Journal of Zoo and Wildlife Medicine 39(4): 562–566, 2008
Copyright 2008 by American Association of Zoo Veterinarians

ASSOCIATION OF WEST NILE VIRUS WITH LYMPHOHISTIOCYTIC PROLIFERATIVE CUTANEOUS LESIONS IN AMERICAN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS*) DETECTED BY RT-PCR

Javier G. Nevarez, D.V.M., Ph.D., Mark A. Mitchell, D.V.M., Ph.D., Timothy Morgan, D.V.M., Ph.D., Dipl. A.C.V.P., Alma Roy, M.S., Ph.D., and April Johnson, D.V.M., Ph.D.

Abstract: West Nile virus (WNV) is known to affect captive populations of alligators and, in some instances, cause significant mortalities. Alligators have been shown to amplify the virus, serve as a reservoir host, and even represent a source of infection for humans. This study describes a cutaneous manifestation of WNV in captive-reared American alligators (*Alligator mississippiensis*), previously described as lymphohistiocytic proliferative syndrome of alligators (LPSA), based on the findings of gross examination, histopathologic evaluation, WNV antibody testing, and WNV reverse transcriptase polymerase chain reaction (RT-PCR). Forty alligators with LPSA and 41 controls were examined. There was a significant difference ($P = 0.01^{-21}$) in the WNV serostatus between the treatment group (100%) and the control group (0%, 95% CI: 0–7.3%). In the treatment group, 97.5% (39/40) (95% CI: 92.7–102.3%) of the LPSA skin lesions were positive for WNV via RT-PCR. Of the skin sections within the treatment group that had no LPSA lesions, 7.5% (3/40) (95% CI: 0–15.7%) were positive for WNV. In the control group, all of the skin samples were negative for WNV (41/41) (0%; 95% CI: 0–7.3%). The LPSA skin lesions were significantly more likely to be WNV positive by RT-PCR when compared to control animals ($P = 0.07^{-20}$) and normal skin sections from affected animals ($P = 0.08^{-16}$). There was no significant difference in the WNV RT-PCR results between control animals and normal skin sections from affected animals ($P = 0.24$). These findings suggest that LPSA is a cutaneous manifestation of WNV in alligators.

Key words: *Alligator mississippiensis*, American alligator, enzyme-linked immunosorbent assay, lymphohistiocytic proliferative syndrome of alligators, reverse transcriptase polymerase chain reaction, West Nile virus.

INTRODUCTION

Lymphohistiocytic proliferative syndrome of alligators (LPSA) and West Nile virus (WNV) have been previously described as diseases affecting captive-reared American alligators (*Alligator mississippiensis*) (Figure 1).[6,12,14] Of these two, WNV presents a public health concern. It has been shown that alligators can amplify WNV and serve as a reservoir for the virus.[9] It is also known that personnel working in alligator farms at the time of a WNV outbreak have been exposed to the disease, and there is one confirmed case of direct exposure to WNV by handling tissues from an infected alligator (Tengelsen 2004, pers. comm.). Until recently, the etiology of LPSA was unknown. Past studies had not found evidence of a bacterial and fungal etiology, but were also unable to conclude whether the disease was viral in origin.[12] Based on the histopathologic findings, herpes viruses were initially suspected; however, polymerase chain reaction assays were inconclusive. An association between exposure to WNV and the development of LPSA was described by Nevarez et al.[13] following reports from Louisiana alligator ranchers about a possible link between WNV and the appearance of LPSA lesions in alligators that had survived WNV outbreaks.

One challenging aspect of studying LPSA is the difficulty in correctly identifying the skin lesions antemortem. There are a number of gross lesions on the skin of alligators that are routinely misidentified as LPSA lesions. For this reason, both gross observation and histopathologic identification were used to determine the presence of skin lesions. In addition, it had also become clear that alligator ranches with a history of WNV would also have a history of LPSA, and vice versa.

The objective of this study was a further char-

From the Department of Veterinary Clinical Sciences, Louisiana State University School of Veterinary Medicine, Skip Bertman Drive, Baton Rouge, Louisiana 70803, USA (Nevarez); Department of Veterinary Clinical Medicine, University of Illinois at Urbana-Champaign College of Veterinary Medicine, 1008W Hazelwood Drive, Urbana, Illinois 61802, USA (Mitchell); Department of Pathobiological Sciences, Louisiana State University School of Veterinary Medicine, Skip Bertman Drive, Baton Rouge, Louisiana 70803, USA (Morgan); Louisiana Animal Disease Diagnostic Laboratory, 1909 Skip Bertman Drive, Baton Rouge, Louisiana 70803, USA (Roy); and University of Florida College of Veterinary Medicine, Gainesville, Florida 32608, USA (Johnson). Correspondence should be directed to Dr. Nevarez (jnevarez@vetmed.lsu.edu).

