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VALIDATION AND USE OF AN INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODIES TO WEST NILE VIRUS IN AMERICAN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS)* IN FLORIDA

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ABSTRACT: In October 2002, West Nile virus (WNV) was identified in farmed American alligators (*Alligator mississippiensis*) in Florida showing clinical signs and having microscopic lesions indicative of central nervous system disease. To perform seroepidemiologic studies, an indirect enzyme-linked immunosorbent assay (ELISA) was developed to determine exposure of captive and wild alligators to WNV. To validate the test, a group of WNV-seropositive and -seronegative alligators were identified at the affected farm using hemagglutination inhibition (HAI) and the plaque reduction neutralization test (PRNT). The indirect ELISA utilized a rabbit anti-alligator immunoglobulins polyclonal antibody as the secondary antibody, and inactivated WNV-infected Vero cells were used as the coating antigen. For all samples (n=58), the results of the ELISA were consistent with the HAI and PRNT findings. Plasma was collected from 669 free-ranging alligators from 21 sites across Florida in April and October 2003. Four samples collected in April and six in October were positive for WNV antibodies using HAI, PRNT, and the indirect ELISA. This indicated that wild alligators in Florida have been exposed to WNV. These findings can be used as a baseline for future surveys.

Key words: *Alligator mississippiensis*, American alligator, ELISA, HAI, PRNT, serology, West Nile virus.

INTRODUCTION

West Nile virus (WNV) infection was recently identified in farmed American alligators (*Alligator mississippiensis*) in Georgia (Miller et al., 2003) and Florida (Jacobson et al., 2005), USA. During the outbreak in Florida, WNV was isolated from multiple tissues and was confirmed using TaqMan reverse transcription–polymerase chain reaction. Although serology is a powerful tool for determining exposure of animals to specific pathogens, serology was not performed during these outbreaks. Although no deaths were reported, antibodies to WNV were found in Nile crocodiles (*Crocodylus niloticus*) in a commercial farming operation in Israel (Steinman et al., 2003). For arboviruses, the most reliable and usually the most type-specific serologic test is the neutralization test (Beaty et al., 1995). However, certain features of this assay, such as the requirement for using live virus, limit its availability (Ebel et al., 2002). In contrast, the enzyme-linked immunosorbent assay (ELISA) has gained popularity as a screening assay because of its relative ease of operation and rapidity. Recently, an indirect ELISA was developed for detecting antibodies to WNV in chickens (Johnson et al., 2003) and multiple species of wild birds (Ebel et al., 2002; Blitvich et al., 2003). To determine WNV exposure in large numbers of samples from wild and farmed alligators, we developed an indirect ELISA using a rabbit polyclonal antibody produced against alligator immunoglobulins as the secondary antibody (Brown et al., 2001). Here we present our validation findings using plasma of captive alligators that survived an epizootic of WNV infection at an alligator farm in Florida (USA). We also present findings of a serologic survey of free-rang-

TABLE 1. Sampling sites in Florida and numbers of American alligators seropositive by ELISA for West Nile virus antibodies in April and October 2003.

Site number	Sampling site	Latitude	Longitude	April 2003	October 2003	Recaptures[b]
1	Lake Seminole	30°48′N	84°56′W	0/6[a]	0/21	0
2	Lake Iamonia	30°38′N	84°14′W	0/12	0/14	0
3	Lake Miccosukee	30°36′N	83°0′W	0/9	0/11	1
4	Wacissa River	30°12′N	83°57′W	1/4	0/2	0
5	Lake Sampson	29°54′N	82°11′W	1/12	1/8	3
6	Wildlife Research Laboratory	29°36′N	82°20′W	0/9	0/10	0
7	Rodman Reservoir	29°31′N	81°53′W	0/15	0/21	1
8	Orange Lake	29°27′N	82°12′W	0/25	0/20	0
9	Suwannee River	29°19′N	83°05′W	0/2	0/15	0
10	Lake Woodruff	29°06′N	81°24′W	1/19	0/41	0
11	Lake Rousseau	29°01′N	82°34′W	0/5	0/1	0
12	Lake Panasoffkee	28°47′N	82°07′W	0/5	0/15	1
13	Lake Monroe	28°49′N	81°13′W	1/21	0/38	0
14	St. Johns River at Hwy 50	28°31′N	80°52′W	0/19	1/19	2
15	Lake Tohopekaliga	28°09′N	81°20′W	0/20	0/20	0
16	Lake Kissimmee	27°56′N	81°17′W	0/20	1/19	0
17	Blue Cypress Wildlife Conservation Area	27°41′N	80°38′W	0/20	0/22	0
18	Lake Istokpoga	27°23′N	81°18′W	0/20	0/20	0
19	Lake Okeechobee	26°53′N	81°0′W	0/20	1/17	0
20	Lake Trafford	26°25′N	81°29′W	0/20	1/20	1
21	Water Conservation Areas 2 and 3	26°15′N	80°20′W	0/21	0/20	0

[a] Number seropositive per site/total number sampled per site.
[b] Number of alligators captured in April that were recaptured in October.

ing alligators from multiple sites in Florida.

