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ASSESSING FLAVIVIRUS, LENTIVIRUS, AND HERPESVIRUS EXPOSURE IN FREE-RANGING RING-TAILED LEMURS IN SOUTHWESTERN MADAGASCAR

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ABSTRACT: The ring-tailed lemur (*Lemur catta*) is an endangered species found in southwestern Madagascar, and understanding infectious disease susceptibility is an essential step towards the preservation of wild and captive lemur populations. Lemurs are primates that are widely dispersed throughout the island of Madagascar and may serve as hosts or reservoirs for zoonotic infections. The aim of this study was to determine the prevalence of antibodies to West Nile virus (WNV), simian immunodeficiency virus (SIV), and herpes simplex virus type 1 (HSV-1) in a population of free-ranging ring-tailed lemur from the Beza Mahafaly Special Reserve, Madagascar. Samples were collected from 50 animals during field capture studies in June and July 2004 and assayed for presence of viral antibodies during the 12 mo following collection. Forty-seven of the 50 lemurs sampled had antibodies against WNV detectable by epitope-blocking enzyme-linked immunosorbent assay (ELISA). In addition, 50 of 50 samples had titers against WNV ranging from 80 to ≥1,280 using plaque reduction neutralization test ($PRNT_{90}$). Ten lemurs had antibodies against lentiviral antigens as determined by Western blot analysis. None of the lemurs had antibodies against HSV-1 using ELISA.

Key words: Herpes simplex virus type 1, *Lemur catta*, Madagascar, ring-tailed lemur, serologic status, simian immunodeficiency virus, West Nile virus.

INTRODUCTION

Lemurs are ranked among the most endangered primates due to unprecedented levels of habitat loss and fragmentation (Flügger and Pfeiffer, 1992). One consequence of a rapidly increasing human population is that wild animals and human beings are coming into more frequent contact, potentially resulting in increased transmission of infectious agents between lemurs and man.

In 1978, the Beza Mahafaly Special Reserve was established in southwestern Madagascar. This reserve includes birds, mammals, reptiles, and insects in a deciduous and semideciduous forest (Sauther, 1991). Several lemur species reside in the Beza Reserve including the endangered ring-tailed lemur (*Lemur catta*). Since 1987, ring-tailed lemurs have been the focus of a long-term study of this species' demographics, socioecology, and general health (Sussman, 1991; Sauther et al., 1999). Until recently, however, little has been done to characterize the disease profile of this species. In June and July of 2003, complete health assessments were performed on 70 ring-tailed lemurs and diagnostic samples were collected for bacterial and parasitic analysis at Beza Mahafaly (Sauther et al., in press). Similar assessments were performed on 20 free-ranging adult ring-tailed lemurs from another reserve in Madagascar (Dutton et al., 2003). Screening of lemurs within reserve habitats for evidence of human viral infections (adenovirus group, influenza A and B, parainfluenza 1, rotavirus group, hepatitis A, hepatitis B surface antigen) produced negative results (Dutton et al., 2003; Junge and Louis, 2005). Madagasy lemur sera collected from 1978 to 1980 were screened for antibodies to arboviruses. Positive reactions to flavivirus antigens, mainly West Nile virus (WNV), were detected in 39 of the 209 animals. Additionally, nine of 22 ring-tailed lemurs

had been previously exposed to one or more flaviviruses (Rodhain et al., 1982). In a subsequent study, it was found that brown lemurs (*L. fulvus*) were susceptible to clinically inapparent infection by both WNV and yellow fever virus (Rodhain et al., 1985). Experimental infection of brown lemurs with WNV resulted in viremia titers of sufficient magnitude and duration to infect blood-feeding *Aedes aegypti* mosquitoes. Yellow fever virus–infected lemurs had lower viremia titers. Another serological survey conducted over the whole island of Madagascar on sera from 563 animals and 626 human beings collected between 1965 and 1982 demonstrated that WNV had the highest seroprevalence among nine different arboviruses tested (Fontenille, 1989).

