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ENDEMIC ORTHOPOXVIRUS CIRCULATING IN PROCYONIDS IN MEXICO

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Abstract

Limited serosurveillance studies suggested that orthopoxviruses (OPXV) are widespread in the US (e.g., *Raccoonpox virus, Skunkpox virus, Volepox virus*) and Brazil (*Vaccinia virus*); however, their animal reservoir(s) remain unconfirmed. Mexican mammal diversity includes several species related to those in which evidence for OPXV infections has been found (*Oryzomys, Peromyscus, Microtus,* and Procyonidae). The presence of these groups of mammals in Mexico and the evidence of their possible involvement in the maintenance of OXPV in nature suggest the same or similar OPXV are circulating in Mexico. We tested 201 sera from 129 procyonids via modified enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) to estimate OPXV antibody prevalence in these animals. We detected a prevalence of 16.67% in *Nasua narica* (white-nosed coati), 35% in *Procyon lotor* (raccoon), and 30.4% in *Bassariscus astutus* (ring-tailed cat) when tested by either ELISA or WB. Western blot results presented protein bands consistent with the size of some OXPV immunodominant bands (14, 18, 32, 36, and 62 kDa). These results support the hypothesis that OPXV circulate in at least three genera of Procyonidae in central and Southeast Mexico.

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Keywords

Antibody prevalence; *Orthopoxvirus*; procyonids; serosurvey

INTRODUCTION

The genus *Orthopoxvirus* (OPXV) contains species that have been associated with severe febrile rash illness in humans (e.g., smallpox and monkeypox; Damon 2007). Three species of this genus have been recognized to be endemic to North America: *Skunkpox virus, Raccoonpox virus,* and *Volepox virus.* Other than their initial descriptions (in 1978, 1961, and 1985, respectively), few studies have been conducted regarding the ecology, geographic distribution, pathology, and pathogenicity of these North American Orthopoxviruses (NA OPXV; Regnery 1987; Knight et al. 1992; Emerson et al. 2009; Gallardo-Romero et al. 2012). Additionally, recent evidence suggests that *Variola virus*, the causative agent of human smallpox, was initially a rodent-borne virus before evolving into an exclusively human pathogen (Li et al. 2007). Other rodent-borne OPXV are cowpox, monkeypox, and vaccinia viruses (Khodakevich et al. 1987b; Crouch et al. 1995; Chantrey et al. 1999; Trindade et al. 2007; Abrahão et al. 2009; Schatzmayr et al. 2011; Peres et al. 2013).

The voles and mice (*Microtus californicus* and *Peromyscus truei*) in which volepox virus was found are noncommensal species that have little contact with humans; thus, it is possible that this virus infects humans but has had little chance for transmission. However, raccoons (*Procyon lotor*), white- and brown-nosed coatis (*Nasua* spp.), ring-tailed cats (*Bassariscus astutus*), cacomixtles (*Bassariscus sumichrasti*), and skunks (*Mephitis* spp.) are frequently in contact with humans, and NA OPXV have been isolated from raccoons and skunks (Alexander et al. 1972; Emerson et al. 2009).

Due to our limited knowledge of the natural history of the NA OPXV and their close relationship to the OPXV that cause disease in humans (i.e., monkeypox, cowpox, vaccinia, and variola virus), we consider these viruses to be a risk for human heath (Emerson et al. 2009; Gallardo-Romero et al. 2012).

Orthopoxviruses have been studied and isolated in Brazil since the 1960s, but their vaccinia-like strains are divided into two monophyletic groups. One theory is that some Brazilian strains have an independent origin from the vaccines used during the smallpox eradication, suggesting that these OPXV have been endemic in Brazil since before the smallpox outbreaks and vaccination campaigns occurred (Trindade et al. 2007). Anti-OPXV antibodies have been identified in serum from domestic animals (pets and livestock), wild animals (small and medium sized mammals, monkeys, and marsupials), and humans in Brazil (Abrahão et al. 2009; Abrahão et al. 2010; Schatzmayr et al. 2011; Peres et al. 2013). Recently, a 30% of prevalence of OPXV antibodies was reported in Mexican grey squirrels (*Sciurus aureogaster*) from Mexico City parks (Martinez-Duque et al. 2014).

