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SEROLOGIC EVIDENCE FOR CIRCULATING ORTHOPOXVIRUSES IN PERIDOMESTIC RODENTS FROM RURAL UGANDA

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ABSTRACT: The prevalence of orthopoxviruses (OPXV) among wildlife, including monkeypox virus (MPXV), remains largely unknown. Outbreaks of human monkeypox in central Africa have been associated with hunting, butchering, and consuming infected forest animals, primarily rodents and primates. Monkeypox cases have not been reported in east Africa, where human contact with wildlife is more limited. Whether this lack of human disease is due to the absence of MPXV in rodents is unknown. However, testing of wildlife beyond the known geographic distribution of human cases of monkeypox has rarely been conducted, limiting our knowledge of the natural distribution of MPXV and other OPXV. To improve our understanding of the natural distribution of OPXV in Africa and related risks to public health, we conducted a serosurvey of peridomestic rodents (*Rattus rattus*) in and around traditional dwellings in Kabarole District, Uganda, from May 2008 to July 2008. We tested for OPXV antibody in areas free of human monkeypox. Sera from 41% of the *R. rattus* individuals sampled reacted to OPXV-specific proteins from multiple, purified OPXV samples, but did not react by enzyme-linked immunosorbent assay. The specific OPXV could not be identified because poxvirus DNA was undetectable in corresponding tissues. We conclude that an OPXV or a similar poxvirus is circulating among wild rodents in Uganda. With the known geographic range of OPXV in rodents now increased, factors that dictate OPXV prevalence and disease will be identified.

Key words: ELISA, monkeypox, *Orthopoxvirus*, poxvirus, *Rattus*, rodent, Uganda, Western blot.

INTRODUCTION

Orthopoxvirus is a genus within the viral family *Poxviridae*. Orthopoxviruses (OPXV) include several pathogens of human health concern, such as monkeypox, cowpox, and variola (the causative agent of smallpox) (Damon, 2006). Orthopoxviruses were originally defined by their similar immunologic properties. Once an individual is infected and develops a humoral immune response to an OPXV infection, anti-OPXV immunoglobulin G (IgG) will be present in this individual's serum. In addition to providing cross-protection, these antibodies provide serologic evidence of a previous exposure to an OPXV (Karem et al., 2005).

Since the eradication of smallpox and the discontinuation of vaccination programs in 1979 (Breman and Henderson, 1998), monkeypox has emerged as a threat to human health. In endemic zones of central and west Africa, mortality associated with monkeypox virus infections reaches 10% (Heymann et al., 1998). Although monkeypox occurs throughout the Democratic Republic of Congo (DRC), no human cases have been reported across the eastern political border shared between DRC and Uganda (Levine et al., 2007). This restricted spatial distribution could be due to several factors, including the absence of viable rodent reservoirs, environmental conditions, or cultural norms resulting in

lower human contact with potential wildlife reservoirs beyond the zone of monkeypox endemism (Levine et al., 2007).

The roof rat (*Rattus rattus*) is considered a highly invasive, opportunistic species, thought to have been first introduced to Uganda in 1910 (Delany, 1975). Roof rats are known to transmit zoonotic pathogens, such as *Yersinia pestis* (Eisen et al., 2008), *Leishmania* spp. (Quinnell and Courtenay, 2009), *Cowpox virus* (Martina et al., 2006), and hantaviruses (Cueto et al., 2008). In western Uganda, roof rats are usually found in areas associated with human dwellings (Delany, 1975). It is common for people in western Uganda to find rodents in food storage areas and fecal contamination on food for human consumption (Gillespie, pers. obs.). Because roof rats typically have an overlapping habitat with humans, their infection status can provide evidence of zoonotic disease risk in the human population.

Although the rodent reservoir species is not known for many OPXVs, members of this viral genus infect a broad range of rodent hosts (Damon, 2006). Despite much effort, MPXV has been isolated only once from the wild (Khodakevich et al., 1986); therefore, serosurveys are valuable for evaluating OPXV circulation among wildlife in the absence of viral isolation. We hypothesized that the evaluation of serum from roof rats for the presence of OPXV IgG would provide evidence of potential zoonotic disease risk at the interface of human-wildlife interactions.