While there is no documented evidence of enzootic viral disease in ring-tailed lemurs, lentiviruses have been identified in many African nonhuman primate species (Hussain et al., 2003; Apetrei et al., 2004). While lentiviruses have not been identified in wild primates outside of the African continent, the unique phylogeny of lemurs with respect to continental African primates provides a basis for analysis of these species for potential lentiviral infections. Many Old and New World primate species are susceptible to herpesviruses, which are generally chronic and relatively apathogenic for species-adapted strains (Henkel et al., 2002). In contrast, transmission of herpesviruses between primate species results in enhanced morbidity for the nonadapted strain (Kornegay et al., 1993; Mansfield et al., 1998) including one report describing an epizootic resulting from human herpesvirus transmission to lemurs (Kemp et al., 1972). Therefore, this virus family is another logical candidate for investigation of enzootic infections in lemur populations. The objective of this study was to determine the prevalence of antibodies to WNV, simian immunodeficiency virus (SIV), and herpes simplex virus type 1 (HSV-1) in a population of free-ranging ring-tailed lemurs from Beza Mahafaly Special Reserve (Madagascar). Information obtained is relevant for management of this endangered species, as a measure of health assessment and as a method to evaluate the role of this primate in serving as a host species for potential zoonotic pathogens.

MATERIALS AND METHODS

Plasma samples were obtained from 50 ring-tailed lemurs during ongoing studies of health and nutrition in the reserve in June and July 2004. Procedures were approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC). Lemurs were immobilized using a blow dart containing ketamine (60 mg), and blood samples were collected in heparin tubes via femoral venipuncture. Blood samples were centrifuged at 800 × G for 10 min, and plasma was collected and frozen in 1 ml aliquots and stored in liquid nitrogen. Samples were shipped to Colorado State University, where they were stored at −70 C until testing from August 2004 to August 2005. Control sera were obtained from archival samples collected from three ring-tailed lemurs at the St. Louis Zoological Park prior to 2000.

Sera were tested for antibodies to flaviviruses by epitope-blocking enzyme-linked immunosorbent assay (ELISA) following the standard protocol (Blitvich et al., 2003). Briefly, ELISAs were performed using the WNV NS1 protein–specific monoclonal antibody (MAb) 3.1112G (Chemicon International, Temecula, California, USA) or the flavivirus E protein–specific MAb 6B6C-1 (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA). The ability of the test sera, diluted 1:10, to block the binding of the MAbs to WNV antigen was compared to the blocking ability of a pooled suspension of control serum from three captive lemurs without antibody to WNV. Data were expressed as percentage of inhibition and values ≥30% were considered indicative of the presence of antibodies to WNV. All sera were tested by plaque reduction neutralization test (PRNT) using WNV (strain NY99-35261-11) according to standard methods (Beaty et al. 1995). Plaque reduction neutralization tests were performed using African green monkey kidney (Vero) cells. Two-fold dilutions of sera were tested, starting at a dilution of 1:20. Titers were expressed as the reciprocal of serum dilutions

yielding ≥90% reduction in the number of plaques ($PRNT_{90}$).

