Molecular epidemiology of paramyxoviruses in Zambian wild rodents and shrews

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Summary

Rodents and shrews are known to harbour various viruses. Paramyxoviruses have been isolated from Asian and Australian rodents, but little is known about them in African rodents. Recently, previously unknown paramyxovirus sequences were found in South African rodents. To date, there have been no reports related to the presence and prevalence of paramyxoviruses in shrews. We found a high prevalence of paramyxoviruses in wild rodents and shrews from Zambia. Semi-nested RT-PCR assays were used to detect paramyxovirus RNA in 21% (96/462) of specimens analyzed. Phylogenetic analysis revealed that these viruses were novel paramyxoviruses and could be classified as Morbillivirus- and Henipavirus-related viruses, and previously identified rodent paramyxovirus-related viruses. Our findings suggest the circulation of previously unknown paramyxoviruses in African rodents and shrews, and provide new information regarding the geographical distribution and genetic diversity of paramyxoviruses.
Main Text

Paramyxoviruses are well-known infectious agents of humans and animals. The Paramyxoviridae family contain non-segmented negative-strand RNA viruses, and can be divided into the subfamilies Paramyxovirinae and Pneumovirinae. The Paramyxovirinae subfamily contains the genera Avularivirus, Rubulavirus, Respirovirus, Henipavirus, and Morbillivirus, along with some unclassified members. Rodents are classified in the order Rodentia and are the most diverse and abundant mammals worldwide. They harbour a wide range of viruses and can be reservoirs of zoonotic viruses such as Hantavirus, Lassa virus and tick-borne encephalitis virus (Meerburg et al., 2009). At least seven paramyxoviruses (Sendai virus, Nariva virus, Mossman virus, J-virus, Beilong virus, Tailam virus and Murine pneumonia virus) are thought to have originated in rodents.

Sendai virus, a member of the genus Respirovirus, is an etiological agent of pneumonia in rodent species and is distributed worldwide (Faisca & Desmecht, 2007). Murine pneumonia virus is classified within the Pneumovirus genus, in the Pneumovirinae subfamily. It was isolated from the lung tissues of laboratory mice (Dyer et al., 2012). Nariva virus was isolated from Zygodontomys b. brevicauda in Trinidad. In Australia, Mossman virus was isolated from Rattus leucopus and Rattus fuscipes, and J-virus was found in Mus musculus (Jack et al., 2005; Jun et al., 1977; Lambeth et al., 2009; Miller et al., 2003). Beilong virus was initially isolated from human kidney mesangial cell line in the laboratory, but the origin of Beilong virus was expected to be rat kidney mesangial cell line (Li et al., 2006). Beilong virus variants have since been found in Rattus norvegicus and Rattus rattus from China (Woo et al., 2012). These findings would indicate that the natural host of Beilong virus is a rodent. The complete genome sequence of another paramyxovirus identified in
Rattus andamanensis from China was designated Tailam virus (Woo et al., 2011). With the exception of Sendai virus and Murine pneumonia virus, these aforementioned paramyxoviruses are phylogenetically distinct from other mammalian paramyxoviruses and remain unclassified at the genus level.

Shrews are small mole-like mammals in the family Soricidae, order Soricomorpha. Although they are similar in size and appearance to rodents, phylogenetic analyses based on mitochondrial cytochrome b gene sequences has shown a clear genetic difference between shrews and rodents (Guo et al., 2013; Kang et al., 2011). To date, there have been no reports related to the presence and prevalence of paramyxoviruses in shrews. A single paramyxovirus has been isolated from a treeshrew, however the animal is classified within the different order, Scandentia (Tidona et al., 1999).

The application of RT-PCR assays, using degenerate primers targeting the consensus region of the paramyxovirus L gene, has revealed previously unrecognized paramyxoviruses, particularly in bats (Baker et al., 2012; Drexler et al., 2012; Kurth et al., 2012; Lau et al., 2010; Sasaki et al., 2012; Weiss et al., 2012; Wilkinson et al., 2012). However, paramyxovirus diversity in rodents and shrews remains poorly understood. In this study we aimed to investigate the presence and prevalence of paramyxoviruses in rodents and shrews from Zambia.

Our study was conducted with permission from the Zambia Wildlife Authority. We analyzed 431 wild rodents and 31 wild shrews collected across four locations in Zambia: 256 rodents and 5 shrews from Lusaka (Eastern area: 15°26'6.12"S, 28°26'9.93"E; Northern
area: 14°58'6.12"S, 28°14'8.33"E; and Southern area: 15°34'6.88"S, 28°16'5.13"E); 122 rodents and 2 shrews from Livingstone (17°50'8.72"S, 25°43'59.19"E); 48 rodents and 24 shrews from Mpuungu (08°45'5.45"S, 31°06'8.43"E); and 5 rodents from Kasanka (15°34'6.88"S, 28°16'5.13"E). Rodents and shrews were captured around houses and fields using Sherman traps and cage traps from 2010–2012. The captured animals were euthanized with diethyl ether and their kidneys removed. Rodent and shrew species were verified using nucleotide sequence analysis of the mitochondrial cytochrome $b$ gene, as described previously (Ishii et al., 2012). Around 61% of the animals analyzed were *Mastomys natalensis*, referred to as Natal multimammate mice. All captured shrews were members of the genus *Crocidura*. At least 19 species of rodent and 3 species of shrew were included in this study (Table 1).

