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PREVALENCE OF ANTIBODIES TO ORTHOPOXVIRUS IN WILD CARNIVORES OF NORTHWESTERN CHIHUAHUA, MEXICO

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ABSTRACT: The distribution of orthopoxviruses (OPXVs) across the North American continent is suggested to be widespread in a wide range of mammalian hosts on the basis of serosurveillance studies. To address the question of whether carnivores in northwestern Mexico are exposed to naturally circulating OPXVs, wild carnivores were collected by live trapping within four different habitat types during fall of 2013 and spring of 2014 within the Janos Biosphere Reserve in northwestern Chihuahua, Mexico. A total of 51 blood samples was collected for testing. Anti-OPXV immunoglobulin G enzyme-linked immunosorbent assay, western blot, and rapid fluorescent focus inhibition test (RFFIT) assays were conducted. About 47% (24/51) of the carnivores tested were seropositive for anti-OPXV binding antibodies and had presence of immunodominant bands indicative of OPXV infection. All samples tested were negative for rabies virus neutralizing antibodies by RFFIT, suggesting that the OPXV antibodies were due to circulating OPXV, and not from exposure to oral rabies vaccine (vaccinia-vectored rabies glycoprotein vaccine) bait distributed along the US–Mexico border. Our results indicated that there may be one or more endemic OPXV circulating within six species of carnivores in northwestern Mexico.

Key words: Antibody, carnivores, infectious disease, northwestern Mexico, orthopoxvirus, prevalence, serosurveillance.

INTRODUCTION

Members of the genus *Orthopoxvirus* (OPXV) infect a variety of mammalian taxa as both enzootic and zoonotic pathogens, with some species associated with severe febrile rash illness in humans (monkeypox virus). Other OPXVs are known to cause significant economic losses from infected livestock (vaccinia virus), or are recognized to infect pets and zoo animals (cowpox virus; Damon 2007). The distribution of OPXV is worldwide, with this naturally endemic virus genus occurring on all continents except Australia and Antarctica (Emerson et al. 2009). Three species of this genus have been recognized to be endemic to North America: skunkpox virus (Emerson et al. 2009), raccoonpox virus (Alexander et al. 1972), and volepox virus (Regnery 1987). In 2017 a human poxvirus infection from a novel OPXV was discovered in Alaska; however, it is possible that this may have been the result of an introduction from the Old World (Springer et al. 2017). This is the latest in an increasing number of reports of novel emerging OPXVs (Osadebe et al. 2014; Hoffman et al. 2015; Vora et al. 2015) that add to the increase in detected cases of human monkeypox disease in Africa (Rimoin et al. 2010; Durski et al. 2018). Administration of routine smallpox vaccinations ceased shortly after the eradication of smallpox in the 1980s. The smallpox vaccination afforded human populations with cross-protection to other related OPXV infections such as cowpox, vaccinia, and monkeypox viruses (Fulginiti et al. 2003). There is an increased proportion of unvaccinated people as the global population grows, further increasing the risks of human infection from new and emerging OPXVs.

Although the natural reservoir hosts of North American OPXVs remain unknown, anti-OPXV antibodies have been detected in many mammal species across the continent, and rodents are thought to be the primary reservoir hosts. North American OPXVs have been identified and isolated from many animals including rodents such as pinyon mouse (*Peromyscus truei*), California vole (*Microtus californicus*), and northern pygmy mouse (*Baiomys taylori*; Regnery 1987; Martínez-Duque et al. 2014; Gallardo-Romero et al. 2016; Hodo et al. 2018). Isolates of OPXVs have been obtained from different carnivores such as raccoons (*Procyon lotor*) and skunks (*Mephitis* spp.); seropositive samples have also been collected from coatis (*Nasua* spp.), and ring-tailed cats (*Bassariscus* spp.).

There are many predator–infected-prey interaction studies that have concluded that predators (carnivores) take a disproportionate number of prey that are diseased and infected with parasites (Hethcote et al. 2004). Infected prey are typically sick and more vulnerable to predation, and in some cases infected prey will alter their behavior, making them more easily preyed upon (Lafferty 1992). An increase in vulnerability of the infected prey, coupled with the tendency for predators to forage for easily available prey, can lead to the spread of disease to and among the susceptible predator population (Krebs 1978). Carnivores adapt well to urban and periurban environments (Gehrt et al. 2010) and can facilitate cross-species disease transmission with domestic dogs (*Canis lupus familiaris*) and cats (*Felis catus*) and their owners (Deem et al. 2001; Millan et al. 2016). There could be a potential risk to the human population in a given region where a carnivore population exhibits a high OPXV seroprevalence.