FIGURE 1. Roof rats (*Rattus rattus*) were collected in and around human dwellings near Kibale National Park in Uganda to test for antibodies against orthopoxviruses such as monkeypox virus. Although monkeypox occurs throughout the Democratic Republic of Congo (DRC), no human cases have been reported across the eastern political border between DRC and Uganda.

MATERIALS AND METHODS

Acquisition of animal samples

From May 2008 to July 2008, 44 roof rats were live-trapped inside and within close proximity of traditional dwellings near Kibale National Park in the Kabarole District of Uganda (0°13′–0°41′N, 30°19′–30°32′E; Fig. 1) using Sherman live-capture traps (3×3.5×9; H.B. Sherman Traps, Inc., Tallahassee, Florida, USA). We targeted areas with high human population densities, such as villages and trading centers. Traps were baited with various types of local human food and set overnight. In the morning, traps were picked up and transported a short distance to a field laboratory. Roof rats were removed from traps and anesthetized with 5% isoflurane. While the rats were in a deep plane of anesthesia, 2 mL of blood was collected from each rat via cardiocentesis, followed by humane euthanasia. Each rat was photographed, and standard measurements were recorded and used for species confirmation (Delany, 1975). Samples of the lung, liver, spleen, and any apparent skin lesions were collected postmortem. All samples were shipped on dry ice to the Centers for Disease Control and Prevention (Atlanta, Georgia, USA). All procedures and animal collection were approved by the University of Illinois Urbana-Champaign institutional animal care and use committee (06037).

PCR analysis

DNA was extracted from the spleen tissue of all 44 rats and from skin lesions of two rats with the DNA EZ1 tissue kit (Qiagen, Valencia, California, USA). All DNA samples were screened for poxvirus DNA using guanine-cytosine (GC) content-based pan-pox

universal PCR assay (Li et al., 2010). These assays should detect all known viruses within the subfamily *Chordopoxviridae*, except members of the genus *Avipoxvirus*. In addition, DNA extracted from skin lesions was analyzed using more-specific RT-PCR assays to identify OPXV genus-specific DNA (Li et al., 2007).

Enzyme-linked immunosorbent assay (ELISA)

Serum from each rodent was assessed for IgG antibodies to OPXV using a modification of a previously published ELISA (Hutson et al., 2009; Keckler et al., 2011). Specifically, we tested each sample against *Vaccinia virus*, a member of the OPXV genus. The ELISA plates were prepared with crude *Vaccinia virus* (Dryvax strain) with two modifications made from the published assay (Keckler et al., 2011). The first modification was that each serum sample was run at a 1:100 dilution in quads (four times each run) to ensure the quality of results and to reduce false-positive results because of variability among individual wells. The second modification was the use of a *R. rattus*–specific peroxidase-labeled, affinity-purified antibody, goat–anti-rat IgG at a 1:1,000 dilution in blocking buffer (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland, USA). The final optical density (OD) values were obtained by averaging the OD values from the four wells containing *Vaccinia virus* minus the average OD and 2 SDs of the OD values from the corresponding lysate wells (Keckler et al., 2011).