Total RNA was extracted from the kidneys of all rodents and shrews using TRIzol reagent and a PureLink RNA Mini Kit (Life Technologies, Invitrogen). Kidney has high prevalence rate and high viral load of paramyxoviruses in rodents, therefore it is considered to be the relevant organ to detect rodent paramyxoviruses (Drexler et al., 2012). We screened 462 RNA samples by semi-nested RT-PCR as described previously (Sasaki et al., 2012). We used degenerate primers (PAR-F, PAR-F2 and PAR-R) that were specific for the $L$ gene in *Paramyxovirinae* subfamily members (Tong et al., 2008). Amplicons were electrophoresed on 1.6% (w/v) agarose gels and purified with a QIAquick Gel Extraction Kit (Qiagen). Sequences were determined by direct cycle sequence analysis in both directions using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Applied Biosystems). All obtained sequences were subjected to BLAST analysis.
Based on our RT-PCR results, approximately 21% (96/462) of the RNA samples were positive for presence of the L gene. Among the 96 positive samples, 84 were from rodents and 12 were from shrews. Amino acid sequence similarities to Paramyxovirinae members ranged from 67–90%, with no previously known paramyxoviruses identified in this study. All sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB844333–AB844429 (Supplementary Table S1).

Phylogenetic analysis of the deduced amino acid sequences for the L gene fragments amplified in this study was conducted. We generated a phylogenetic tree using MEGA5 software and the maximum likelihood method with complete deletion option and the WAG+G+I substitution model (Tamura et al., 2011). LR, LivR, MpR and KasR represent rodents or shrews from Lusaka, Livingstone, Mpulungu and Kasanka, respectively. The tree highlights the diversity of paramyxoviruses circulating in rodents and shrews from Zambia (Figure 1). All detected viruses could be grouped into four clades. Paramyxoviruses from 1 rodent and 11 shrews branched from the lineage leading to the genus Henipavirus to form genotype 1. Tailam virus-related and J virus-related paramyxoviruses were detected in 30 rodents that formed genotype 2, while Mossman virus-related paramyxoviruses were detected in 47 rodents to form genotype 3 (Figure 1). Genotype 4 comprised paramyxoviruses from 6 rodents and 1 shrew, and a member of the Morbillivirus genus. In genotype 3, the virus sequences obtained from multiple Mastomys natalensis captured across different geographical locations were almost identical (99%). Our results suggest that novel paramyxoviruses are endemic in Zambian rodents and shrews.

We amplified a different region of the L gene by semi-nested RT-PCR using degenerate
primers (RES-MOR-HEN-F1, RES-MOR-HEN-F2 and RES-MOR-HEN-R). Based on the sequence of each obtained fragment, virus-specific primers were designed and used to amplify a longer L gene fragment between regions targeted by the RES-MOR-HEN and PAR primers. Conventional two-step RT-PCRs were performed with Superscript III reverse transcriptase (Life Technologies, Invitrogen) and PrimeSTAR GXL DNA polymerase (Takara Bio), according to the manufacturer’s instructions. We obtained 31 L gene fragments, ranging 1586–1793 bp, from 22 rodents and 9 shrews. Phylogenetic analyses were conducted using representative nucleotide sequences with the maximum likelihood method and the GTR+G+I substitution model (Figure 2). In parallel with the maximum likelihood method, we also applied a Bayesian method to construct phylogenetic trees using MrBayes software, version 3.2.2 (Ronquist et al., 2012) (Supplementary Figure S1). Both maximum likelihood and Bayesian trees resulted in the same topologies. The four clades shown in Figure 1 also appeared in these trees, supporting the phylogenetic relationships in Figure 1 and providing a deeper understanding of rodent and shrew paramyxovirus phylogeny.

Virus isolation was conducted using Vero and BHK cells, which are used for propagation of known rodent paramyxoviruses (Jack et al., 2005; Lambeth et al., 2009; Li et al., 2006; Miller et al., 2003). Tissue homogenates [10% (w/v)] in Eagle’s minimum essential medium (MEM) were prepared from 18 kidney tissues that were positive for paramyxovirus RNA, and used to infect Vero and BHK cells. Cells were maintained in MEM containing 2% fetal bovine serum (FBS) and 2% antibiotic-antimycotic solution (Life Technologies, Gibco). Supernatants were passaged onto fresh cells every 7 days; no cytopathic effect was observed for 25 days post-inoculation. Paramyxovirus RNA was not
detected in culture supernatants when we used semi-nested RT-PCR assays. Consequently, no paramyxoviruses were isolated from any of the tested tissues.