We tested sera collected from white-nosed coatis, raccoons, and ring-tailed cats from an eco-park and a natural reserve in Mexico to detect exposure by OPXV. We used modified

enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) assays to detect anti-OPXV antibodies in these animals.

METHODS

Collection sites

Sera samples were collected from two localities. 1) The eco-park "Parque Museo de La Venta," (hereafter La Venta), located in central Tabasco State, is one of Mexico's eco-archaeological attractions because of its collection of famous colossal Olmec heads. Animal samples from this site were collected with permit FAUT-0250 issued by Secretaría de Medio Ambiente y Recursos Naturales (Semarnat). 2) The "Reserva Ecológica del Pedregal de San Angel (REPSA)" ecologic reserve belongs to the Universidad Nacional Autónoma de México (UNAM) and is located in Southeastern Mexico City. The reserve covers over 237.3 ha of volcanic rock terrain, and about 39 wild mammal species have been reported there (Hortelano-Moncada et al. 2009). Animal samples from the REPSA were collected with permit REPSA/410/2009 issued by the Universidad Nacional Autónoma de México (UNAM).

Trapping protocol

In La Venta, white-nosed coatis were trapped by anesthetic dart delivery and raccoons were trapped using Tomahawk live traps (Tomahawk Live Trap Company, Hazelhurst, Wisconsin, USA) baited with sardines. Four trapping periods were conducted between 2011 and 2012. In REPSA, ring-tailed cats were trapped using Tomahawk traps baited with a combination of sardines in tomato sauce, yogurt, raisins, strawberry jelly, and banana. Sixteen trapping periods were conducted between 2009 and 2011. In both sites, traps were baited before sunset and checked early the next morning. The animals were confined to the trap <12 h and were immediately released after sampling.

Animal identification, sample collection, and transportation: Animals from La Venta were anesthetized by intramuscular injection with a mixture of 10 mg/kg of ketamine hydrochloride (HCl) and 0.5 mg/kg of xylazine HCl. Blood was drawn by jugular venipuncture using a 5-mL syringe with a 21-G needle. Animals were tattooed with an individual identification number and released when completely ambulatory. Blood was stored at 4 C until centrifuged (about 3 h later). Serum was collected in 1.5-mL sterile microtubes and stored at −20 C until shipment.

Ring-tailed cats from the REPSA were anesthetized via intramuscular injection with a combination of 3.6 (±0.8) mg/kg of ketamine HCl and 59.4 (±13.8) μg/kg of dexmedetomidine HCl. Blood samples were collected from the jugular vein and the animals received an injection of 293.3 (±70.1) μg/kg of atipamezole to reverse the anesthesia. Prior to release, animals were marked with ear tags for future identification on recapture. Samples were stored at 4 C for 24 h and serum was separated and stored at −70 C until shipment. The sera were part of a capture–recapture study and some animals were trapped and sampled more than once.

Sample processing: The sera were shipped on dry ice to the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA) Poxvirus laboratory for serologic analysis and kept at −20 C until tested. For initial screening of sera, a modified ELISA was used for detection of OPXV immunoglobulin G antibodies (IgG). Crude vaccinia virus Western Reserve at a concentration of 0.01 μg/well, diluted in carbonate buffer, was used for coating half of each microtiter plate and the other half was coated with an equal concentration of BSC-40 cell lysate. Animal sera were tested at a 1:100 dilution in duplicate. A volume of 100 μL/well at a dilution of 1:10,000 of anti-raccoon IgG (H+L) labeled with horseradish peroxidase (A140-123 P; Bethyl Laboratories, Montgomery, Texas, USA) was diluted in blocking buffer and used as conjugate. SureBlue™ TMB Microwell Peroxidase Substrate (KPL 52-00-01, KPL, Inc., Gaithersburg, Maryland, USA) and TMB Stop Solution (KPL 50-85-05) were used to develop the plates. Finally, plates were shaken for 5 s and read at 450 nm on a spectrophotometer (Spec-traMAX® 190; Molecular Devices, LLC, Sunny-vale, California, USA) to obtain the optical density (OD) values. The average of the duplicates from the corresponding sample in the cell lysate half of the plate plus two standard deviations was used to generate a cut-off value (COV). A sample was considered positive if the average of the experimental sample duplicates was 0.05≥COV.