MATERIALS AND METHODS

Alligators and serum/plasma samples

In September and October 2002, a large commercial alligator farming operation in Florida (28°32′N, 80°56′W) had an epizootic of central nervous system disease in alligators up to 2 yr of age (Jacobson et al., 2005). Approximately 2 mo after the epizootic ended, 10 alligators from a pen where deaths occurred (group 1) and 10 from a pen where there were no deaths (group 2) were moved into separate pens. At 3 mo (30 January 2003), 4 mo (28 February 2003), and 9 mo (17 July 2003) after the end of the outbreak, alligators were manually restrained and 3 ml of blood were collected from the cervical supravertebral vein (Olson et al., 1975) of each alligator using a sterile syringe and a 22-gauge needle. Blood was aliquoted into glass tubes containing no anticoagulant and into tubes coated with lithium heparin. Serum and plasma, respectively, were removed, placed into cryotubes, and frozen at −20 C. Plasma samples were transported to the University of Florida (Gainesville, Florida) on dry ice for indirect ELISA validation. Matched serum samples were transported on dry ice to Florida Department of Health Bureau of Laboratories (Tampa, Florida) for determining presence or absence of antibody against WNV using hemagglutination inhibition (HAI) and the plaque reduction neutralization test (PRNT).

Blood samples were similarly collected from 669 free-ranging alligators from 21 sites in Florida (Table 1) during April (305; 115 males and 190 females) and October (364; 158 males, 204 females, and two of unknown sex) 2003. The alligators ranged in size from 49 cm to 177.5 cm and weighed from 0.3 kg to 17.2 kg. All animals were classified as either juveniles (658; 1 to 3 yr old) or subadults (11; 3.5 to 5 yr old). Juvenile alligators were captured at night by hand. Subadult alligators were captured by hand or with a locking snare. Alligators were placed in a cloth bag for a maximum of 2 hr before having a blood sample removed from the cervical supravertebral vein. Blood samples were collected using sterile syringes

and 18-, 20-, or 22-gauge needles depending on the size of the animal. From 3 ml to 10 ml of blood were collected and placed into serum collection tubes that did not contain an anticoagulant. Tubes were stored on ice for up to 12 hr before being centrifuged. The serum was then stored in liquid nitrogen or a −80 C freezer. All samples were transferred to the University of Florida on dry ice for indirect ELISA testing. An aliquot of serum from each alligator determined by ELISA to be seropositive and 12 determined to be seronegative for exposure to WNV were transported to the Florida Department of Health Bureau of Laboratories for confirming presence or absence of antibody against WNV using the HAI and PRNT. Samples were received with approval of the University of Florida Institutional Care and Use Committee (#D047).

Hemagglutination inhibition and plaque reduction neutralization tests

Sera from group 1 and group 2 alligators from the affected alligator farm were tested by HAI for flavivirus antibodies using St. Louis encephalitis (SLE) virus hemagglutinating antigen (strain P15, a 1964 Florida isolate) prepared by sucrose-acetone extraction (Beaty et al., 1995). Sera from all ELISA-positive free-ranging alligators and from 12 ELISA-negative free-ranging alligators (eight from sites having ELISA-positive alligators and four from sites having no ELISA-positive alligators) were similarly tested by HAI. A micro-adaptation of an HAI antibody test was used (Beaty et al., 1995). A titer of ≥10 was regarded as diagnostic of infection with an agent antigenically similar to that used in the test.

The presence or absence of WNV and SLE virus-neutralizing antibody in samples from the two groups of captive alligators that were tested by HAI was confirmed using PRNT. The methodology has been described elsewhere (Beaty et al., 1995). Sera from all ELISA-positive free-ranging alligators and sera from the 12 ELISA-negative free-ranging alligators tested by HAI also were tested by PRNT. Viruses used were WNV strain Eg101 (CDC, Ft. Collins, Colorado) and SLE virus strain P15. Plaques were counted on days 3 and 4 for WNV and days 6 and 7 for SLE virus. Beginning with the 1:10 dilution, twofold dilutions of serum that caused 90% or greater reduction in the number of plaques, as compared to serum negative controls, were considered antibody positive.

