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NOTE

Virology

Detection of novel orthoreovirus genomes in shrew (*Crocidura hirta*) and fruit bat (*Rousettus aegyptiacus*)

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ABSTRACT. Orthoreoviruses have been indentified in several mammals, however, there is no information about orthoreoviruses in shrews. In this study, we screened wild animals in Zambia, including shrews, rodents, and bats for the detection of orthoreoviruses. Two orthoreovirus RNA genomes were detected from a shrew intestinal-contents (1/24) and a bat colon (1/96) sample by reverse-transcription (RT)-PCR targeting the RNA-dependent RNA polymerase gene of orthoreoviruses. Phylogenetic analyses revealed that each of the identified orthoreoviruses formed a distinct branch among members of the *Orthoreovirus* genus. This is the first report that shrews are susceptible to orthoreovirus infection. Our results suggest the existence of undiscovered orthoreoviruses in shrews and provide important information about the genetic diversity of orthoreoviruses.

KEY WORDS: *Crocidura hirta*, *Orthoreovirus*, *Rousettus aegyptiacus*, Zambia

Orthoreoviruses, belonging to the genus *Orthoreovirus* in the family *Reoviridae*, are non-enveloped, icosahedral, segmented double-stranded RNA (dsRNA) viruses [8]. The orthoreovirus genome consists of 10 dsRNA segments, three large segments (L1–L3), three medium segments (M1–M3), and four small segments (S1–S4). Orthoreoviruses have been identified in vertebrates and invertebrates, describing the prevalence of orthoreoviruses in a wide range of hosts, and the *Orthoreovirus* genus is composed of seven species: *Avian orthoreovirus* (ARV), *Baboon orthoreovirus* (BRV), *Mahlapitsi orthoreovirus* (MAHLV), *Mammalian orthoreovirus* (MRV), *Nelson Bay orthoreovirus* (NBV), *Piscine orthoreovirus* (PRV), and *Reptilian orthoreovirus* (RRV) [3, 8]. The genus *Orthoreovirus* is divided into two distinct phylogenetic subgroups: the fusogenic orthoreoviruses such as NBV, ARV, BRV, MAHLV and RRV; and the nonfusogenic orthoreoviruses such as MRV and PRV [3, 6, 8]. MRV, a prototype of the genus *Orthoreovirus*, is nonfusogenic and has been identified in several mammals, including humans, bats, rodents, dogs, civet cats, cattle, pigs, minks and alpine chamois [1, 2, 4, 7, 17–20, 26]. In contrast, NBV, a bat-origin orthoreovirus, is a prototype

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of fusogenic orthoreovirus, inducing the formation of multinucleated syncytia in cell culture, and have also been isolated from humans with respiratory illness [5, 10, 27, 28, 35]. BRV isolated from baboons genetically differed from MRV and NBV, and assigned as the new species within the genus *Orthoreovirus* [9]. Broome virus, a recently discovered orthoreovirus from a bat, is divergent from any other species, and represents a new although it is not yet formally recognized as the species [29]. These reports have expanded our knowledge about the host range and genetic diversity of orthoreoviruses.

Shrews are small mammals with long, narrow, and pointed snouts, and insectivore, feeding on many invertebrates on the ground. The family *Soricidae* (shrews), belonging to order *Soricomorpha*, consists of three subfamilies: *Crocidurinae* (white-toothed shrews), *Soricinae* (red-toothed shrews) and *Myosoricinae* (African white-toothed shrews) [34]. Shrews are similar in size and appearance to rodents classified to order *Rodentia*, but there is a clear genetic and biological difference between these animals [11, 13]. Althougt tree shrews classified within order *Scandentia* are similar name to shrews, they are bigger than shrews and the animals inhabit forests on the tree [34]. Phylogenetic analyses based on mitochondrial cytochrome b gene sequences have shown a clear genetic difference between shrews, rodents and bats, and these animals are phylogenetically distinct from each other [11]. To date, various viruses identified in shrews, such as adenoviruses, arteriviruses, bufaviruses, hantaviruses, hepadnaviruses, herpesviruses and nairoviruses, are phylogenetically distinct from those in rodents and bats, indicating the unique shrew virome [11, 13, 21, 24, 31, 32, 36, 37]; however, there has been no report about the presence of orthoreoviruses in shrews.