Western blot (WB) assay

A WB assay was performed to identify specific OPXV protein profiles in the captured animals. Specific viruses included cowpox-Brighton (CPXV-BR), cowpox-Sweden (CPXV-SW), monkeypox-ROC2005 (MPXV), vaccinia-Dryvax (VACV), camelpox (CMLV), and taterapox (TATV). Each virus was propagated in BSC-40 cells (Centers for Disease Control and Prevention, Atlanta, Georgia, USA). Crude virus was purified using a 36% sucrose cushion and standard poxvirus purification protocols (Smallwood et al., 2010). The BSC-40 cell lysate proteins were included in each assay as a control to test for nonspecific binding of antibodies to cellular debris potentially remaining in semi-purified viral stock. In addition to using crude BSC-40 lysate protein, BSC-40 cells were mock-purified using identical methods designed for poxvirus purification and tested against serum samples to ensure adequate purification. Purified viral proteins (15 μg) from CPXV-BR, CPXV-SW, MPXV, VACV, CMLV, and TATV and crude BSC-40 lysate were incubated with Laemmli buffer (Bio-Rad, Hercules, California, USA) containing 2-mercaptoethanol at 5% and boiled for 5 min. Each sample was separated by 4–20% gradient polyacrylamide gel electrophoresis (Ready Gel Tris-HCL; Bio-Rad), and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad). Molecular-weight standards were run on each gel (Precision Plus Kaleidoscope 1Kb; BioRad). After the protein transfer, PVDF membranes were blocked for 2 hr using 200 mg of dry milk in 100 mL of phosphate-buffered saline and 0.1% Tween (PBST). Membranes were then washed with PBST three times each for 10 min and were probed with rodent serum at a dilution of 1:1,000 in blocking buffer at 4 C overnight. Next, membranes were washed with PBST three times each for 10 min and exposed to goat–anti-rat secondary antibodies, alkaline phosphatase (AP) conjugate IgG at a dilution of 1:3,000–1:6,000 in blocking buffer for 1 hr (Promega, Madison, Wisconsin, USA), followed by three washings as described above. Immunoreactive interactions between rodent serum antibodies and OPXV proteins were identified using autoradiography detection (Immun-Star AP substrate, Bio-Rad). Immunodominant bands present on each blot were recorded, and the molecular weights in kilodaltons (kDa) of each of these bands were determined from a linear regression on the log scale of the molecular weight standards for each blot (Turner and Baxby, 1979). Any viral protein band that matched in size to those found in the BSC-40 lysate were considered negative. Antiserum from rodents with confirmed exposure to MPXV and from rodents with no known OPXV exposure were used as positive and negative controls respectively.

RESULTS

PCR and ELISA

Extracted DNA from spleens ($n=44$) and skin-lesion samples (from 2 of the 44 rodents) were negative for poxvirus DNA using Pan-Pox Universal PCR assays (data not shown) (Li et al., 2007). The DNA from skin lesions was also negative for OPXV DNA using reverse-transcription PCR. Thus, all animals were negative for current infection with a poxvirus at the time of euthanasia. All samples had ELISA OD values <0.01 and were considered antibody-negative by ELISA (data not shown).

Western blot assay for anti-OPXV antibodies

Antiserum collected from an MPXV-infected prairie dog (*Cynomys ludovicianus*)