ELISA procedure

West Nile virus isolated from a pool of *Culex* sp. mosquitoes collected in Staten Island, New York in 2000 and grown in Vero cells (CCL-8, American Type Culture Collection, Manassas, Virginia) was used as the antigen in the assay. To produce this antigen, Vero cells infected with WNV were processed into inactivated antigen as previously described (Ebel et al., 2000). Each well of a high-protein-binding microplate (Maxisorp F96; Nunc, Kamstrup, Denmark) was coated overnight at 4 C with 50 μl of 1:500 (approximately 36.5 ng) dilution of either the positive (infected; P) or negative (uninfected; N) Vero cells diluted in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.02% NaN_3 (phosphate buffered saline [PBS]/azide). Wells were then washed four times in ELISA wash buffer (PBS/azide with 0.05% Tween 20). This washing process was repeated in between all of the following steps. Wells were then blocked with 300 μl of 5% nonfat dry milk diluted in ELISA wash buffer (blocking buffer) for 1 hr at room temperature. All remaining steps were incubated for 1 hr at room temperature. Plasma samples were then added in 50-μl volumes at twofold dilutions from 1:100 to 1:6400 diluted in blocking buffer. The secondary antibody used was a biotin-conjugated rabbit anti-alligator immunoglobulins polyclonal antibody (Brown et al., 2001). The conjugate was added at a volume of 50 μl at a dilution of 1:1,000 in blocking buffer for each sample. Alkaline phosphatase-conjugated streptavidin (Zymed Laboratories, Inc., San Francisco, California, USA) was then applied to each well at 50 μl of a 1:2,000 dilution in PBS/azide. Next, 100 μl of a 1.0 mg/ml *P*-nitrophenyl phosphate prepared in 0.01 M sodium bicarbonate buffer containing 2 mM $MgCl_2$ was added to each well and the plates were then stored in the dark. The absorbance of each well was read at A_{405} using a StatFax 3200 microplate reader (Awareness Technology, Palm City, Florida) at 10 min.

Each alligator plasma sample was tested in duplicate on positive (WNV antigen infected) Vero cells and, because of limited quantities of alligator plasma, once on negative (WNV antigen free) Vero cells. The average absorbance reading of the replicate was calculated. The positive/negative (P/N) value of each sample was calculated by dividing the mean absorbance of each duplicate pair of positive antigen-containing wells by the absorbance reading of the wells containing negative (uninfected) Vero cell lysate. Samples with a P/N value ≥2 were considered positive for anti-WNV antibody and samples with a P/N value <2 were considered negative for anti-WNV antibody (Beaty et al., 1995; Martin et al., 2000).

Sensitivity and specificity

Using the PRNT as the gold standard, the 10 alligators in group 1 were considered true positives and the 10 alligators in group 2 were considered true negatives. Sensitivity (proportion of positive samples that were classified as seropositive by ELISA) and specificity (proportion of negative samples that were classified as seronegative by ELISA) of the indirect ELISA were determined.

Reproducibility of the assay

Intra-assay and interassay reproducibility were determined by performing three precision runs. One plasma sample was selected from the seropositive group (group 1) and one from the negative group (group 2) and each sample was assayed 24 times on three separate days. The mean A_{405}, the standard deviation (SD), and the coefficient of variation (CV) for the three precision runs were calculated using the same batch of processed WNV-infected Vero cells, plasma, and conjugated secondary antibody. All assays were performed at a plasma dilution of 1:200.

RESULTS

ELISA Validation

Hemagglutination inhibition and PRNT findings for groups 1 and 2 alligators used in the ELISA validation are reported in Table 2. All group 1 alligators had HAI titers ≥20, indicating that they were exposed to a flavivirus. All group 1 alligators had PRNT titers that ranged from 20 to ≥320, indicating that they had anti-WNV antibody. Hemagglutination inhibition and serum-neutralizing antibodies in the seropositive alligators persisted up to the last sampling time, approximately 6 mo after the first blood samples were collected. One group 1 alligator had a titer ≥1:320 for both WNV and SLE. Group 2 alligators had HAI and PRNT titers <1:10, indicating that they did not have antibody against WNV.

All group 1 alligators from the affected alligator farm were seropositive by ELISA for exposure to WNV, having P/N ratios ≥2 and all group 2 alligators were considered negative, having P/N ratios <2 (Table 2). The optimum dilution, where the mean positive absorbance had the greatest difference from the mean absorbance of the negative group, was 1:200. As with HAI and PRNT, ELISA was able to detect antibodies against WNV in the seropositive alligators up to the last sampling time, approximately 6 mo after the first blood samples were collected.