Discovery of novel orthoreovirus in mammals, such as bats and baboons suggests that there may be a unrecognized orthoreoviruses circulating in a wide range of animal hosts [9, 29]. As part of the activities aimed at identifying potential pathogens in Zambian wildlife, we screened shrews, rodents and bats for the detection of orthoreoviruses and performed phylogenetic analysis of detected viruses.

With permission from Zambia Wildlife Authority, now the Department of National Parks and Wildlife (Act No. 12 of 1998), Ministry of Tourism and Arts [23–25], we captured 24 shrews and 48 rodents around houses and fields using sherman traps and cage traps in Mpulungu in 2012, and 96 bats captured using a harp trap or a shotgun in Lusaka, Ndola, Monze, Livingstone, and Kasanka National Park in Zambia during the period 2014–2015 (Supplementary Fig. 1). We collected intestinal contents from the shrews and the rodents, and colon samples from the bats . We used intestinal contents and colon samples for screening of the orthoreovirus genome, because some orthoreoviruses were isolated from intestine, feces, intestinal contents and rectal swab in various animals [1, 7, 12, 14, 16–20, 22, 26, 27]. These samples were kept at −80°C, and we isolated RNA from them to screen for the orthoreovirus genome (Table 1). In our previous studies, bat species were identified by morphology and sequencing of ribosomal RNA and the mitochondrial cytochrome b gene; sequencing of the latter was also used to identify rodent and shrew species [23, 25].

Table 1. Information of samples and reverse-transcription (RT)-PCR results

Animal species	No. of positive/ No. tested sample	Sampling place	Sampling year
Shrew			
Crocidura hirta	1/23	Mpulungu	2012
Crocidura luna	0/1		
Total	1/24		
Bat			
Eidolon helvum	0/9	Kasanka NP	2014
Eidolon helvum	0/9	Ndola	
Epomophorus crypturus	0/20	Monze	
Rousettus aegyptiacus	1/9	Lusaka	
Hipposideros sp.	0/10	Lusaka	
Miniopterus schreibersii	0/9	Lusaka	
Nycteris sp.	0/20	Livingstone	
Rousettus aegyptiacus	0/10	Lusaka	2015
Total	1/96		
Rodent			
Aethomys chrysophilus	0/6	Mpulungu	2012
Cricetomys gambianus	0/3		
Gerbilliscus leucogaster	0/1		
Grammomys sp.	0/1		
Mastomys natalensis	0/28		
Paraxerus cepapi	0/2		
Rattus rattus	0/3		
Saccostomus sp.	0/3		
Steatomys sp.	0/1		
Total	0/48		

Intesitinal-contents suspensions in phosphate-buffered saline [10% (w/v)] and colon homogenates in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 4% antibiotic–antimycotic solution (Life Technologies, Gibco, Waltham, MA, USA) [10% (w/v)] were prepared and briefly centrifuged. Total RNA was extracted from the supernatants of intesitinal-content suspensions and colon homogenates using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) and QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions, respectively. Nested reverse-transcription (RT)-PCR was performed using degenerate primers designed from a conserved sequence within the RNA-dependent RNA polymerase gene (*RdRp*) as previously described [33]. Briefly, viral gene fragments were amplified with PrimeScript One Step RT-PCR Kit Ver.2 (Takara Bio Inc., Kusatsu, Japan) using the forward primer 1607F (5′-CARMGNCGNSCHMGHTCHATHATGCC-3′) and the reverse primer 2608R (5′-TAVAYRAAVGWCCASMHNGGRTAYTG-3′) for first-round RT-PCR according to the manufacturers' protocol. Nested PCR was performed with Takara Ex Taq hot start version (Takara Bio Inc.) using the forward primer 2090F (5′-GGBTCMACNGCYACYTCBACYGAGCA-3′) and the reverse primer 2334R (5′-CDATGTCRTAHWYCCANCCRAA- 3′) according to the manufacturers' protocol. NBV RNA [35] was used as a positive control for the RT-PCR assay. PCR products of 226 bp were purified from an agarose gel and subjected to direct sequencing using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