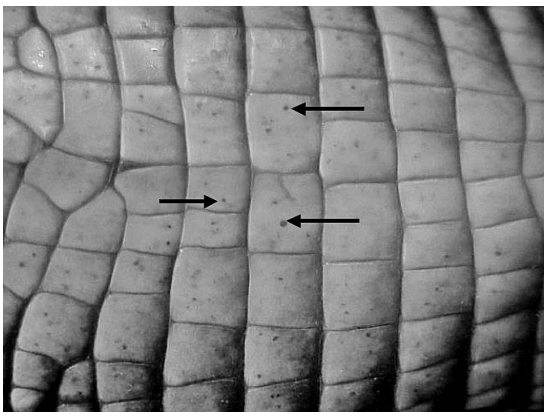

Figure 1. Typical LPSA lesions as observed grossly on the ventral skin of alligators (arrows).

acterization of LPSA as a cutaneous manifestation of WNV in captive-reared American alligators. The specific hypotheses for this study were that animals with LPSA skin lesions would be significantly more likely to have 1) WNV exposure based on antibody testing, and 2) WNV-positive skin lesions based on RT-PCR tests.

MATERIALS AND METHODS

This study was approved by the Louisiana State University Institutional Animal Care and Use Committee. A total of 81 6- to 9-mo-old captive-hatched and -reared American alligators were used for the study. All alligators were obtained from a private rancher in Louisiana between March and June of 2006. The facility had a previous history of WNV and LPSA. The most recent WNV outbreak in the state was diagnosed in October of 2005 in the same facility. Alligators were housed in rectangular buildings with appropriate stocking densities, as recommended by the Louisiana Department of Wildlife and Fisheries, at water temperatures between 29–32°C (85°–90°F), and were fed a commercial alligator diet that was occasionally mixed with whole ground chicken. Husbandry practices at the facility were comparable to other facilities in the state. One building contained animals with LPSA lesions while another contained animals without LPSA lesions. Within each building, alligators were selected at random. Animals were selected based on the presence (treatment group) or absence (control group) of LPSA skin lesions, as identified grossly by one of the authors (JGN) and later confirmed via histopathology. The forty alligators selected for the treatment group (LPSA positive) originated from the only building in the facility in which LPSA had been observed. This also happened to be the same (and only) building where the WNV outbreak occurred in October of 2005. The additional 41 alligators representing the control group (LPSA negative) were obtained from a separate building in which no WNV or LPSA had been reported or diagnosed. All alligators from the treatment and control groups were collected and processed on different occasions to minimize the chance of cross-contamination. A gross examination was performed on each animal to document the presence or absence of LPSA skin lesions.

Prior to euthanasia, each alligator was bled from either the supravertebral sinus or the lateral occipital sinus. Blood was placed in blood tubes with no preservative. The tubes were centrifuged at 5,000 *g* for 5 min before removing the serum and placing it in sterile vials. The serum was then stored at −70°C until being shipped with ice packs to the University of Florida (Gainesville, Florida USA) for WNV antibody testing. The WNV antibody tests were performed using an indirect enzyme-linked immunosorbent assay for detection of antibodies to West Nile virus in American alligators, as reported by Jacobson et al.[7] After the blood collection, alligators were euthanized with Beuthanasia-D Special (Schering Plough Animal Health, Union, New Jersey 07083, USA) at a dosage of 1ml/2.5kg, administered in the supravertebral sinus. Following euthanasia, a complete necropsy was performed on each alligator, and tissue samples were collected and saved in 10% neutral buffered formalin for histopathologic examination. Preserved tissues were embedded in paraffin, sectioned to 5-μm thickness, adhered to a glass slide, and stained with hematoxylin and eosin using standard histologic procedures. Using standard immunohistochemical staining, additional 5-μm sections of paraffin-embedded tissues were tested for the presence of WNV.