Preliminary sensitivity and specificity values of the indirect ELISA were determined to be 100% (95% confidence interval=95% to 100%). The mean A_{405}, SD, and CV for three precision runs on three separate days are presented in Table 3.

WNV antibodies in free-ranging alligators

Of the 669 alligators sampled, four of 305 (1.3%) from the following four locations sampled in April 2003 were positive by ELISA: Wacissa River, Lake Woodruff, Lake Sampson, and Lake Monroe. Six of 364 (1.6%) sampled in October 2003 from the following five collections were positive by ELISA: Lake Sampson, St. Johns River at Highway 50, Lake Trafford, Lake Okeechobee, and Lake Kissimmee. Lake Sampson was the only location where were two seropositive alligators were identified. Of the alligators sampled in April, nine were recaptured in October. Of these, one was ELISA-positive in both April and October.

The four ELISA-positive alligators that were sampled in April and the six ELISA-positive alligators sampled in October also were positive by PRNT; one of the alligators sampled in April that was positive by ELISA and PRNT was negative by HAI. The 12 ELISA-negative alligators selected as controls were negative by HAI and PRNT.

DISCUSSION

Two groups of alligators from an alligator farm in Florida that had an outbreak of WNV (Jacobson et al., 2005) were used to validate an indirect ELISA to determine exposure of American alligators to WNV. Ten alligators (group 1) from a pen where alligators died during an outbreak of WNV in September to October 2002 were positive by ELISA, HAI, and PRNT. All alli-

TABLE 2. Serologic findings for group 1 (G1) and group 2 (G2) alligator serum or plasma samples assayed by hemagglutination inhibition (HAI), plaque reduction neutralization test (PRNT), and indirect enzyme-linked immunosorbent assay (ELISA) for determining exposure of alligators to St. Louis encephalitis (SLE) virus or West Nile virus (WNV).

	Flavivirus HAI			PRNT						ELISA P/N[b]		
				WNV			SLE					
Group 1	30 January 2003	28 February 2003	13 July 2003	30 January 2003	28 February 2003	13 July 2003	30 January 2003	28 February 2003	13 July 2003	30 January 2003	28 February 2003	13 July 2003
G1-1	>40	>40	>40	160	80	>40	<10	10	<10	11.8	15.9	10.2
G1-2	>40	>40	ND[a]	160	≥320	ND	1:20	≥320	ND	12.2	16.0	12.0
G1-3	>40	>40	>40	20	80	>40	<10	10	<10	10.1	12.0	12.7
G1-4	>40	>40	>40	40	60	20	<10	<10	<10	7.1	11.0	11.5
G1-5	>40	>40	>40	40	80	>40	<10	<10	<10	10.4	11.7	10.7
G1-6	>40	>40	>40	80	≥320	>40	1:10	20	10	12.4	12.4	12.8
G1-7	>40	>40	>40	80	160	>40	1:20	10	10	10.7	13.6	12.5
G1-8	>40	>40	ND	80	80	ND	<10	<10	ND	9.6	13.4	ND
G1-9	>40	>40	20	40	40	>40	<10	<10	<10	11.6	11.4	11.9
G1-10	>40	>40	>40	80	40	>40	<10	<10	<10	15.9	12.6	12.5
Group 2												
G2-1	<10	<10	ND	<10	<10	ND	<10	<10	ND	0.9	0.9	1.1
G2-2	<10	<10	R[c]	<10	<10	<10	<10	<10	<10	1.1	1.0	0.9
G2-3	<10	<10	<10	<10	<10	<10	<10	<10	<10	0.9	0.9	0.9
G2-4	<10	<10	<10	<10	<10	<10	<10	<10	<10	1.1	1.1	1.0
G2-5	<10	<10	<10	<10	<10	<10	<10	<10	<10	0.9	0.9	0.9
G2-6	<10	<10	R	<10	<10	<10	<10	<10	<10	0.9	1.1	ND
G2-7	<10	<10	<10	<10	<10	<10	<10	<10	<10	0.9	1.0	1.0
G2-8	<10	<10	<10	<10	<10	<10	<10	<10	<10	1.0	1.0	1.0
G2-9	<10	<10	<10	<10	<10	<10	<10	<10	<10	1.0	0.9	1.0
G2-10	<10	<10	<10	<10	<10	<10	<10	<10	<10	1.0	1.1	1.0

[a] ND = not determined.
[b] P/N = positive/negative.
[c] R = reactive but not positive at 1:10.