Because we anticipated that a lemur-specific lentivirus would be significantly divergent both genetically and antigenically from African primate SIV, we designed a multiplex immunoblot assay using both simian and felid lentiviral antigens to maximize the possibility of detecting antibodies. We adapted a Western blot procedure that has been useful for detection of serum antibodies from a large number of feline species against nonspecies-specific lentiviral antigens (VandeWoude et al., 1997), and used a method for detecting lemur antibodies that did not rely on cross-reactivity between anti-macaque antibodies and lemur IgG. Sera were tested by immunoblot analysis for antibodies that reacted with SIV (strain SIVmac251), three unique feline immunodeficiency viruses (FIV strain B-2546 from a domestic cat [FIV]; FIV-Pco, puma lentivirus strain PLV-1695 from *Felis concolor* [PLV]; and FIV-Ple, strain LLV-458 from *Panthera leo* [LLV]), or growth medium control as previously described (VandeWoude et al., 1997). Antigens were purified from virus-infected cell culture medium by sucrose gradient centrifugation. Purified viral proteins were quantified using the Pierce bicinchoninic acid protein determination assay (Pierce Biotechnology, Inc., Rockville, Illinois, USA). Forty micrograms of each antigen preparation were separated by 12% polyacrylamide gel electrophoresis and transferred electrostatically to a polyvinylidene fluoride membrane (Milipore, Billerica, Massachusetts, USA). Nylon strips were exposed to 1:50 dilutions of lemur plasma in blocking buffer (phosphate-buffered saline, pH 7.2 containing 5% dehydrated milk), and antibody binding to the immobilized antigens was detected using protein A conjugated to horseradish peroxidase (protein A-HRP, Southern Biotech, Birmingham, Alabama, USA) in blocking buffer. Strips were developed using substrate, peroxidase, and membrane enhancer solutions (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, USA). Known positive serum from a reference species (pigtail macaque J88324 or PLV-infected cat) was used as a positive control (Israel et al., 1993; VandeWoude et al., 2003).

Sera were tested by ELISA for antibodies to HSV-1 using HSV-1–precoated plates (Viral Antigens Inc., Memphis, Tennessee, USA). Sera were diluted 1:10 in blocking buffer and then added to each well. Plates were washed in blocking buffer containing 0.5% Tween-20 (Sigma-Aldrich, St. Louis, Missouri, USA). Protein A conjugated to horseradish peroxidase (Southern Biotech, Birmingham, Alabama, USA) was added to each well, incubated, and plates were washed and developed using the KPL substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland, USA) and peroxidase solutions. Optical densities (OD) were measured using a multiscan spectrum spectrophotometer (ThermoElectron Corp., Waltham, Massachusetts, USA). Goat anti–HSV-1 antibodies conjugated to horseradish peroxidase (Biodesign International, Saco, Maine, USA) were used as a positive control. Sera obtained from six individual cats from a specific pathogen-free colony were used as negative controls, and goat anti–HSV-1 served as a positive control. All lemur sera were diluted 1:10 and run in duplicate. Samples were considered positive if the OD>negative control+3 SD.

TABLE 1. Seroprevalence of antibodies to West Nile virus (WNV) and other flaviviruses as detected by a flavivirus-blocking enzyme-linked immunosorbent assay (ELISA).[a]

Blocking antibody	Positive/total wild	Positive/total control
WNV	47/50 (94%)	0/3
Flavivirus	50/50 (100%)	0/3

[a] ELISA inhibition ≥30% was considered positive. Monoclonal antibody (MAb) 3.1112G is WNV-specific, MAb 6B6C-1 is flavivirus specific. All wild lemurs had antibodies that competed with flavivirus E protein MAb, and 94% had antibodies that competed with WNV NS1 protein MAb.

RESULTS

Epitope-blocking antibodies to the flavivirus E glycoprotein were detected by ELISA in all 50 lemurs (Table 1). Forty-seven (94%) lemurs were also positive in the WNV-specific ELISA, and their WNV $PRNT_{90}$ titers ranged from 80 to ≥1280 (Table 1, 2). Though negative by ELISA, the three other lemurs also had neutralizing antibodies to WNV; two had WNV $PRNT_{90}$ titers of 320, and one had a WNV $PRNT_{90}$ titer of 640. Antibodies to WNV were not detected by blocking ELISA or PRNT in any control sera (Table 2).