In this study, we detected various paramyxoviruses from different species of rodents in Zambia. These viruses were related to the members of the *Morbillivirus* genus and unclassified rodent paramyxoviruses (Mossman virus, Tailam virus and J-virus). Over the last 50 years, six paramyxoviruses have been identified in rodents from Asian countries and Australia (Faisca & Desmecht, 2007; Jun *et al.*, 1977; Lambeth *et al.*, 2009; Li *et al.*, 2006; Miller *et al.*, 2003; Woo *et al.*, 2011). Drexler *et al.* detected paramyxoviruses that were phylogenetically related to Morbilliviruses and Beilong virus in *Rhabdomys pumilio* from South Africa using the different primer sets (Drexler *et al.*, 2012). These results give us an indication of the distribution and genetic diversity of rodent paramyxoviruses in Africa.

Genome sequence analyses of rodent paramyxoviruses have revealed that they are not phylogenetically grouped into any established genera (Jack *et al.*, 2005; Lambeth *et al.*, 2009; Li *et al.*, 2006; Miller *et al.*, 2003; Woo *et al.*, 2011). Our phylogenetic analysis showed that many of paramyxoviruses detected from rodents in Zambia were distinct from any established genera and related to previously identified rodent paramyxoviruses. These paramyxoviruses could be classified into a new genus, or genera, of the *Paramyxovirinae* subfamily.

We also identified unique paramyxovirus sequences from wild shrews. This is the first report describing the prevalence of paramyxoviruses in shrews. The prevalence of henipavirus-related paramyxoviruses in lesser red musk shrews (*Crocidura hirta*) was
found to be high in this study. Shrew paramyxoviruses appear to be distinct from previously
identified rodent paramyxoviruses, suggesting the presence of novel unique species within
the subfamily Paramyxovirinae. It is possible there are some differences in the genetic
diversity of paramyxoviruses among rodents and shrews. Results from previous studies
have described a clear phylogenetic division between hantaviruses from rodents and shrews
(Guo et al., 2013; Kang et al., 2011).

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Japan. This work was also supported by Japan Society for the Promotion of Science (JSPS)
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Figure Legend

Figure 1. Diversity of paramyxoviruses detected in wild rodents and shrews from Zambia.

A phylogenetic tree was generated based on a 176 amino acid sequence from a conserved region of the paramyxovirus L gene corresponding to positions 13910-14439 in Nipah virus genome (GenBank/EMBL/DDBJ accession number, NC_002728). We detected 84 rodent paramyxoviruses (RodentPV) and 12 shrew paramyxoviruses (ShrewPV). These were indicated by grey shading and analyzed alongside known paramyxoviruses. Genotype 1, 2, 3, and 4 represent Henipavirus-, Tailam virus-, Mossman virus- and Morbillivirus-related paramyxoviruses, respectively. Previously identified rodent paramyxoviruses are marked with an asterisk. Bootstrap values calculated from 1000 replicates are indicated at each tree root. The horizontal scale bar represents a distance of 0.1 substitutions per site. Viruses and their respective accession numbers are listed in Table S1. Species abbreviations are as follows: *Acomys subspinosus* (Aco sub), *Aethomys chrysophilus* (Aet chr), *Arvicanthis* sp. (Arv sp.), *Gerbilliscus leucogaster* (Ger leu), *Grammomys* sp (Gra sp.), *Hylomyscus* sp. (Hyl sp.), *Mastomys natalensis* (Mas nat), *Mus minutoides* (Mus min), *Rattus rattus* (Rat rat), *Saccostomus* sp. (Sac sp.), *Steatomys* sp. (Ste sp.), *Crocidura hirta* (Cro hir), *Crocidura* sp. (Cro sp.).

Figure 2. Extended phylogenetic analysis of a portion of the L gene from paramyxoviruses.

Larger L gene fragments (1586–1793 bp) were detected in rodent and shrew specimens, corresponding to nucleotides 12629–14439 in the Nipah virus genome (NC_002728). A phylogenetic tree was generated using representative nucleotide sequences with the maximum likelihood method and the GTR+I+G substitution model. Paramyxoviruses detected in this study are indicated by shading (grey). Genotype 1, 2, 3, and 4 represent...
Henipavirus-, Tailand virus-, Mossman virus- and Morbillivirus-related paramyxoviruses, respectively. Bootstrap values calculated from 1000 replicates are indicated at each tree root. The scale bar represents a distance of 0.5 substitutions per site. Viruses and their respective accession numbers are listed in Table S1.
## Table 1. RT-PCR screening results

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<th>Lusaka</th>
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<th>Kasanka</th>
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*Number of RT-PCR-positive individuals per number of rodents or shrews captured
†Genotypes were defined according to phylogeny results based on a portion of the paramyxovirus L gene.