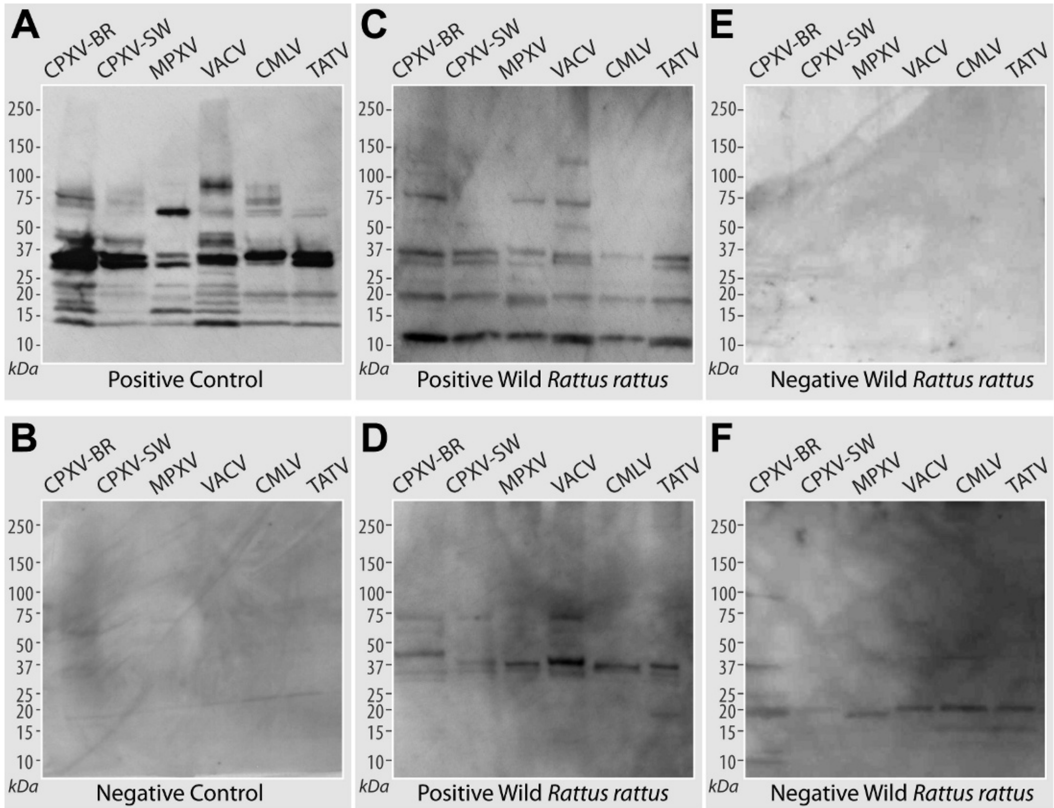

FIGURE 2. Specific purified viruses used for the orthopoxvirus (OPXV) Western blot assay were cowpox-Brighton (CPXV-BR), cowpox-Sweden (CPXV-SW), monkeypox-ROC2005 (MPXV), vaccinia-Dryvax (VACV), camelpox (CMLV), and taterapox (TATV). Shown are blots are of serum from (A) an MPXV-infected prairie dog (positive control), (B) an uninfected prairie dog (negative control), (C and D) two individual roof rats (*Rattus rattus*) representing positive serum samples for OPXV reactivity, and (E and F) two roof rats considered negative for OPXV reactivity.

was used as a positive control and detected multiple viral proteins from all OPXVs tested (Fig. 2A). Although many bands are present, a select few were consistently more immunodominant (darker) than others. This positive control antiserum detected an immunodominant doublet in the range of 30–39 kDa from all purified OPXVs surveyed. This homologous doublet is consistent with two immune-dominant proteins characterized in mature OPXV virions: the 32-kDa D8 envelope protein and the 39-kDa A4 core protein (sizes given are in accordance to VACV) (Demkowicz et al., 1992). The MPXV-infected prairie dog serum also detected a high molecular-weight band consistently immunodominant in lanes containing VACV and MPXV proteins and often present but less dominant against other OPXVs. This third immunodominant homologous protein was 62 kDa in VACV and is consistent with the molecular weight of A10, a well-characterized viral core protein and supports findings in previous VACV studies (Demkowicz et al., 1992). Serum from uninfected prairie dogs (Fig. 2B) produced no such reaction, suggesting that the bands detected in the positive control were due to bona fide reactions between anti-OPXV antibodies and viral antigens. The mock-purified lysate antigen did not produce any reactive bands on the membranes when tested against the controls and sample serum. Therefore, we concluded that the purified virus used in this

study was of very high quality with no detectable cell lysate material in the viral stocks.

The A10, A4, and D8 proteins exhibit profound immunodominant characteristics on WB using serum from humans inoculated with VACV and testing of that serum against VACV proteins (Demkowicz et al., 1992), and this pattern is identical to WB patterns against VACV and MPXV found using serum from both prairie dogs (Fig. 2A) and humans recovered from monkeypox (Dubois and Slifka, 2008). Thus, the ability of antiserum to detect these bands is considered an indicator of a past OPXV infection. This method for defining the positive protein banding patterns of OPXV has been used and accepted in previous studies (Azwai et al., 1996) and is more specific and informative than simply identifying positive samples as any sample with nonspecific reaction to viral proteins (Goldberg et al., 2008). Thus, we defined a serum sample from wild rodents as positive for OPXV antibodies if the serum detected the immunodominant doublet in most of the immunoblotted denatured OPXV. Because the A10 (62 kDa in VACV) protein is only immunodominant in a select few OPXVs (specifically MPXV and VACV), we noted the immunodominant bands present but did not require this immunodominant band to be present for a blot to be considered positive.