Positive sera were subsequently tested with serial 2-fold dilutions (1:100 to 1:3,200) and the COV was calculated using the OD value from the cell lysate part of the plate minus the value from the corresponding sample/dilution in the viral side of the plate. No duplicates were run because six dilutions of the same sample were tested. An animal was considered positive for anti-orthopoxvirus antibodies if the serum sample was positive in at least two consecutive dilutions (i.e., 1:100 and 1:200).

Western blots were performed following the standard protocol for polyacrylamide gradient gels and polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, California, USA) as described (Gallardo-Romero et al. 2012). Purified vaccinia virus proteins (Western Reserve strain) at a concentration of 1.5 μg/μL were loaded on the gel. Either the BenchPro™ 4100 Western Processing System (WP0001, WP1001, Invitrogen, Carlsbad, California, USA,) or the Mini-PROTEAN II Multiscreen Apparatus (part number 170-4017, Bio-Rad Laboratories) were used. The experimental serum was diluted in blocking buffer at 1:100. Recombinant Protein A/G Alkaline Phosphatase Conjugated (part number 32391, Pierce™ Thermo Fisher Scientific, Waltham, Massachusetts, USA) at a dilution of 1:5,000 in blocking buffer was used a secondary antibody. Following the incubation with the substrate Immun-Star™ AP (no. 170-5018, Bio-Rad Laboratories), the membrane was placed on the Bio-Rad ChemiDoc™ system for chemiluminescent detection. The band molecular weight in kilo-daltons (kDa) was recorded for future analysis. A sample was considered positive if it presented at least the 14- or the 62-kDa immunodominant bands. The 14-kDa band has been observed in survivors from OPXV infections who manifest an immune response. This band size is consistent with the previously described envelope protein encoded by the A27 gene ortholog of vaccinia virus Copenhagen (VV-Cop). This gene has an important role in allowing mature virus to bind to cell surface glycosaminoglycans and stimulates a cellular immune response (Demkowicz et al. 1992; Che-Sheng et al. 1998; Gallardo-Romero et al. 2012). The 62-kDa band is likely a major core protein encoded by the A10 gene (VV-Cop

ortholog) derived from the P4a precursor. It is the most abundant core protein found in the virion and plays an important role in its assembly. It is also important in stimulating memory B-cells and the humoral immune response (Vanslyke et al. 1991).

There is no commercially available serum from procyonids infected with OPXV to be used as a positive control for the assays. However, rabbit naive serum and rabbit hyperimmune anti-vaccinia serum were used as assay controls on every plate and blot. The rabbit hyperimmune serum was developed in our lab specifically to be used as a positive control in several assays and has been tested to confirm cross-reactivity with the anti-bodies we used in this study. To be consistent with the previously validated protocols, we used different antibodies and antigen purities for our distinct serologic assays.

RESULTS

We obtained 201 serum samples from white-nosed coatis (n=113), raccoons (n=58), and ring-tailed cats (n=30). Some animals were captured up to four times. In summary, sera from 129 individuals were assayed (66 white-nosed coatis, 40 raccoons, and 23 ring-tailed cats). When samples were tested for anti-vaccinia IgG by ELISA, sera from 13 white-nosed coatis, 17 raccoons, and 9 ring-tailed cats were positive. Using WB, we found 22 white-nosed coati, 21 raccoon, and 12 ring-tailed cat sera with protein bands consistent with the size of several OPXV-specific bands (14, 18, 32, 36, and 62 kDa).

Raccoon sera

Twenty-six of 40 raccoons were sampled only once during the study. Eleven raccoons were captured twice, two were captured three times, and one was captured on four occasions. Fourteen (35%) raccoons had detectable antibody to OPXV.

We tested 58 samples from the 40 raccoons and 53 samples had concordant results in both assays (36 negative and 18 positives), showing 93% correlation. Four samples were positive only by WB (Table 1). Females and males were collected equally (20 of each); however, adults were more predominant than juveniles (29/40). Positive results are presented in Table 1. Two animals that were negative on first capture were positive on subsequent recapture. Three animals positive on initial capture were negative by ELISA on recapture but remained positive by WB.

