added 4 days post-infection, and plates were observed for plaques up to 10 days post-infection. Plaque-positive wells were harvested into 1 ml Dulbecco's modified Eagle's medium + 10 % FBS and clarified by centrifugation and the infected supernatant was stored at −80 °C. The viral RNA from plaque-positive samples was extracted from 200 µl of the supernatant and eluted into a final volume of 140 µl AE buffer (composition not disclosed by Qiagen) using the Qiagen BioRobot EZ1 Workstation with the EZ1 Virus Mini kit v2.0.

Virus isolates were subjected to next generation sequencing. High-quality viral nucleic acid was prepared by digesting extracted RNA with DNase I (Invitrogen/Life Technologies) according to the manufacturer's instructions followed by purification using the Qiagen RNeasy mini kit. The cDNA was generated from 100 ng digested, purified viral RNA using the Ovation RNA-Seq System V2 kit (NuGEN Technologies). A cDNA library was then prepared from 400 ng purified cDNA using the Ion Xpress Plus gDNA and Amplicon Library Preparation kit (Ion Torrent, Life Technologies) according to the manufacturer's instructions and using an enzymic shear-time of 8 min. The amplified cDNA library was quantified on the 2100 Bioanalyser (Agilent Technologies) and diluted to a concentration of 280 million molecules ml^{-1} in deionized water. Template-positive Ion Sphere Particles were generated and enriched using the Ion OneTouch 200 Template kit v2 or the v2 DL kit (Ion Torrent, Life Technologies). Enriched Ion Sphere Particles were then sequenced on the Ion Personal Genome Machine using the Ion PGM Sequencing 200 kit or Ion PGM Sequencing 300 kit and an Ion 316 Chip. *De novo* sequence assemblies were performed using both the Lasergene Genomics Suite (DNA STAR) and CLC Genomics Workbench 5.1.1 (CLC Bio) software packages. Contigs were searched against the GenBank database to identify the closest sequence relatives, genomic location and orientation.

Using these methods, a novel rhabdovirus was identified from the liver of *H. vittatus*. This virus was named Fikirini rhabdovirus after the forests immediately surrounding the caves where the infected bat was captured (Fig. 1). The complete 11 139 nt genome sequence of the rhabdovirus (GenBank accession number KC676792) was confirmed via conventional reverse-transcription (RT-PCR) and direct sequencing using novel primers (given in Tables S1 and S2,

available in JGV Online). Sequences of the rhabdovirus genome termini were determined using the 5′/3′ RACE kit, 2nd generation (Roche) as per the manufacturer's instructions. Virus-specific primers used for RACE are available upon request. The genome of Fikirini rhabdovirus contained the expected rhabdovirus ORFs: nucleoprotein, phosphoprotein, matrix, glycoprotein and polymerase (Fig. 2). There were no additional accessory proteins or ORFs in alternate reading frames.

Maximum-likelihood phylogenetic trees were generated in MEGA 5.05 (Tamura *et al.*, 2011) for different fragments of rhabdovirus genomes available in GenBank. Among these, only N sequences were available for the viruses most closely related to Fikirini rhabdovirus. The analysis was performed using a complete deletion substitution model and 1000 bootstrap replicates (Fig. 3).

From the infected bat, additional sections of lung, kidney, brain and intestine, and oral and faecal swabs were processed for virus isolation as described above and screened for viral nucleic acids. The samples were screened by RT-PCR using primers designed from the nucleoprotein gene: KEN352-00046F ACGGTCTTTCGACTGGCTTC/KEN352-00729R AAGAGCCGAGCAATCCTTGA and polymerase gene: KEN352-10555F GTTGGGAGCTGGCTATTGGA/KEN352-11036R CCCACGGAGTTTGAGATCCT. All tested tissues and the bat faeces contained infectious rhabdovirus as well as viral RNA. Only the oral swab from this bat was negative for both infectious virus and viral RNA.

