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Orthopoxvirus infection among wildlife in Zambia

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Summary

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 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 four 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.

Main text

The genus *Orthopoxvirus* contains variola virus, the causative agent of smallpox in humans, and several zoonotic orthopoxviruses, which have broad host ranges among mammalian species, including monkeypox virus (MPXV), cowpox virus (CPXV), and vaccinia virus (VACV) (Essbauer *et al.*, 2010). Taterapox virus is another orthopoxvirus (OPXV), which was isolated from a rodent (*Tatera kempii*) in West Africa (Lourie *et al.*, 1975). In Africa, human monkeypox cases have been reported in rural regions of the Congo Basin and West Africa close to tropical rainforest. The majority of human monkeypox cases have been reported in the Democratic Republic of the Congo (DRC) (Rimoin *et al.*, 2010), while outbreaks have also been reported in the Republic of the Congo and Sudan, both of which neighbor DRC (Formenty *et al.*, 2010; Learned *et al.*, 2005). The natural cycle of MPXV infection in wild animals and the exact reservoir of the virus are currently unknown. 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 *Funiscirus*, *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 and 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°51’31.0”S, 27°45’41.0”E), Mpulungu (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 and 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 vaccinia virus proteins, as reported previously with some modification (Morikawa *et al.*, 2005; Saijo *et al.*, 2006). Briefly, 96-well plates were coated with lysates of vaccinia virus 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 horseradish peroxidase (HRP)-conjugated Protein A/G (Thermo Scientific) for rodent and shrew samples, or with HRP-conjugated anti-human immunoglobulin G (Jackson Immunoresarch) for primate samples, followed by a 3,3′,5,5′-tetramethylbenzidine substrate (SurModics). The final optical density (OD) values were obtained by subtracting the OD of wells with mock lysate from the OD of each sample well. The cutoff values for NHPs samples were defined as the average OD of NHPs samples plus three times the standard deviation, with outliers excluded by Smirnov-Grubbs’ test. The cutoff values for rodent and shrew samples were defined as the average OD of ten uninfected mouse sera plus ten times the standard deviation.

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 seroprevalance in serum samples from rodents and shrews. In Africa, rope squirrels (*Funiscirus* spp.), sun squirrels (*Heliosciurus* sp.), Gambian rats (*Cricetomys* spp.), 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 pouched rats (*Cricetomys gambianus*), five African dormice (*Graphiurus* sp.), and two African bush squirrels (*Paraxerus cepapi*). Of these rodents, only one Gambian pouched rat had OPXV antibodies (Table 2). Meanwhile, we found that Natal multimammate mice (*Mastomys natalensis*, 33 of 173 = 19.1%) and tiny fat mice (*Steatomys 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 Mpulungu 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: Mpulungu, 14.6%; Namwala, 20.6%; Solwezi, 10.0%; and Mazabuka, 15.1% ($p = 0.42$, chi-square 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 have been reported (Laakkonen *et al.*, 2006; Tryland *et al.*, 1998).

We further attempted to detect OPXV DNA from 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′-CGCATATTATCACGTTGGGG-3′) and rpo18-442R (5′-TACCTTGTTGAGCCTCCATTAG-3′), 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 Liberia_1970_184, CPXV Brighton Red, VACV Lister, camelpox virus (CMLV), ectromelia virus (ECTV), taterapox virus (TATV), and variola virus (VARV) (Fig. S1). 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 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. 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-kD protein gene *A27L* of VACV (Scaramozzino *et al.*, 2007), the DNA polymerase gene *E9L* (Reynolds *et al.*, 2010), the hemagglutinin gene *J7R* (Kulesh *et al.*, 2004), and the insulin metalloproteinase-like protein gene *G1L* (Li *et al.*, 2010). In our assay using the 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 monkeypox (Liberia), cowpox (Brighton Red), vaccinia (Lister), camelpox (J1), and ectromelia (Hampstead) virus genomes using both conventional and real-time PCR (Fig. S1). Next, OPXV DNA screening was attempted using DNA samples