TABLE 3. Reproducibility of the enzyme linked immunosorbent assay[a] to test alligators for exposure to West Nile virus (WNV).

Precision runs	Positive plasma sample			Negative plasma sample		
	Mean A_{405}[b]	SD[c]	CV[d]	Mean A_{405}	SD	CV
P1-IVC[e]	0.792	0.079	9.974	0.068	0.009	13.235
P2-IVC	1.068	0.05	4.681	0.071	0.006	8.451
P3-IVC	0.928	0.065	7.035	0.071	0.011	16.099
P1-CVC[f]	0.059	0.006	10.470	0.061	0.004	5.866
P2-CVC	0.073	0.004	5.455	0.066	0.067	10.593
P3-CVC	0.065	0.006	8.660	0.065	0.005	7.049

[a] Reproducibility is based on three precision runs. Each precision run consisted of 12 duplicate tests (24 observations) of a positive (G1-2) and negative (G2-3) alligator plasma.
[b] Mean A_{405} values for 24 observations for each precision run.
[c] Standard deviation.
[d] Coefficient of variation.
[e] Vero cells containing killed WNV (IVC) was used as the antigen.
[f] Singlets of control (noninfected) Vero cells (CVC) also were tested to calculate positive/negative ratios.

gators (group 2) from a pen where no deaths occurred were seronegative by the same tests. One alligator in group 1 also had equivalent titers to SLE. When distinguishing between WNV and SLE, specimens are confirmed positive if their 90% neutralization titer against WNV is at least fourfold greater than against SLE, a closely related flavivirus that cross-reacts with WNV in serologic assays (Johnson et al., 2003). Since the titers for WNV and SLE were ≥320, greater dilutions of serum would be necessary to see if a fourfold difference was present. A second explanation might be that an alligator was exposed to both SLE and WNV. Although exposure of alligators to SLE has not previously been reported, there are reports of exposure in other orders of reptiles (Shortridge and Oya, 1984).

The indirect ELISA findings were consistent with PRNT. Using P/N ratios for each plasma sample that was assayed, all group 1 alligators had ratios >7.0 and all group 2 alligators had ratios ≤1.1. Using previously established criteria for arbovirus samples tested by ELISA (Beaty et al., 1995; Martin et al., 2000), the ratio of group 1 alligators was well above a P/N of ≥2, a value that is required to categorize a sample as positive. With the PRNT as the gold standard, the confidence levels of sensitivity and specificity of this test were 95% to 100%. The lower limit of the interval of the ELISA sensitivity and specificity indicated that some alligators classified as seronegative or seropositive could be false negatives and false positives, respectively. Since cross-reaction to other flavivirus antibodies would be expected, this would reduce specificity of the test, leading to false positives. Testing of plasma/serum samples collected in the current study using other flaviviruses as the antigen would help determine the specificity of the ELISA. Experimental transmission studies using different flaviviruses in alligators also would help sort out this potential limiting feature of the indirect ELISA. Still, the indirect ELISA has merit as an initial screening test for determining exposure to flaviviruses. This should be followed by PRNT for more specific confirmation.

This study represents the first report of antibodies to WNV in free-ranging alligators. Of 305 alligators sampled from 21 sites in Florida in April 2003, four alligators that were positive by HAI and PRNT also were positive by ELISA. Of 364 alligators sampled in October 2003, six alligators that were positive by HAI and

PRNT also were positive by ELISA. All alligators negative by HAI and PRNT also were negative by ELISA. The overall correlation indicated that the indirect ELISA may be used to initially screen free-ranging alligators for exposure to WNV.

Although free-ranging alligators in Florida had antibodies against WNV, seroprevalence in 2003 was low. Four of 21 sites sampled in April 2003 and five of 21 sites sampled in October 2003 had alligators with antibody against WNV. This indicated either a low level of exposure of alligators across the state of Florida or an inability of alligators to uniformly develop antibodies against this virus after exposure. Transmission studies with WNV-infected mosquitoes that feed on alligators or studies where live virus is injected into alligators are needed to better understand these findings. Although we suspect that mosquitoes are capable of transmitting WNV to alligators, there are no reports indicating what species feed on alligators and how efficiently they can infect alligators. A study to identify the species of mosquitoes occurring at the affected farm in Florida and the species that feed on alligators has been started (S. Garrett, pers. comm.). Further surveys also will be needed to determine the impact of WNV on free-ranging alligator populations in Florida.

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