Orthopoxvirus infection among wildlife in Zambia

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Human monkeypox is a viral zoonosis caused by monkeypox virus, an orthopoxvirus (OPXV). The majority of human monkeypox cases have been reported in moist forested regions in West and Central Africa, particularly in the Democratic Republic of the Congo (DRC). In this study we investigated zoonotic OPXV infection among wild animals in Zambia, which shares a border with DRC, to assess the geographical distribution of OPXV. We screened for OPXV antibodies in sera from non-human primates (NHPs), rodents and shrews by ELISA, and performed real-time PCR to detect OPXV DNA in spleen samples. Serological analysis indicated that 38 of 259 (14.7 %) rodents, 14 of 42 (33.3 %) shrews and 4 of 188 (2.1 %) NHPs had antibodies against OPXV. The OPXV DNA could not be detected in spleens from any animals tested. Our results indicated that wild animals living in rural human habitation areas of Zambia have been infected with OPXV. A rope squirrel (Funisciurus anerythrus) is the only wild-caught animal from which MPXV has been isolated in DRC (Khodakevich et al., 1986). Several species of rodents, including those from the Funisciurus, Heliosciurus, Cricetomys and Graphiurus genera, and primates are susceptible to MPXV infection (Hutin et al., 2001; Parker et al., 2007).

Although the Republic of Zambia borders DRC, there have been no reported cases of human monkeypox in the country. To determine the possible distribution of MPXV and other OPXVs in Zambia, we conducted an OPXV serosurvey and genome detection in samples from non-human primates (NHPs), rodents and shrews. This study was authorized by the Zambia Wildlife Authority. We collected tissues and serum samples from baboons and vervet monkeys killed for pest management purposes, with the permission of the Zambia Wildlife Authority (certificate no. 2604). Samples were collected from 88 vervet monkeys (Chlorocebus pygerythrus) from the Mfuwe (Eastern Province, 13°16’30.2” S 31°40’00.4” E) and Livingstone...
(Southern Province, 17°50’ 8.72” S 25°43’ 59.19’’ E) districts, and from 50 yellow baboons (*Papio cynocephalus*) and 50 chacma baboons (*Papio ursinus*) from the Mfuwe and Livingstone districts of Zambia, respectively (Fig. 1, Table 1). A total of 259 rodents and 42 shrews were caught using Sherman traps or cage traps in the Lusaka (Lusaka Province, 15°29’ 19.89’’ S 28°26’ 43.14’’ E), Namwala (Southern Province, 15°49’ 38.74’’ S 26°51’ 1.09’’ E), Mazabuka (Southern Province, 15°51’ 3.10’’ S 27°45’ 41.0’’ E), Mpuungu (Northern Province, 8°46’ 52.81’’ S 31°5’ 30.05’’ E) and Solwezi (North-Western Province, 12°24’ 20.84’’ S 26°14’ 52.76’’ E) districts of Zambia (Fig. 1, Table 1). Species were identified by morphology and by sequencing of the cytochrome *b* or recombination activating gene 1 regions using DNA obtained from spleen tissue, as previously described (Orba et al., 2011; Sasaki et al., 2013).

Serum samples from the animals were tested for IgG antibodies to OPXV by ELISA using the entire VACV proteins, as reported previously with some modifications (Morikawa et al., 2006; Sasaki et al., 2013). Briefly, 96-well plates were coated with lysates of VACV Lister-infected or mock-infected HeLa cells. After blocking with 5% (w/v) skim milk powder, diluted serum samples were added to the plates. Anti-OPXV antibodies were detected with HRP-conjugated Protein A/G (Thermo Scientific) for rodent and shrew samples, or with HRP-conjugated anti-human IgG (Jackson Immunoresarch) for primate samples, followed by a 3,3’-5,5’-tetramethylbenzidine substrate (SurModics). The final OD₄₅₀ values were obtained by subtracting the OD₄₅₀ of wells with mock lystate from the OD₄₅₀ of each sample well. The cut-off values for NHP samples were defined as the mean OD₄₅₀ of NHP samples plus three times the SD, with outliers excluded by Smirnov-Grubbs’ test. The cut-off values for rodent and shrew samples were defined as the mean OD₄₅₀ of ten uninfected mouse sera plus ten times the SD.