For virus isolation, shrew intestinal-content suspensions and bat colon homogenates were prepared from orthoreovirus nested RT-PCR-positive samples and inoculated onto monolayers of African green monkey kidney cells (Vero E6) and baby hamster kidney (BHK) cells previously described for the isolation of orthoreoviruses [9, 17–19, 27, 29, 35].

Among 24 shrew intestinal-contents and 96 bat colon samples, one from a shrew (*Crocidura hirta*) and one specimen from a fruit bat (*Rousettus aegyptiacus*) were positive for orthoreovirus by nested RT-PCR assay (Table 1). We next attempted to amplify a different region of *RdRp* using semi-nested PCR with the first RT-PCR product as a template, and using degenerate primers and specific primers designed from the obtained sequence. A longer region of *RdRp* was amplified by semi-nested PCR from Zambian shrew orthoreovirus using the degenerate forward primer 1607F and the specific reverse primer 2140R (5′-GAAGAGCATTGTCTAGATGG-3′), and using the specific forward primer 2125F (5′-TACTATGATGCAATGCTTCC-3′) and the degenerate reverse primer 2608R with KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan). Finally, a 1,000 bp length fragment of orthoreoviral *RdRp* was obtained from the shrew specimen. However, despite several attempts, we failed to amplify a longer *RdRp* fragment from the bat specimen. The two partial sequences of *RdRp* obtained from the bat colon and the shrew intesitinal-contents were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers (DDBJ/EMBL/GenBank LC486243 and LC486244, respectively).

BLAST analyses indicated that the identified *RdRp* sequence of the Zambian shrew orthoreoviruses showed the highest similarity to *RdRp* of NBV. Pairwise comparison of the partial *RdRp* sequence from the shrew specimen showed 69.6% nucleotide sequence identity to that of NBV Miyazaki-Bali/2007 (GenBank accession no. AB908279), which was identified from humans returning to Japan from Bali, Indonesia [35]. The highest amino acid identity was 78.4% to ARV Pycno-1 (GenBank accession no. BAQ19494), which was identified from *Hypsipetes amaurotis* in Japan [22]. Phylogenetic analyses of the partial *RdRp* nucleotide sequences and amino acid sequences predicted based on obtained nucleotide sequences were conducted with representative orthoreovirus sequences available in GenBank (Supplementary Table 1). The sequences were aligned using the ClustalW protocol [30], and phylogenetic trees were generated using the maximum likelihood method based on the Tamura-Nei and JTT matrix-based model with 1,000 bootstrap replications by MEGA7 software [15]. In the phylogenetic tree based on 1,000 bp sequences from orthoreovirus *RdRp*, the Zambian shrew orthoreovirus segregated from other orthoreoviruses (Fig. 1). The Zambian shrew orthoreovirus shared a common origin with NBV and was located independently from any known orthoreoviruses. According to the phylogenetic analysis based on predicted 333 amino acid sequence of orthoreovirus *RdRp*, the Zambian shrew orthoreovirus formed an independent branch from the ancestor of NBV and ARV (Supplementary Fig. 2). To date, no information about orthoreoviruses in the shrew is available. Our results suggest that shrews are susceptible to orthoreovirus infection.