Alternatively, the presence of antirabies and anti-OPXV antibodies in carnivores of northwestern Mexico could be indicative of an exposure to oral rabies vaccine (ORV, a vaccinia-vectored rabies glycoprotein vaccine) because of the proximity to the US Department of Agriculture's (USDA) bait drop zones along the US–Mexico border, and not to an exposure to a natural endemic OPXV. In this study, we tested sera collected from wild carnivore species in northwestern Mexico to further assess the distribution and prevalence of OPXV in North America, and to investigate differential species-specific exposures in this region.

MATERIALS AND METHODS

Collection site

We collected blood from wild carnivores during fall 2013 (October, November) and spring 2014 (April, May) from five areas (Monte Verde, La Bascula, Ojitos, Rancho San Pedro, and El Cuervo) within the Janos Biosphere Reserve of northwestern Chihuahua, Mexico (30°51′50″N, 108°30′09″W; Fig. 1). Sampling sites were selected in multiple habitat types to maximize the species diversity captured within the region.

Trapping protocol and sample collection

Within each of the five areas, 16 trapping stations were set at 0.5- to 0.8-km intervals along a 10-km transect. Each station contained one cage-style live trap (Tomahawk Live Trap Inc., Hazelhurst, Wisconsin, USA; size 76×76×178 cm or 152×51×71 cm) and one leg-hold trap (#1.75 or #3 soft catch coilspring, Oneida Victor Inc., Euclid, Ohio, USA) with at least 30 m between traps. The traps were baited with sardine, chicken, and commercial lures, such as coon digger I, coyote urine, and bobcat gland (Kishel's®, East Aurora, New York, USA). Traps were set for nine consecutive days per site and checked at least once a day. Once captured, each individual was chemically restrained with an intramuscular injection of ketamine hydrochloride (Anesket®, Pisa, Atitalaquia, Hidalgo, Mexico) and xylazine hydrochloride (Procin®, Pisa) according to the recommended doses for wild carnivores (Kreeger et al. 2012). Animals were identified, sexed, and ear tagged. Blood samples were collected (2–6 mL) in serum separator tubes from cephalic or femoral veins; tubes were centrifuged at 1,400 × G for 5 min to obtain serum. Serum samples were transferred into Microtainer® (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) tubes and stored in liquid nitrogen in the field. Samples were later transferred to a –70 C freezer until laboratory testing. All procedures for trapping and handling carnivores followed the guidelines of the American Society of Mammalogists (Sikes and Gannon 2011) and were approved by the Animal Care Committee of the Veterinary School of the