Ten lemurs (nine wild lemur and one control sera) had antibodies that bound with relatively strong reactivity to Gag p25

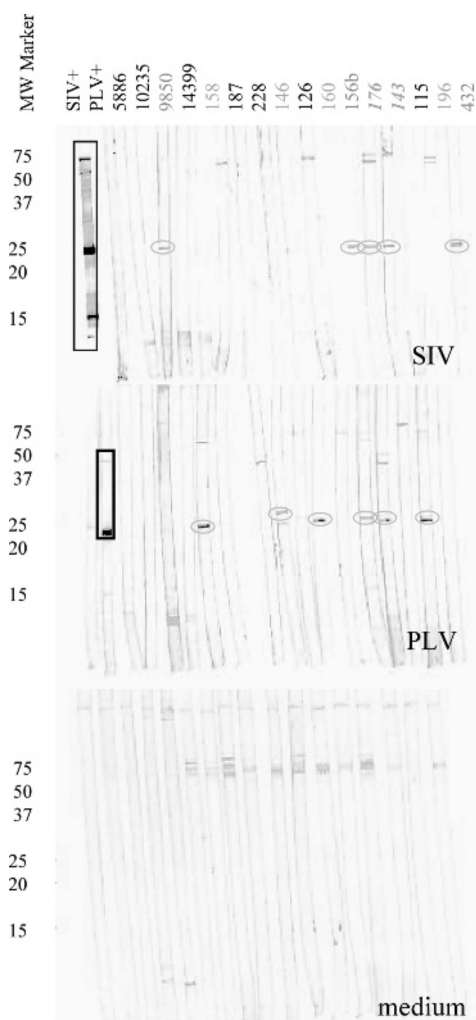

FIGURE 1. Immunoblot assay with feline immunodeficiency virus (FIV) and simian immunodeficiency virus (SIV) antigens detects seroreactivity to Gag p25 in lemur sera. Sera collected from 50 wild *L. catta* were subjected to Western blot analysis against SIV and three types of FIV (domestic cat origin, FIV; *Felis concolor* origin, PLV; *Panthera leo* origin, LLV) antigens as described. Sera were also reacted with proteins isolated from tissue culture supernatant to control for nonspecific binding. Control sera consisted of serum from a macaque infected with SIV (SIV+, banding pattern within black rectangle), and serum from a domestic cat infected with PLV (PLV+, banding pattern within black rectangle). Sera from three captive *L. catta* (5886, 10235, 985) were also tested against all antigens. Naïve cat and primate sera were also negative (data not shown). Molecular weight (MW) marker indicates location of MW band for each individual gel. Grey numbers indicate individuals with antibodies to SIV p25; red indicates antibodies to PLV p25. Antibody positive results are outlined by round ovals. Two individuals indicated by red italics (176, 143) tested positive for antibodies to both PLV and SIV antigens. Sera did not react against media control antigens, demonstrating that reactions were specific for antigens present in infected tissue culture supernatant.

TABLE 2. Distribution of West Nile virus (WNV) $PRNT_{90}$ titers for lemurs with epitope-blocking ELISA antibodies to WNV.[a]

WNV $PRNT_{90}$ titer[b]	Number/total
<20	0
40	0
80	5
160	12
320	11
640	13
≥1,280	6
Total	47

[a] Titer ≥20 is considered positive. $PRNT_{90}$ = plaque reduction neutralization test (≥90% reduction in the number of plaques); ELISA = enzyme-linked immunosorbent assay.