The identified *RdRp* sequences of the Zambian bat orthoreoviruses was related to that of RRV, and the highest identity is 64.0% to RRV 112-99 (GenBank accession no. EU309698), which was identified from a *Boa constrictor* in Germany [33]. The amino acid sequence of the Zambian bat orthoreovirus showed 60.0% to those of RRV CH1197/96 (GenBank accession no. ABY28252), which was identified from a *Testudo graeca* in Switzerland [33]. To infer phylogenetic relationship with the Zambian bat orthoreovirus, we constructed a phylogenetic tree based on 226 bp and predicted 75 amino acid sequences of orthoreovirus *RdRp*. The Zambian bat orthoreovirus also formed a distinct branch among members of the *Orthoreovirus* genus, and the Zambian shrew orthoreovirus showed the similar topology as shown in Fig. 1 (Fig. 2 and Supplementary Fig. 3). Although the Zambian bat orthoreovirus was phylogenetically located near the clade of RRV or Broome virus isolated from bats in Australia, the nucleotide and amino acid identities were less than 64.0% and 60.0% to any other known orthoreoviruses as described above, respectively. These results suggest that the Zambian bat orthoreovirus is genetically distict from other orthoreoviruses previously identified in bats. Throughout the experiment for virus isolation, no cytopathic effect was observed and no orthoreovirus genome was detected in the culture supernatants by nested RT-PCR.

In this study, we detected two unique orthoreoviruses from a shrew and a bat in Zambia. Orthoreoviruses have been reported in various mammalian hosts, except for shrews [1, 2, 4, 5, 7, 9, 10, 17–20, 26–29, 35]. Although shrews are similar in size and appearance to rodents, they are biologically genetically different from rodents and classified to other order [34]. This is the first report for the detection of orthoreovirus genome in intesitinal contents of shrews captured in Zambia. In previous studies, clear phylogenetic divisions between various viruses from shrews, rodents, and bats were obsearved [11, 13, 21, 24, 31, 32, 36, 37]. The Zambian shrew orthoreovirus was also phylogenetically distinct from any other orthoreoviruses identified in rodents and