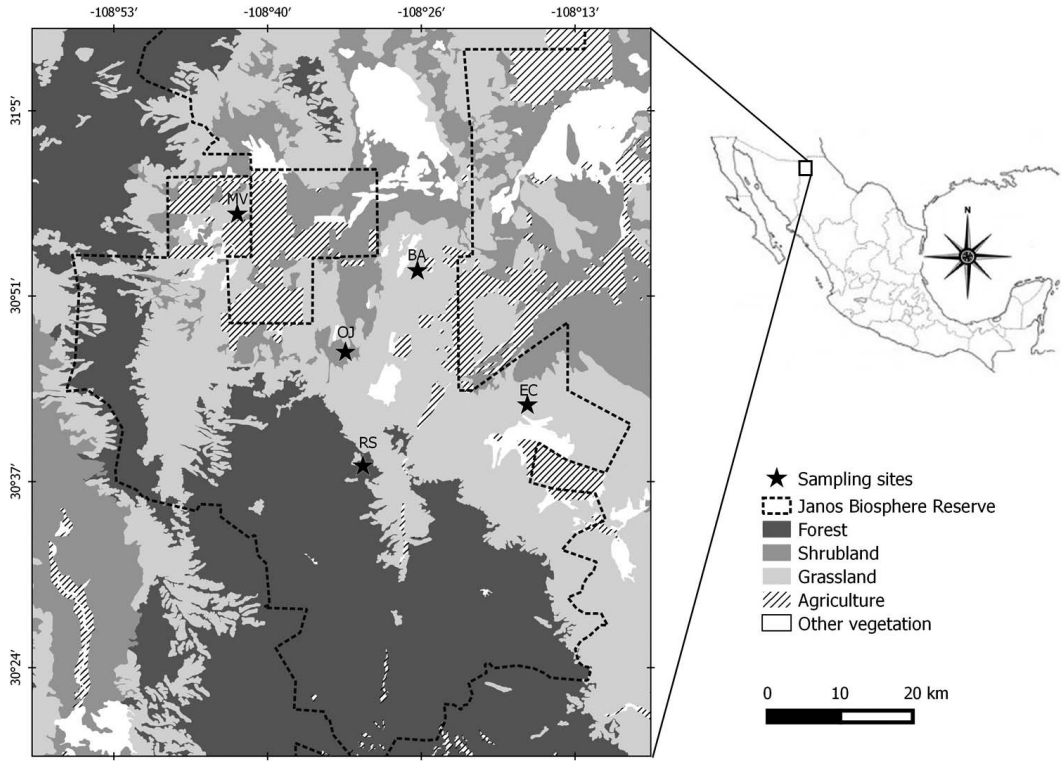

FIGURE 1. Map of Janos Biological Reserve in northwestern Chihuahua, Mexico where wild carnivores were captured in fall of 2013 and spring of 2014 for serologic testing for orthopoxvirus, displaying the five sampling sites (MV=Monte Verde; BA=La Bascula; OJ=Ojitos; RS=Rancho San Pedro; EC=El Cuervo) and habitat types of the region.

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Serologic testing

Serum samples were shipped on dry ice to the Poxvirus and Rabies Branch Laboratory at the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA) for serologic analysis, and placed at –20 C until testing. A modified enzyme-linked immunosorbent assay (ELISA) was used for detection of OPXV immunoglobulin G antibodies (Gallardo-Romero et al. 2016). A crude preparation of the Western Reserve strain of vaccinia virus at a concentration of 0.01 µg/well diluted in carbonate buffer (carbonate–bicarbonate buffer, Sigma-Aldrich Corporation, St. Louis, Missouri, USA; pH 9.6) was used for coating half of the wells of each microtiter plate; the other half of the wells were coated with an equal concentration of BSC-40 cell lysate (CDC Core Facility, Atlanta, Georgia, USA). Animal sera were tested at 1:100, 1:200, and 1:400 dilutions in duplicate. A volume of 100 µL/well of recombinant protein A/G labeled with horseradish peroxidase (Pierce™ Life Technology Corporation, Grand Island, New York, USA) diluted to 1:10,000 in blocking buffer was used as conjugate. SureBlue™ 3,3′,5,5′-tetramethylbenzidine (TMB) microwell peroxidase substrate (KPL, Inc., Gaithersburg, Maryland, USA) and TMB stop solution (KPL) were used to develop the plates. Finally, plates were shaken for 5 s and analyzed at 450 nm on a spectrophotometer (SpectraMAX® 190; Molecular Devices, LLC, Sunnyvale, California, USA) to obtain the optical density (OD) values.

The average of the OD values from the duplicate of a sample in the virus half of the plate minus the average plus 2 SDs of the duplicates from the same sample in the lysate half of the plate was used to generate a cutoff value. A sample was considered positive by ELISA to the presence of anti-OPXV antibodies if the average of the duplicates of the OD values was above the cutoff value.

Following the initial ELISA screening, western blots (WBs) were performed using the Wes™ technology (ProteinSimple, San Jose, California,

USA) following the manufacturer's recommendations for a 25-well plate protocol using a 12–230-kDa kit. Briefly, purified Western Reserve vaccinia virus at a concentration of 0.2 μg/μL was used, sera were tested at a 1:50 dilution, and recombinant protein A/G labeled with horseradish peroxidase (Pierce) was used at a 1:10,000 dilution for the experimental samples. Naïve rabbit serum was used as negative control at a 1:50 dilution, and rabbit anti-vaccinia hyperimmune serum was used as a positive control of the assay at a 1:100 dilution. The anti-rabbit conjugate included in the kit was used with the positive and negative rabbit sera. A sample was considered positive by WB if at least one of the four immunodominant bands: 25, 39, 49 or 62 kDa, was present, as these bands are known to be associated with OPXV infection (Demkowicz et al. 1992; Hutson et al. 2007; Gallardo-Romero et al. 2012, 2016; Salzer et al. 2013).