Worldwide, bats have been found associated with a diversity of rhabdoviruses (Calisher *et al.*, 2006; Kuzmin *et al.*, 2009; Aznar-Lopez *et al.* 2013). Many of these bat rhabdoviruses have been classified in the genus *Lyssavirus*, including Shimoni bat virus, which was also isolated from coastal Kenya from *H. vittatus* (Kuzmin *et al.*, 2010a). Based on the available N gene sequences, Mount Elgon bat virus, Kern Canyon virus and Oita virus constitute a distinct monophyletic clade (Kuzmin *et al.*, 2006) (Fig. 3). Viruses in this group appear to be associated with insectivorous bats throughout the world, with Oita virus found in *Rhinolophus cornutus* in Japan (Iwasaki *et al.*, 2004), Mount Elgon bat virus in *R. hildebrandtii eloquens* in Kenya (Metselaar *et al.*, 1969) and Kern Canyon virus in *Myotis yumanensis* in the USA (Murphy & Fields, 1967). Fikirini rhabdovirus also falls in this taxanomic group and is associated with an insectivorous bat host (Fig. 3).
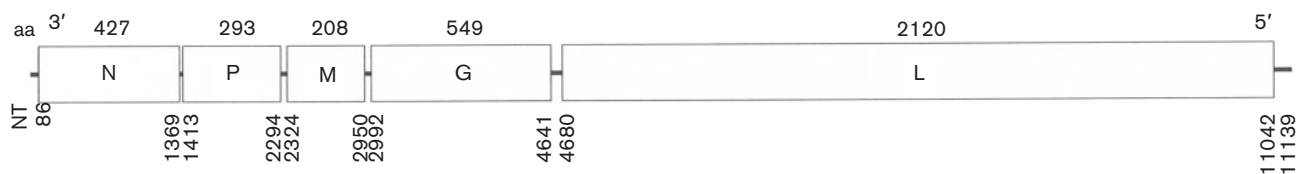
Fig. 2. Schematic diagram of the genome and ORFs of Fikirini rhabdovirus. N, Nucleoprotein; P, phosphoprotein; M, matrix; G, glycoprotein; L, polymerase.
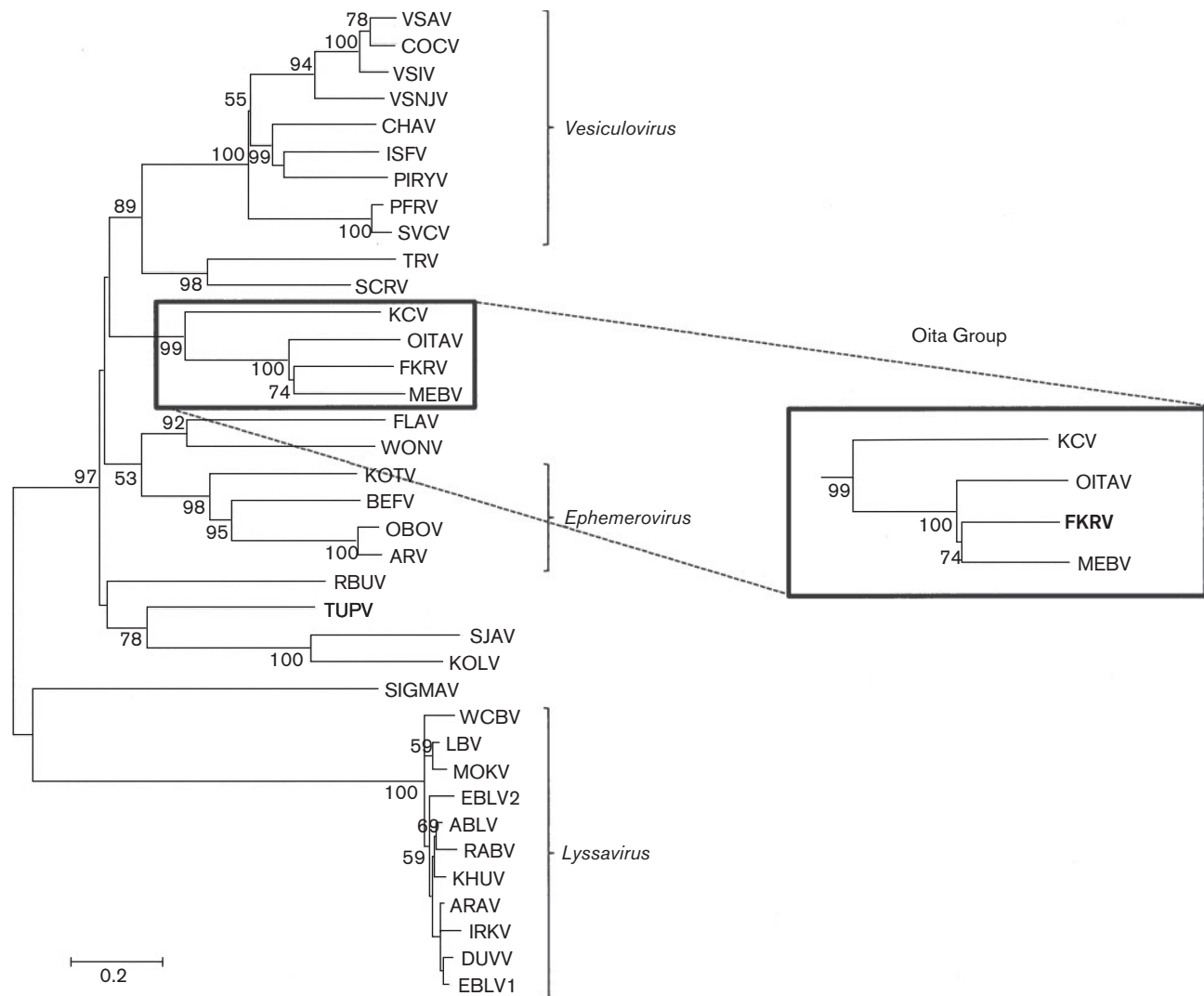
Fig. 3. Maximum-likelihood tree based on partial amino acid nucleoprotein gene sequences (complete gap deletion). The shortest nucleoprotein sequence used to construct the phylogeny was Mount Elgon bat virus which was 302 aa in length. Bar, 0.2 amino acid substitutions per site. Bootstrap values >50 % are shown (1000 replicates). Fikirini rhabdovirus (FKRV), Adelaide River virus (ARV) Q65111, Aravan virus (ARAV) Q6X1D8, Australian bat lyssavirus (ABLV) AAD01267, bovine ephemeral fever virus (BEFV) NP_065398, Chandipura virus (CHAV) P11211, Cocal virus (COCV) ACB47434, *Drosophila melanogaster* sigma virus (SIGMAV) ACV67011, Duvenhage virus (DUVV) Q66453, European bat lyssavirus 1 (EBLV1) AAX62875, European bat lyssavirus 2 (EBLV2) YP_001285393, Flanders virus (FLAV) AAN73283 Irkut virus (IRKV) Q5VKP6, Isfahan virus (ISFV) Q5K2K7, Kern Canyon virus (KCV) ABE69215, Khujand virus (KHUV) Q6X1D4, Kolongo virus (KOLV) ABE69214, Kotonkon virus (KOTV) ABE69213, Lagos bat virus (LBV) ABF56214, Mokola virus (MOKV) YP_142350, Mount Elgon bat virus (MEBV) ABE69217, Obodhiang virus (OBOV) ABE69212, Oita rhabdovirus (OITAV) BAD13431, pike fry rhabdovirus (PFRV) ACP27998, Piry virus (PIRYV) P26037, rabies virus (RABV) ACN51666, Rochambeau virus (RBUV) ABE69218, Sandjimba virus (SJAV) ABE69216, Siniperca chuatsi rhabdovirus (SCRV) YP_802937, spring viraemia of carp virus (SVCV) ABW24033, trout rhabdovirus 903/87 (TRV) AAL35756, Tupaia virus (TUPV) YP_238528, vesicular stomatitis Alagoas virus (VSAV) ACB47439, vesicular stomatitis Indiana virus (VSIV) P11212, vesicular stomatitis New Jersey virus (VSNJV) P04881, West Caucasian bat virus (WCBV) Q5VKP2, Wongabel virus (WONV) YP_002333271.