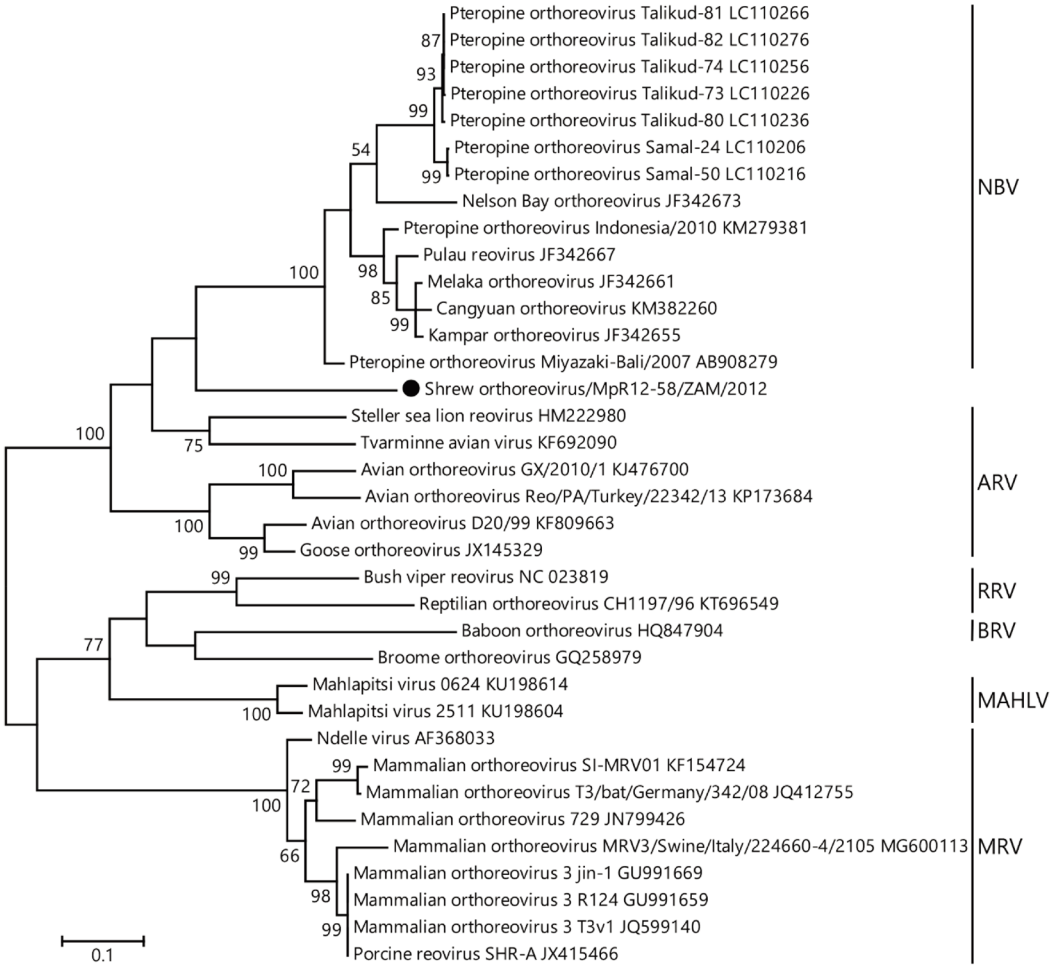

Fig. 1. A phylogenetic tree based on 1,000 bp sequences from orthoreovirus *RdRp* corresponding to position 1601–2606 of the NBV L2 genome was constructed using the maximum likelihood method with 1,000 bootstrap replications. Bootstrap values greater than 50% based on 1,000 replications are shown on the interior nodes, and species names are indicated on the tree. Bar, 0.1 substitutions per site. A black circle represents the Zambian shrew orthoreovirus (GenBank accession no. LC486244) detected in this study. Species abbreviations of the genus *orthoreovirus* are as follows: ARV, *Avian orthoreovirus*; BRV, *Baboon orthoreovirus*; MAHLV, *Mahlapitsi orthoreovirus*; MRV, *Mammalian orthoreovirus*; NBV, *Nelson Bay orthoreovirus*; RRV, *Reptilian orthoreovirus*.

bats (Fig. 1). Thus, there may be some differences in the genetic diversity of orthoreoviruses between shrews and other mammal. Bat-derived orthoreoviruses discovered in Australia, Asia and Europe belong to the clade of either NBV or MRV except Broome virus [5, 10, 20, 27–29, 35]. However, the Zambian bat orthoreovirus was located outside of the clades of NBVs and MRVs and formed a distinct branch among the members of *Orthoreovirus*. Our results suggest that orthoreoviruses in bats are genetically devided to at least four groups, showing the genetic diversity of bat orthoreoviruses. According to the species demarcation criteria in the genus *Orthoreovirus* of the International Committee on Taxonomy of Viruses [3], these two orthoreoviruses do not belong to any known orthoreovirus species, and we suppose that they may be novel orthoreovirus species. However, we need to obtained more information about their genomes. Additionally, phylogenetic analyses revealed that the Zambian shrew orthoreovirus shared a common origin with NBV, which was a causative agent of repiratory illness in humans (Fig. 1). Further epidemiological studies in humans and animals are required to clarify the distribution and risk of orthoreovirus infection in Zambia.