Definition of a true positive

We defined a sample as a true positive (with previous exposure to an OPXV) by assessing the aggregate results of the WB and ELISA testing. An animal was considered seropositive for OPXV if either 1) two ELISA dilutions were positive and one or more OPXV-associated immunodominant bands were detected, or 2) one ELISA dilution was positive and two OPXV-associated immunodominant bands were detected (Demkowicz et al. 1992; Hutson et al. 2007; Gallardo-Romero et al. 2012, 2016; Salzer et al. 2013).

The presence of rabies virus neutralizing antibodies (rVNA) was determined by the rapid fluorescent focus inhibition test (RFFIT; Smith et al. 1996). Before testing, sera were thawed and heat-inactivated at 56 C for 30 min. The rVNA titers of individual samples were calculated by the Reed-Muench method (Reed and Muench 1938) and converted to international units (IU/mL) by comparison with a standard rabies immunoglobulin control containing 2 IU/mL. Positive rVNA titers were defined by at least 50% neutralization of the rabies virus challenge dose (50 focus-forming doses) at a 1:5 dilution (i.e., $\geq$0.06 IU/mL). Titers $<$0.06 IU/mL were considered negative for the presence of rVNA.

Habitat classification

Vegetative land-cover classifications present at Janos Biosphere Reserve include forest, shrubland, grassland, agriculture, and other vegetation (Fig. 1). Sampling sites that were located at the junction of two habitat types are classified as an ecotone, a transitional vegetation type. The habitat classification was based on a 1:250,000-scale land-cover geographic information system database (National Institute of Statistics and Geography, Aguascalientes, Mexico). Habitat types were visually ground truthed within a 100-m radius of each trapping site.

RESULTS

Trapping efforts yielded a total of 51 carnivores representing seven species (Table 1) including: coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), kit fox (*Vulpes macrotis*), bobcat (*Lynx rufus*), raccoon, American badger (*Taxidea taxus*), and striped skunk (*Mephitis mephitis*). Of the samples tested by WB, 59% (30/51) showed bands consistent with bands commonly found in OPXV infections (Demkowics et al. 1992; Hutson et al. 2007; Gallardo-Romero et al. 2012, 2016; Salzer et al. 2013). The 62-kDa band was present in 43% (22/51) of animals, the 39-kDa bands in 25% (13/51), and 49-kDa band in 35% (18/51). The bands at 75 kDa and 95 kDa were observed in six animals.

Our results showed 51% (26/51) of animals with anti-OPXV antibodies by ELISA. On the basis of our definition of a true positive, 47.1% (24/51) of the animals were suspected to have had previous exposure or a current infection by a circulating OPXV. Of these 24 positive samples, all had at least two immunodominant bands present, 33% (8/24) had only one ELISA dilution positive, and the remaining 67% (16) had both ELISA dilutions positive. Species-specific seroprevalence rates were calculated (Table 1). Raccoons displayed the highest seroprevalence rates (75%), followed by coyotes (67%) and kit foxes (55%). Seroprevalence rates in each habitat type sampled are as follows in descending order: forest and grassland ecotone (60%), grassland (59%), shrubland (36%), and forest (20%). One kit fox from the grassland showed high OD values in both ELISA dilutions, but was negative by WB. One coyote from the grassland was positive by ELISA only in the 1:100 dilution, and showed the 62-kDa band. This animal was considered negative because it did not meet the requirements to be considered a true positive according to our definition, even though it presented low

TABLE 1. Species-specific orthopoxvirus (OPXV) seropositivity of wild carnivores captured during fall of 2013 and spring of 2014 at the Janos Biosphere Reserve in northwestern Chihuahua, Mexico. A sample was considered seropositive for orthopoxvirus if either 1) two enzyme-linked immunosorbent assay (ELISA) dilutions were positive and one or more OPXV-associated immunodominant bands were detected by western blot assay (WB), or 2) one ELISA dilution was positive and two OPXV-associated immunodominant bands were detected by WB.

Common name	Species	Total samples	Total seropositive	Percent seropositive
Raccoon	*Procyon lotor*	4	3	75
Coyote	*Canis latrans*	15	10	67
Kit fox	*Vulpes macrotis*	11	6	55
American badger	*Taxidea taxus*	5	2	40
Gray fox	*Urocyon cinereoargenteus*	6	2	33
Striped skunk	*Mephitis mephitis*	5	1	20
Bobcat	*Lynx rufus*	5	0	0
Total		51	24	47

positive values in both assays. Four wild carnivores (one bobcat, one raccoon, and two gray foxes) were negative by ELISA but presented one or more immunodominant bands. However, because of our definition of a true positive, these animals were considered negative.