Using the guidelines described above to define a sample as positive for anti-OPXV antibodies, we identified 18 of the 44 wild *R. rattus* samples as having IgG antibodies reactive to the 39-kD (putative A4) and 32-kD (putative D8) proteins. Figures 2C and 2D are examples of wild rodent antisera from two *R. rattus* samples that were considered positive with immunodominant bands against A4 and D8 proteins (39 and 32 kDa in VACV). In addition, these blots are examples of immunodominant reaction to A10 protein measuring 62 kDa in VACV. Figures 2E and 2F are two examples of wild rodent antisera considered negative for anti-OPXV antibodies because of a lack of immunodominant doublet bands between 32 and 39 kDa on most OPXV proteins.

We concluded that 40.9% (18 of 44) of the roof rats sampled in this study had previous exposure to an OPXV or a related virus within the *Poxviridae*. Of the 18 positive samples, 15 (34.1% of rats tested) also reacted with a homolog 62-kDa band that is representative of protein A10 (Fig. 2C, D) (Demkowicz et al., 1992; Dubois and Slifka, 2008). Although the banding patterns of these blots appear similar to patterns expected from MPXV- and VACV-exposed individuals, we cannot definitively say these viruses are responsible for the antibody-positive results in this sample set.

DISCUSSION

We identified anti-OPXV IgG antibodies in serum from peridomestic roof rats in rural western Uganda—an area with no previous reports of OPXV in rodents and no reports of MPXV in humans. Despite the substantial field effort that has been made in central Africa to identify the reservoir of MPXV, a viral isolate has only been identified in one wild animal (Khodakevich et al., 1986). In addition to the historic difficulties in identifying viral isolates from wildlife, the small sample size precluded us from isolating a specific OPXV from these wild rodents; however, our confirmation of OPXV antibodies in this system highlights the need for ongoing research at this interface.

We also observed that the OPXV WB assay was a more-sensitive diagnostic than ELISA when screening serum samples from roof rats for antibodies to specific OPXVs. This sensitivity difference could be because ELISA methods use whole-virus particles, and ELISA, therefore, tests the ability of IgG antibodies to bind primarily to membrane proteins, whereas a WB assay uses denatured proteins, consisting of membrane and core proteins,

and tests the ability of IgG antibodies to bind to both membrane and core proteins. Our results are consistent with historic studies that conclude that two of the three most immunodominant proteins are core proteins, specifically A4 and A10; therefore, IgG would have the ability to bind to those proteins more readily using a WB assay as opposed to an ELISA (Moss, 2006). Another potential explanation for the difference in sensitivity between these two assays is that the antiserum reaction seen in these samples may be due to exposure to an unknown non-OPXV member of the *Poxviridae* with similar, conserved core proteins as those in OPXVs but variation in the less-conserved membrane proteins.

Although our WB assay results do not identify the specific OPXV responsible for the immunologic reaction seen in our study, we can conclude that the rats have been infected and mounted an immune response to a member of the *Orthopoxvirus* genus or an unknown relative in the *Poxviridae* family. Currently, MPXV is the only known zoonotic OPXV in sub-Saharan Africa. Historically, monkeypox outbreaks have occurred in the Congo basin where handling of infected animal tissue is thought to be the main source of infection, with human-to-human transmission occurring less frequently (Khodakevich et al., 1987; Jezek et al., 1988). Areas where previous outbreaks of monkeypox have occurred consist primarily of a population of people that rely on hunting and bush meat as a primary food source (Khodakevich et al., 1988). The people of western Uganda rely mainly on subsistence farming and domestic cattle (*Bos primigenius*), goats (*Capra aegagrus hircus*), and sheep (*Ovis aries*) (Struhsaker, 1997). This difference in primary food sources and subsequent wildlife contact indicates a critical control point at which the people of western Ugandan are possibly not exposed to zoonotic pathogens transmitted through tissue handling to the same level as individuals in DRC. Therefore, the human population of western Uganda may be at lower risk for transmission of MPXV or novel zoonotic OPXVs.

Our results expand the known geographic range of poxviruses in wild rodent populations. Future work examining factors contributing to the absence of poxvirus infection in Ugandan human populations despite geographic overlap with infected small mammals may help us understand how endemic African OPXVs have remained contained and may help us to determine future poxvirus-related risks to human health.

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