154 extracted from the spleens of animals using a QIAamp DNA Mini kit (Qiagen). We selected
155 spleen tissues to screen for OPXV DNA because it has been reported that OPXV DNA is
156 detected with PCR at a higher rate in spleen tissues than in other tissues in rodents (Reynolds
157 *et al.*, 2010). The results of OPXV DNA screening using the *rpo18* real-time PCR assay
158 showed that the viral DNA could not be detected in any of the DNA samples extracted from
159 spleen tissues of the tested animals (Table 1).
160
161 Because OPXV genome sequences could not be detected in the animals examined in this
162 study, we were unable to determine whether the seropositive animals had been infected with
163 MPXV or another OPXV. It is possible that more than one species of OPXV is circulating
164 depending on the area or animal species. To determine the true situation regarding OPXV
165 infection in Zambia, further study on viral identification is needed.
166 Ecological niche modeling studies of the ecology and geography of MPXV transmission
167 predicted a potential distribution extending across most of the humid tropical evergreen forest
168 areas of Africa (Ellis *et al.*, 2012; Levine *et al.*, 2007). Interestingly, these studies did not
169 predict Zambia as being a potentially favorable habitat for human monkeypox infection.
170 However, as occurred with a monkeypox outbreak in Sudan in 2005, an outbreak may occur
171 in an environmentally unsuitable area following importation of infected animals or humans
172 from the endemic area (Nakazawa *et al.*, 2013). Although OPXV antibodies in NHPs samples
173 were only detected in chacma baboons in Livingstone in the current study, the seropositive
174 rodents were distributed across a wide area of Zambia. In addition, OPXVs are likely
175 circulating near areas of human habitation, as the rodents and shrews examined in this study
176 were trapped around a house and field in a rural area.
177
178 In conclusion, serological evidence of OPXV infection was found in several species of wild
179 animals living in Zambia. These animal species likely interact with humans. Our findings

provide data for determining potential outbreak risks of zoonotic OPXV infection in Zambia.

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Figure legend

Fig. 1

Map of Zambia showing the sampling location. 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), Mpulungu (Northern Province), and Solwezi (North-western Province).

284 **Table 1.** Prevalence of orthopoxvirus antibody measured by ELISA, and the orthopoxvirus
285 *rpo18* gene detection by real-time PCR in vervet monkeys, baboons, rodents, and shrews.
286

Animal	Year	Location		OPXV ELISA*		OPXV PCR†
			n	positive (n)	positive (%)	positive (n)
Vervet Monkey	2009	Mfuwe	50	0	0	0
	2010 -2011	Livingstone	38	0	0	0
Yellow Baboon	2009	Mfuwe	50	0	0	0
Chacma Baboon	2010 -2011	Livingstone	50	4	8.0	0
		Monkeys total	188	4	2.1	
Rodent	2012	Lusaka	5	0	0	0
	2012	Mpulungu	48	7	14.6	0
	2012	Namwala	63	13	20.6	0
	2013	Solwezi	70	7	10.0	0
	2013	Mazabuka	73	11	15.1	0
		Rodent total	259	38	14.7	
Shrew	2012	Mpulungu	20	6	30.0	0
	2012	Namwala	2	1	50.0	0
	2013	Solwezi	16	7	43.7	0
	2013	Mazabuka	4	0	0	0
		Shrew total	42	14	33.3	

287

288 *Orthopoxvirus (OPXV) IgG antibodies measured by ELISA using vaccinia virus as antigen.
289 Serum samples that were positive in a 1:100 dilution were determined by setting the cutoff
290 value for each experiment.
291 †A SYBR Green I-based real-time PCR assay based on a conserved region of the OPXV
292 *rpo18* sequence.
293

294 **Table 2.** Orthopoxvirus antibody titer in sera from shrew and rodent species

295

Species	Location	n	Number of positive for OPXV ELISA							
			positive	1:100	1:200	1:400	1:800	1:1600	1:3200	>1:51200
(Shrew)										
Crocidura	Mpulungu	20	6	5	1					
	Solwezi	16	7	2		1	3	1		
	Namwala	2	1	1						
	Mazabuka	4	0							
	total	42	14	8	1	1	3	1		
(Rodent)										
Mastomys	Mpulungu	28	5	3	2					
	Solwezi	56	7	2		2		1		2
	Namwala	29	11	1	4		2	2	2	
	Mazabuka	58	10	1	7		2			
	Lusaka	2	0							
	total	173	33	7	13	2	4	3	2	2
Steatomys	Mpulungu	1	1	1						
	Namwala	2	2				1	1		
	Mazabuka	2	1		1					
	total	5	4	1	1		1	1		
Cricetomys	Mpulungu	3	1						1	
	Lusaka	2	0							
	total	5	1						1	
Paraxerus		2	0							
Graphiurus		5	0							
Gerbilliscus		27	0							
Aethomys		11	0							
Saccostomus		9	0							
Other species*		22	0							

296

297 *Other species from the genera *Mus, Lemniscomys, Grammomys, Arvicanthis, Rattus,*

298 *Pelomys,* and unidentified species.


DEMOCRATIC REPUBLIC
OF THE CONGO
Mpulungu
Solwezi
Mfuwe
ZAMBIA
Lusaka
Namwala
Mazabuka
Livingstone
0
150 km

Fig. 1