The 62-kDa band is likely a protein encoded by the gene *A10* (VV-Cop ortholog), the most abundant major core protein important for viral assembly and the gene responsible for stimulation of memory B-cell production. The 49-kDa band may be encoded by the *A36R* gene that codes for proteins from 43 to 50 kDa that accumulate late during infection, present on the surface of the extracellular viral envelope. The 39-kDa band is a protein encoded by the *G9R* gene, highly conserved throughout the Poxvirus family and is required for efficient viral replication (Ojeda et al. 2006).

All samples were negative (<0.5 IU/mL) for rVNAs by RFFIT. World Organisation for Animal Health guidelines stipulate that values >0.5 IU/mL represent an adequate response to rabies vaccination (World Organisation for Animal Health 2013).

DISCUSSION

The observed prevalence of anti-OPXV antibodies and immunodominant bands consistent with OPXV infection within the carnivores we captured in northwestern Mexico suggests that OPXV infections in this area are widespread and relatively common. Ecological studies with OPXV elsewhere in the world show that rodents are the primary reservoir for this genus of virus (Crouch et al. 1995; Begon et al. 1999; Chantrey et al. 1999; Hazel et al. 2000; Doty et al. 2017). Our results indicate either that circulation of OPXV exists within many carnivore species in northwestern Mexico, or that OPXV infection may potentially have been transmitted to these carnivore species by interaction with host reservoirs (e.g., ingestion of prey), particularly rodents.

The exposure of OPXV in carnivores of our sampling sites may be a result of the dietary habits of these species and trophic ecology. Some ecoepidemiologic disease models have demonstrated that predators may indirectly promote epidemics of infectious diseases, and disease epidemics may taper off in an environment that becomes free of predators (Morozov 2011). Given that rodents are one of the primary prey items for these predators, predator–prey–pathogen interactions may play a role in the natural disease transmission linkages of OPXV in North America. In a population of infected prey, the presence of a predator has been shown to enhance the success of a pathogen (Baker and Smith 1997;

Caceres et al. 2009; Morozov 2011). This predator-dependent dynamic may exist in northwestern Mexico; however, further research into the ecologic aspects of OPXV transmission in this region is needed. The seroprevalence found in the forest and grassland ecotone and grassland habitat was comparatively higher than the seroprevalence of the forest and shrubland; however, this result may potentially be due to the majority of animals being captured in these two habitats (62%, 32/51). Additional research is needed to delineate the role that habitat may play in the circulation of OPXV in the natural environment.

The USDA Animal and Plant Health Inspection Service has conducted ORV bait drops in Arizona as well as in far-west Texas, which is approximately 210 km from our study site (Blanton et al. 2009). On rare occasions, coyotes have been documented traveling long distances; the longest recorded distance was 310 km by a male coyote in the Rocky Mountains (Kolbe and Squires 2004). Given the small likelihood that these animals had a potential exposure to the ORV bait in the US, all samples were additionally tested for presence of rabies antibodies by RFFIT. All samples were negative for rabies virus antibodies, which indicated that all the OPXV positives we observed in this study are not a result of exposure to ORV baits.

Antibodies to OPXV have been identified in previous studies with rodents, skunks (*Mephitis* spp.), and three genera of Procyonidae from central and southeast Mexico (Regnery 1987; Emerson et al. 2009; Martínez-Duque et al. 2013; Gallardo-Romero et al. 2016; Hodo et al. 2018). Our findings supported the previous studies on OPXV in Procyonidae, as we found raccoons to harbor the highest seroprevalence rates in this region of northwestern Mexico. Our results also introduced new evidence of OPXV infection in other carnivores, including the coyote, gray fox, kit fox, and American badger, and indicated that OPXVs can infect or circulate within widely diverse mammalian taxa in Mexico, and should prompt an increased awareness for OPXV in North America and the potential risks to human health that these viruses may pose. Apart from assessing previous OPXV exposure from serologic studies of carnivores, further studies would benefit from additional utilization of molecular and phylogenetic techniques to determine infection status and taxonomy of OPXV.

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