[b] All three negative controls had titers <20.

antigens of at least one lentivirus by Western blot analysis (Fig. 1, Table 3). One lemur had antibodies that reacted with all four types of viral antigens, and four reacted with more than one antigen. Overall, five lemurs had antibodies that reacted with SIV (four wild lemur, one control) and three had weak or indeterminate reactions against these antigens. Six animals had antibodies that reacted with PLV strongly, with one indeterminate reaction. One animal had antibodies that reacted with FIV, and four had antibodies that reacted with LLV antigens (Table 3). One of the control captive lemur samples also recognized the p25 SIV antigen (Fig. 1). All sera were negative in control Western blots that contained uninfected cell culture medium proteins (Fig. 1). Positive control sera from an SIV-infected macaque showed weak cross-reactivity with FIV and LLV isolates, demonstrating

TABLE 3. Western blot Gag p25 reactivity in wild lemur serum samples.[a]

Lemur ID[b]	SIV	PLV	FIV	LLV	Medium
115	+/−	+/−	—	—	—
143	+	+	—	—	—
146	—	+	—	+	—
156b	+	—	—	—	—
158	—	+	—	—	—
160	+/−	+	—	—	—
176	+	+	+	+	—
196	+/−	+	—	+	—
228	—	—	—	+	—
432	+	—	—	—	—

[a] Supernatant antigen preparations from simian immunodeficiency virus (SIV); puma lentivirus, FIV-Pco (PLV); feline immunodeficiency virus, FIV (FIV); lion lentivirus, FIV-Ple (LLV); and cell culture medium alone. +/− indicates faint or indeterminate reactions. One of three samples obtained from zoo animals was also seroreactive to SIV p25 (not shown).

[b] Forty samples without antibodies to SIV, PLV, FIV, and LLV are not shown. One sample (176) had antibodies that reacted with all four antigens (italicized); three samples (143, 146, 196, bolded) reacted strongly to more than one antigen preparation.

the potential for high titer primate lentiviral antibodies to cross-react with feline lentiviral antigens (Fig. 2).

Antibodies to herpes simplex virus were not detected by ELISA in any lemur serum samples, even when the positive threshold value was lowered to OD>negative control+1 SD. The positive control (goat anti-HSV-1) consistently resulted in ELISA absorbance values >0.9, whereas samples were in the range of <0.3, which was similar to that observed for the negative control sera.

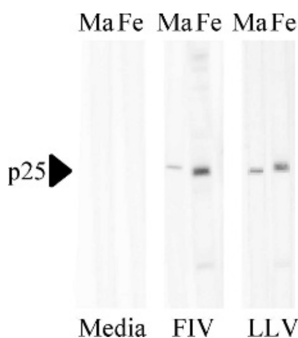

FIGURE 2. Sera from simian immunodeficiency virus positive (SIV+) macaque seroreacts with feline immunodeficiency virus (FIV) antigens. Sera collected from an SIV-infected pigtail macaque (Ma, *Macaca nemestrina*), or PLV-infected cat (Fe), was reacted against media precipitated proteins (media), domestic cat FIV (FIV), or FIV-ple isolated from a lion (LLV). Macaque sera detected Gag p25 antigen in both FIV and LLV preparations as demonstrated. Figure 1 illustrates reaction of sera from this macaque against SIV antigens.

DISCUSSION

The WNV-specific blocking ELISA demonstrated that 47 of 50 lemurs had antibodies to WNV, while the flavivirus blocking ELISA detected antibodies in 50 of 50 lemurs. Sera from captive ring-tailed lemurs collected prior to detection of WNV in the USA were seronegative by both methods. To further investigate this finding, sera were tested by $PRNT_{90}$. All sera were able to neutralize WNV by $PRNT_{90}$, some with titers ≥1280, which may indicate a recent infection. The finding that sera from three lemurs were negative in the WNV blocking ELISA but positive for neutralizing antibodies in the $PRNT_{90}$ could be due to exposure to another flavivirus (e.g., Dengue virus) that cross-reacted with WNV in the $PRNT_{90}$. Testing sera for the specificity of such antibodies will require comparison of $PRNT_{90}$ titers with other flaviviruses, since ring-tailed lemur immunoglobulins were not reliably cross-reactive with anti-human secondary antibodies in direct ELISA (data not shown). However, dengue and yellow fever viruses have thus far not been isolated from vectors in Madagascar (Vazeille et al., 2001). Alternatively, it could be reasoned that these three lemurs were exposed to WNV and that antibody titers to WNV NS1 protein had declined below the designated cut-off level. It is also possible that seropositive results could indicate exposure to dengue, yellow fever, Japanese encephalitis, or an as yet undescribed cross-reactive virus. These results suggests that *L. catta* in zoological collections might be a useful sentinel species for WNV in endemic

areas; a finding reinforced by the report that seropositive *L. catta* were detected at the Bronx Zoo/Wildlife Conservation Park during the 1999 WNV outbreak (Ludwig et al., 2002).