Overall, Fikirini rhabdovirus and Oita virus share 57 % amino acid identity and 71 % similarity across the nucleoprotein with long regions of extremely high identity between these two viruses (aa 272–348 of N show 91 % identity and 95 % similarity). Serologically, Nkolbisson,

Barur and Fukuoka viruses also fall within this group of rhabdoviruses (Calisher *et al.*, 1989), although sequence data supporting this classification are limited. Aznar-Lopez *et al.* (2013) recently published a rhabdovirus phylogeny based on a short fragment of the polymerase gene. In this

analysis, Nkolbisson, Barur and Fukuoka viruses also clustered in the same group with Kern Canyon, Oita and Mount Elgon bat viruses. However, more complete sequence data are needed to further resolve their phylogenetic relationships, and ascertain the exact placement of Fikirini rhabdovirus within this complex.

The finding of infectious virus and viral nucleic acid in multiple tissues of the bat provides further insight into host tropisms and potential mechanisms of transmission, although we did not confirm virus presence in specific tissues through histopathology. Infectious rhabdovirus particles and viral RNA were present in every tissue tested and in the bat faeces, but not the oral swab. This finding suggests that the main exit portal for this virus may be through faeces as opposed to saliva via a bite. The finding of lyssavirus nucleic acid in the faeces has been reported previously (Allendorf et al., 2012). However, the presence in saliva of lyssaviruses is intermittent and depends on the stage of the disease (WHO, 2013). Arthropod transmission of Fikirini rhabdovirus remains a possibility worthy of exploration. Nkolbisson virus was isolated from mosquitoes in Cameroon, Barur virus from a rat and ticks in India, mosquitoes in Kenya and ticks in Somalia, and Fukuoka virus from midges and cattle in Japan (Calisher et al., 1989; Noda et al., 1992). A Barur-like virus was also isolated from ticks in Kenya (Sang et al., 2006). While Nkolbisson virus has also been isolated from a human in the Central African Republic (Ndiaye et al., 1987), the broader public health significance of these rhabdoviruses, and the potential of Fikirini rhabdovirus to be transmitted by arthropods are unknown.

The extent of human exposure to Fikirini rhabdovirus is also unclear. H. vittatus is a cave-roosting bat. At present, the caves are visited by guano collectors, tourists and researchers, which could each serve as potential opportunities for exposure especially with regard to viral shedding in the faeces. Fikirini rhabdovirus grew to high titres and formed plaques in Vero cells. In vivo, Oita virus and Mount Elgon bat virus caused lethal encephalitis in mice (Murphy et al., 1970; Catalog of Arboviruses, 1970; Iwasaki et al., 2004), suggesting that Fikirini rhabdovirus is likely to be similarly pathogenic, although these studies have yet to be completed. Because this virus causes pathology in non-human primate cells and is closely related to viruses that cause morbidity and/or mortality in other vertebrates, further study is warranted to assess its zoonotic potential.

Acknowledgements

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