The OPXV ELISA showed that among the 88 vervet monkeys and 100 baboons tested, four chacma baboons in Livingstone District had OPXV IgG antibodies (Table 1). We further investigated OPXV seroprevalence in serum samples from rodents and shrews. In Africa, rope squirrels (*Funisciurus* sp.), sun squirrels (*Heliosciurus* sp.), Gambian rats (*Cricetomys* sp.) and the African dormouse (*Graphiurus* spp.) are naturally infected with MPXV (Hutin et al., 2001; Parker et al., 2007). In Zambia, we collected samples from five Gambian pocketed rats (*Cricetomys gambianus*), five African dormice (*Graphiurus* sp.) and two African bush squirrels (*Paraxerus cepapi*). Of these rodents, only one Gambian pocketed rat had OPXV antibodies (Table 2). Meanwhile, we found that Natal multimammate mice (*Mastomys natalensis*, 33 of 173=19.1%) and tiny fat mice (*Steatotmys parvus*, 4 of 5=80%) showed high seropositivity rates (Table 2). Shrews (*Crocidura* spp.), which belong to the order *Soricomorpha* and are taxonomically separated from rodents, also had OPXV antibodies (14 of 42=33.3%). We caught a relatively large number of shrews in the Northern part of Zambia, in Mpuungu and Solwezi, while only a few shrews were caught in the south. Sera from shrew samples showed a higher seropositivity rate than that of the total rodent samples (rodent total: 38 of 259=14.7%) (Table 1). There were no regional differences for rodents, with no noticeable differences in OPXV seroprevalence among sampling locations: Mpuungu, 14.6%; Namwala, 20.6%; Solwezi, 10.0%; and Mazabuka, 15.1% (P=0.42, chi-squared for independence test). The serological evidence of OPXV exposure indicates that several species of common African wild animals are susceptible to OPXV infection. MPXV, CPXV and VACV are also known to naturally infect very broad ranges of mammalian species (Essbauer et al., 2010; Haller et al., 2014) and CPXV infection of shrews has been reported (Laakkonen et al., 2006; Tryland et al., 1998).

We further attempted to detect OPXV DNA in wild animals in Zambia. A SYBR Green I-based real-time PCR assay was developed to detect the *pan*-OPXV DNA. The primers for OPXV real-time PCR were designed based on a conserved region of the DNA-dependent RNA polymerase subunit 18 gene (*rpo18*, *E7R* from OPXVs. The primers used were rpo18-228F (5’-CGCATATTATCAGTTGAGG-3’) and rpo18-442R (5’-TACCTTGTTGAGCCTCCATT-A3’), which were designed based on the gene sequence of MPXV strain Congo_2003_358 (GenBank accession number DQ011154). One or two nucleotides varied in the region of the OPXV genes corresponding to the rpo18-228F primer. However, the nucleotides corresponding to the rpo18-442R primer sequence were conserved among the OPXVs, including MPXV Zaire 1979-005, MPXV Zaire 2003-358, and MPXV Congo 2003-358.

**Fig. 1.** Map of Zambia showing the sampling locations. Vervet monkeys and baboons were collected from the Mfuwe (Eastern Province) and Livingstone (Southern Province) areas. Rodents and shrews were collected from Lusaka (Lusaka Province), Namwala (Southern Province), Mazabuka (Southern Province), Mpuungu (Northern Province) and Solwezi (North-Western Province).
Liberia_1970_184, CPXV Brighton Red, VACV Lister, camelpox virus (CMLV), ectromelia virus (ECTV), tater-apox virus (TATV) and variola virus (VARV) (Fig. S1, available in the online Supplementary Material). The real-time PCR was performed using 100 ng of extracted DNA, 400 nM of each primer and SYBR Premix Ex Taq II (Takara Bio) in 20 μl reaction mixtures, and detected with a StepOnePlus real-time PCR system (Life Technologies). The cycling protocol was incubation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. PCR products were confirmed by melt curve analysis to ensure reaction specificity.