Positive immunoblot reactions were noted against all four lentiviral antigen preparations, while no samples cross-reacted with tissue culture medium, suggesting that the seroreactivity noted was specific. The variation in reactivity of the lemur sera to the antigens and the relatively weak binding patterns (i.e., one band versus 2–6 bands of substantially greater intensity in positive feline or primate control) indicate low reactivity to the viral antigens tested. The amino acid sequence composition among feline lentiviruruses diverges 20–30% in conserved regions of the polymerase gene, (Olmsted et al., 1992; Troyer et al., 2005) and ~40% in the regions of Gag which have been analyzed (Langley et al., 1994; Barr et al., 1997). Homologous amino acid sequence identity between SIV and FIV Gag regions is approximately 60% (Olmsted et al., 1992), but as demonstrated in Figure 2, enough secondary structure and homology overlap exists to result in the ability to detect seroreactivity between anti-SIV antibodies and FIV p25 Gag. Our results thus suggest that lentiviral exposure in lemurs is due to infection with a novel virus with significant divergence from either SIV or FIV. Due to the variable nature of these results, it will be important to correlate these data with isolation or genetic identification of virus from lemur peripheral blood mononuclear cells; however, these data are intriguing as no lentiviruses have been isolated from primate species other than Old World (catarrhine) anthropoid species from Africa (Apetrei et al., 2004).

Most nonhuman primates are infected with species-specific alpha-, beta-, or gamma-herpesviruses. Generally, alpha-herpesviruses induce the strongest humoral antibody response, thus in this study we developed a test using HSV-1 antigens in an attempt to detect reactivity in lemur sera (K. Mansfield, pers. comm.). We developed an assay using a primary antibody detection method that used *Staphylococcus* protein A, a general IgG-binding reagent, with a goat anti–HSV-1 as a positive control, thus diminishing the possibility of false negative results due to poor affinity of the secondary antibody to lemur IgG. Despite this modification, we did not detect antibodies against HSV-1 antigens. While HSV-1 antigens could possibly cross-react with other primate herpesviruses, our results do not preclude the possibility that lemurs are infected with a herpesvirus that we were unable to detect serologically. Given that ulcerative vaginal mucosal lesions have been noted in this population of animals (M. Sauther, unpubl. data), and that Herpesviridae are among the most common viruses infecting primates, this possibility deserves further exploration. This finding also confirms that the lemurs in this population are not endemically infected with a human herpesvirus, though potential of such infection does exist (Kemp et al., 1972).

Future studies will attempt to identify specific flaviviruses that infect other lemur species and *L. catta* from other geographic regions, and verify the presence of a lemur lentivirus using sequence amplification and virus isolation techniques. It would be prudent to perform histologic evaluation and attempt viral isolation and serologic evaluation of *L. catta* exhibiting clinical signs, particularly in animals in zoologic collections with lesions suggestive of active viral infection (i.e., mucous membrane ulceration, vesicular disease, hepatitis). Serosurveys of other Madagascar ring-tailed lemur populations and additional lemur species that occupy other ecologic niches should be conducted to examine the presence of viral infection—particularly WNV and lentiviruses, based on results reported here. Finally, future studies should adapt detection systems for other viral agents in order to test lemur sera for seroreactive antibodies, since we

found that anti-human IgG did not bind to lemur antibodies efficiently.

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