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

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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 hepaviruses and parvoviruses, 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.
(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 cm³) 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

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**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-overlap 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 RNaseasy 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 Bioanalyzer (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 11139 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 ACGGTCTTTGACTGCTTC/KEN352-00729R AA-GAGCCGAGCAATCCTGA and polymerase gene: KEN352-10555F GTTGGAAGCTGGCTATTGGA/KEN352-11036R CCAACGGATTTGAGATC. 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 taxonomic 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.
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

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) Q5K2K7, 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) ABE69214, 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, Rhabdophaga 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) P04681, West Caucasian bat virus (WCBV) Q5K2K2, Wongabel virus (WONV) YP_00233271.
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 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 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 likely to be similarly pathogenic, although these studies have yet to be completed. Because this virus causes pathology in nonhuman 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.

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