White-nosed coati sera

Sixty-six out of the 129 individuals were white-nosed coatis, but a total of 113 sera samples were collected from these 66 individuals. We captured 27 white-nosed coatis once, 31 twice, and eight three times. Antibody prevalence was 17% (11/66). Results for the two assays were 92% concordant; 91 samples were negative and 13 were positive by both assays. Nine serum samples were positive only by WB. We sampled 37 adult females, 21 adult males, and eight juveniles (six males and two females). A summary of the positive results is shown in Table 2. Two animals that were negative on first capture were positive on subsequent recapture. Six that were positive on initial capture were ELISA-negative on recapture but remained positive by WB (Table 2).

Ring-tailed cat sera

The least represented group of animals was ring-tailed cats, with only 23 animals sampled. Eighteen ring-tailed cats were captured once, four were captured twice, and one was captured four times. The antibody prevalence was 30% (7/23). We tested 30 samples from the 23 individuals. Results from 27 samples were concordant between assays (18 negative and nine positive), showing 90% correlation. Three sera were positive only by WB. Ten females and 13 males were collected; however, adults were more predominant than juveniles (20/23). The positive ring-tailed cat samples are shown on Table 3.

DISCUSSION

The majority of samples were either positive or negative by both assays, and most of the animals were represented by only one sample. Nine raccoons, three white-nosed coatis, and five ring-tailed cats were positive by both assays in all samples available as shown in Tables 1–3. The interesting results were observed with those animals that had more than one sampling date. Two raccoons (numbers 11 and 17), four white-nosed coatis (numbers 16, 39, 69, and 74), and two ring-tailed cats (numbers 9 and 37) seroconverted over time. These animals were negative in the first sample with either both assays or by ELISA only, and then positive by both assays in subsequent samples, indicating the animal may have been exposed or re-exposed to an OPXV after the first capture date. Another group of six animals (two raccoons and four white-nosed coatis) were positive by both assays in the first sample tested and negative by ELISA but positive by WB in the subsequent sample; this may indicate a decrease in antibody titer with time.

The WB was more sensitive in detecting OPXV antibodies, and there are two possible explanations for this: 1) crude vaccinia virus was used for ELISA, compared to purified vaccinia virus used for WB; and 2) for ELISA, anti-raccoon IgG was used as the conjugate versus recombinant protein A/G for WB. The conjugate used in ELISA is specific to the detection of raccoon IgG. Cross-reactivity was seen with sera from other genera of the same family, but the sensitivity of the conjugate to IgG of the white-nosed coati and ring-tailed cat is unknown; however, the two genetically close species presented similar prevalences (30% for ring-tailed cats and 35% for raccoons). This compares to 17% in coatis, a species more-distantly related (Koepfli et al. 2007). We consider that there may be a correlation between assay sensitivity and the genetic distance of the species mentioned above. The recombinant protein A/G used for WB contains four Fc-binding domains from Protein A and two from Protein G and binds to all antibody species and subclasses recognized by either Protein A or Protein G. Based on our results, it seems likely the recombinant protein A/G is detecting more than one anti-OPXV immunoglobulin in procyonid sera. Each of these conditions may contribute to the lower sensitivity of the ELISA compared to WB in detecting OPXV antibodies.

Serologic cross-reactivity between species of OPXV allows us to test experimental sera using vaccinia virus while recognizing, however, that the antibodies detected do not indicate the circulation of any particular OPXV. Our results might be explained in several ways. Firstly, the geographic range of NA OPXV may extend more southerly than previously thought. Secondly, vaccinia viruses similar to those seen in Brazil may be circulating in

hosts further north into Central and North America. Finally, this may be evidence of a new OPXV.

Without the cross-protection afforded by smallpox vaccination, a greater proportion of the human population may now be susceptible to OPXV infection. It is therefore prudent to be aware of circulating OPXV that could be potential risks to human health, particularly for individuals with immunosuppressive conditions. Our antibody prevalence results strongly support the hypothesis of enzootic OPXV circulating in at least three species of the family Procyonidae from central and southeastern México. This study demonstrates the need for additional OPXV research in Mexico. Additional serosurveys and virus isolates are needed to assess the zoonotic potential of the Mexican OPXV. Studying the pathogenesis and phylogenetic properties of the virus(es) would contribute to a better understanding of their potential impact on public health. Several zoonotic OXPV are maintained in nature by different species of rodents (Khodakevich et al. 1987a; Crouch et al. 1995; Chantrey et al. 1999; Trindade et al. 2007; Abrahão et al. 2009; Schatzmayr et al. 2011; Peres et al. 2013). Therefore, it is important to test wild rodents sympatric with these three species of procyonids to estimate the prevalence in wild animals and identify the natural reservoir of these Mexican OPXV. Anti-OPXV antibodies were recently reported in squirrels from Mexico City (Martinez-Duque et al. 2014); now we have evidence of OPXV infection in Mexican procyonids.