We validated the sensitivity of detection of the OPXV genome using the rpo18 primers together with primers designed to amplify the 14 kDa protein gene A27L of VACV (Scaramozzino et al., 2007), the DNA polymerase gene E9L (Reynolds et al., 2010), the haemagglutinin gene J7R (Kulesh et al., 2004) and the insulin metalloproteinase-like protein gene G1L (Li et al., 2010). In our assay using a StepOnePlus real-time PCR system, the rpo18 primers were the most sensitive for amplification of the targeted gene from monkeypox (Zr599) virus compared with the other primers (Fig. S1). This assay could detect approximately 10 copies of the rpo18 gene. We also confirmed that the rpo18 primers amplified the targeted region from MPXV (Liberia), CPXV (Brighton Red), VACV (Lister), CMLV (J1) and ECTV (Hampstead) genomes using both conventional and real-time PCR (Fig. S1). Next, OPXV DNA screening was attempted using DNA samples extracted from the spleens of animals using a QIAamp DNA Mini kit (Qiagen). We selected spleen tissues to screen for OPXV DNA because it has been reported that OPXV DNA is detected with PCR at a higher rate in spleen tissues than in other tissues in rodents (Reynolds et al., 2010). The results of OPXV DNA screening using the rpo18 real-time PCR assay showed that the viral DNA could not be detected in any of the DNA samples extracted from spleen tissues of the tested animals (Table 1).

Because OPXV genome sequences could not be detected in the animals examined in this study, we were unable to determine whether the seropositive animals had been infected with MPXV or another OPXV. It is possible that more than one species of OPXV is circulating depending on the area or animal species. To determine the true situation regarding OPXV infection in Zambia, further investigations into viral identification are needed.

Ecological niche modelling studies of the ecology and geography of MPXV transmission predicted a potential distribution extending across most of the humid tropical evergreen forest areas of Africa (Ellis et al., 2012; Levine et al., 2007). Interestingly, these studies did not predict Zambia as a potentially favourable habitat for human monkeypox infection. However, as occurred with a monkeypox outbreak in Sudan in 2005, an outbreak may occur in an environmentally unsuitable area following importation of infected animals or humans from the endemic area (Nakazawa et al., 2013). Although OPXV antibodies in NHPs samples were only detected in chacma baboons in Livingstone in the current study, the seropositive

### Table 1. Prevalence of OPXV antibody measured by ELISA, and the OPXV rpo18 gene detection by real-time PCR in vervet monkeys, baboons, rodents and shrews

<table>
<thead>
<tr>
<th>Animal</th>
<th>Year</th>
<th>Location</th>
<th>n</th>
<th>OPXV ELISA* Positive (n)</th>
<th>OPXV ELISA* Positive (%)</th>
<th>OPXV PCR† Positive (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vervet monkey</td>
<td>2009</td>
<td>Mfuwe</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2010–2011</td>
<td>Livingstone</td>
<td>38</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Yellow baboon</td>
<td>2009</td>
<td>Mfuwe</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chacma baboon</td>
<td>2010–2011</td>
<td>Livingstone</td>
<td>50</td>
<td>4</td>
<td>8.0</td>
<td>0</td>
</tr>
<tr>
<td>Monkeys total:</td>
<td></td>
<td></td>
<td>188</td>
<td>4</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Rodent</td>
<td>2012</td>
<td>Luwaka</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>Mbulungu</td>
<td>48</td>
<td>7</td>
<td>14.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>Namwala</td>
<td>63</td>
<td>13</td>
<td>20.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>Solwezi</td>
<td>70</td>
<td>7</td>
<td>10.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>Mazabuka</td>
<td>73</td>
<td>11</td>
<td>15.1</td>
<td>0</td>
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<tr>
<td>Rodent total:</td>
<td></td>
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<td>259</td>
<td>38</td>
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</tr>
<tr>
<td>Shrew</td>
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<td>20</td>
<td>6</td>
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<td>0</td>
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<tr>
<td></td>
<td>2012</td>
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<td>1</td>
<td>50.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>Solwezi</td>
<td>16</td>
<td>7</td>
<td>43.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>Mazabuka</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Shrew total:</td>
<td></td>
<td></td>
<td>42</td>
<td>14</td>
<td>33.3</td>
<td></td>
</tr>
</tbody>
</table>

*OPXV IgG antibodies measured by ELISA using VACV as the antigen. Serum samples that were positive in a 1:100 dilution were determined by setting the cut-off value for each experiment.
†A SYBR Green I-based real-time PCR assay based on a conserved region of the OPXV rpo18 sequence.
rodents were distributed across a wide area of Zambia. In addition, OPXVs are likely to be circulating near areas of human habitation, as the rodents and shrews examined in this study were trapped around a house and field in a rural area.

In conclusion, serological evidence of OPXV infection was found in several species of wild animals living in Zambia. These animal species are likely to interact with humans. Our findings provide data for determining potential outbreak risks of zoonotic OPXV infection in Zambia.

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