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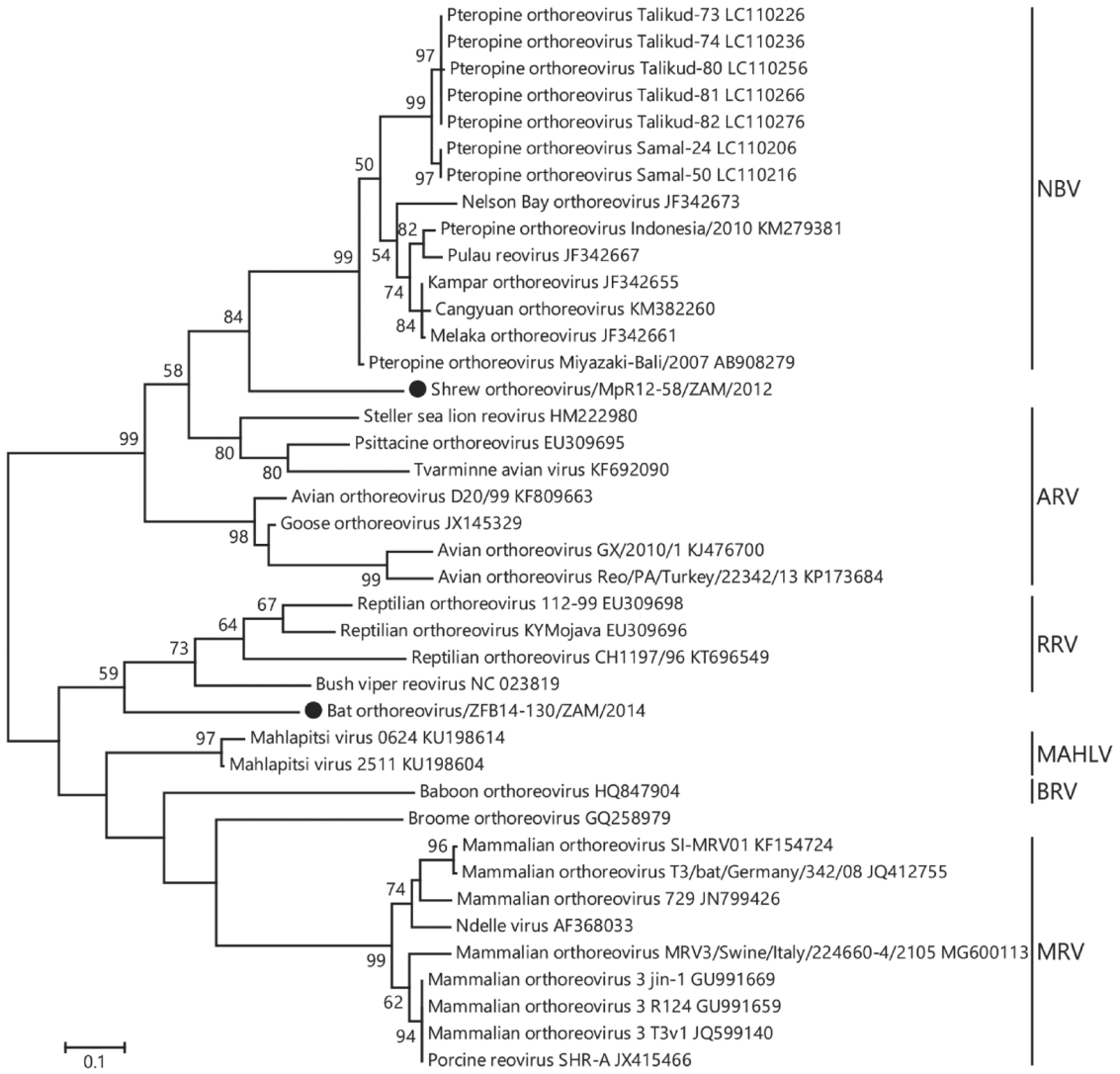

Fig. 2. A phylogenetic analysis based on 226 bp from the orthoreovirus *RdRp* corresponding to position 2084–2309 of NBV L2 genome was performed using the maximum likelihood method with 1,000 bootstrap replications. Bootstrap values greater than 50% based on 1,000 replications are shown on the interior nodes, and the species names are indicated on the tree. Bar, 0.1 substitutions per site. Black circles represent the Zambian shrew orthoreovirus (GenBank accession no. LC486244) and the Zambian bat orthoreovirus (GenBank accession no. LC486243) detected in this study. Species abbreviations of the genus *orthoreovirus* are as follows: ARV, *Avian orthoreovirus*; BRV, *Baboon orthoreovirus*; MAHLV, *Mahlapitsi orthoreovirus*; MRV, *Mammalian orthoreovirus*; NBV, *Nelson Bay orthoreovirus*; RRV, *Reptilian orthoreovirus*.

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