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Table 1.

Results for enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) to detect antibodies to orthopoxviruses in raccoons (*Procyon lotor*) in Mexico, 2009–2011. + = positive; − = negative.

Tattoo	Sex	Age	August 2011	January 2012	August 2012	December 2012
11	Male	Adult	No sample	No sample	ELISA−,WB−;	ELISA+,WB+
17	Female	Adult	ELISA−,WB−	No sample	No sample	ELISA+,WB+
18	Male	Juvenile	No sample	ELISA+,WB+	ELISA−,WB+	No sample
19	Female	Adult	ELISA+,WB+	ELISA−,WB+	ELISA−,WB+	ELISA+,WB+
26	Male	Adult	ELISA+,WB+	No sample	No sample	No sample
32	Female	Adult	No sample	ELISA+,WB+	No sample	ELISA−,WB+
40	Male	Juvenile	No sample	ELISA+,WB+	ELISA+,WB+	ELISA+,WB+
42	Male	Adult	No sample	No sample	ELISA+,WB+	No sample
44	Male	Adult	No sample	No sample	ELISA+,WB+	No sample
48	Female	Juvenile	No sample	No sample	No sample	ELISA+,WB+
49	Female	Adult	No sample	No sample	No sample	ELISA+,WB+
51	Female	Juvenile	No sample	No sample	No sample	ELISA+,WB+
91	Female	Adult	No sample	No sample	No sample	ELISA+,WB+
92	Female	Adult	No sample	No sample	No sample	ELISA+,WB+

Table 2.

Results for enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) to detect antibodies to orthopoxviruses in white-nosed coati (*Nasua narica*) in Mexico, 2011–2012. + = positive; − = negative.

Tattoo	Sex	Age	August 2011	January 2012	August 2012	December 2012
16	Male	Adult	ELISA−,WB−	No sample	ELISA+,WB+	ELISA+,WB+
19	Male	Adult	No sample	ELISA+,WB+	No sample	No sample
20	Male	Adult	ELISA+,WB+	No sample	ELISA—, WB+	No sample
24	Female	Adult	No sample	ELISA+,WB+	No sample	ELISA−, WB+
39	Male	Adult	ELISA−,WB+	ELISA+,WB+	No sample	ELISA−,WB+
42	Female	Adult	No sample	ELISA+,WB+	No sample	ELISA−, WB+
45	Female	Adult	No sample	ELISA+,WB+	No sample	No sample
60	Male	Adult	ELISA+,WB+	ELISA−,WB+	ELISA−,WB+	No sample
69	Male	Adult	No sample	ELISA−,WB−	ELISA+,WB+	ELISA−,WB+
74	Female	Adult	No sample	No sample	ELISA−,WB+	ELISA+,WB+
82	Male	Juvenile	No sample	No sample	ELISA+,WB+	ELISA+,WB+

Table 3.

Results for enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) to detect antibodies to orthopoxviruses in ring-tailed cat (*Bassariscus astutus*) in Mexico, 2009–2011. + = positive; − = negative.

Ear tag	Sex	Age	Spring 2009	Fall 2009	Spring 2010	Summer 2010	Spring 2011
9	Female	Adult	ELISA−,WB+	ELISA+,WB+	No sample	ELISA+,WB+	ELISA−,WB+
29	Male	Adult	ELISA+,WB+	No sample	ELISA+,WB+	No sample	No sample
37	Female	Adult	No sample	No sample	ELISA—,WB+	ELISA+,WB+	No sample
38	Female	Adult	No sample	No sample	ELISA+,WB+	No sample	No sample
66	Male	Adult	No sample	No sample	No sample	No sample	ELISA+,WB+
85	Male	Adult	ELISA+,WB+	No sample	No sample	No sample	No sample
120	Male	Adult	No sample	No sample	No sample	ELISA+,WB+	No sample