A real time reverse transcriptase polymerase chain reaction test (RT-PCR) was performed on the skin and a liver-brain tissue pool from all animals. A 4-mm punch biopsy was used to obtain the skin samples after a determination that this biopsy size provided the 25–30 mg of tissue needed for testing. The same amount (25–30 mg) of liver and brain tissue was obtained for WNV RT-PCR testing. In the treatment group, two skin biopsies were obtained. One biopsy was obtained directly over a scale with an LPSA lesion (TxA) in order to ensure that the lesion itself was being tested. A second skin biopsy was obtained from a scale section over which there was no LPSA skin lesion (TxB) present. This second biopsy was obtained as an additional intrabiopsy control. One potential pitfall of

Table 1. Results of RT-PCR from skin and liver-brain pool from alligators.

Results	TxA[a]	TxB[b]	CxS[c]	Tx L-B[d]	Cx L-B[e]
Positive	39	3	0	27	0
Negative	1	37	40	13	40
Prevalence	97.5%	7.5%	0%	67.5%	0%
95% Confidence interval	93–100%	0–16%	0–7%	53–82%	0–7%

[a] TxA = skin from treatment group with LPSA lesion.
[b] TxB = skin from treatment group without LPSA lesion.
[c] CxS = skin from control group, no LPSA lesion.
[d] Tx L-B = liver-brain pool from treatment group.
[e] Cx L-B = liver-brain pool from control group.

collecting the second skin sample in the treatment animals was that an LPSA lesion that was not grossly visible could have been obtained with the biopsy. In the control group, a skin biopsy (CxS) was obtained from a scale after corroboration that there were no LPSA lesions in the whole ventral skin surface. Multiple biopsies were obtained from each animal in order to have additional tissues for banking. Brain and liver samples were collected from each group during necropsy. All tissue samples (skin, liver-brain) were placed in a sterile plastic vial containing RNAlater® (Ambion Inc., Austin, Texas 78744, USA) at a 5:1 ratio of solution to tissue and stored at −20°C until testing was performed. The RT-PCR procedure was performed according to a previously published protocol.[14]

The 95% binomial confidence intervals were calculated for each proportion. For cases where the prevalence was zero, the technique described by Van Belle was used.[17] A Fisher's exact test was used to compare the treatment group and control group for each diagnostic test (RT-PCR, serology, and histopathology). A $P \leq 0.05$ was considered statistically significant.

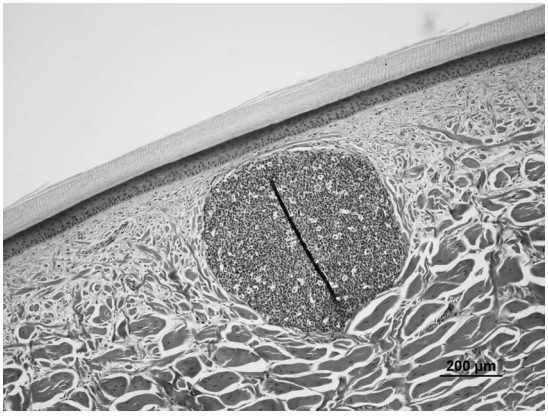

Figure 2. Typical LPSA lesion as observed under the microscope and stained with H&E (20×).

RESULTS

All 40 animals in the treatment group tested positive for WNV antibodies, while all 41 animals in the control group were seronegative. These results are consistent with a previous study that revealed an association between WNV seropositivity and the presence of LPSA lesions.[13] There was a significant difference ($P = 0.01^{-21}$) in the serostatus of the treatment group (100%) when compared to the control group (0%, 95% CI: 0–7.3%).

Results for the RT-PCR are presented in Table 1. In the treatment group, 97.5% (39/40) (95% CI: 92.7–100 %) of the LPSA skin lesions (TxA) were positive for WNV via RT-PCR. Of the skins within the treatment group that had no LPSA skin lesions (TxB), 7.5% (3/40) (95% CI: 0–15.7%) were positive for WNV. In the control group, all of the skin samples (CxS) were negative for WNV (41/41) (0%; 95% CI: 0–7.3%). All alligators in TxA were significantly more likely to have RT-PCR WNV-positive skin than those in TxB ($P = 0.08^{-16}$) and CxS ($P = 0.07^{-20}$). There was no significant difference in the findings of WNV from the skins of alligators between TxB and CxS ($P = 0.24$).

For the liver and brain, pooled samples in the treatment group (Tx L/B), 67.5% (27/40) (95% CI: 53–82%), were positive for WNV via RT-PCR. In the control group, all (41/41) (0%; 95% CI: 0–7.3%) of the liver and brain pool samples (Cx L/B) were negative for WNV. There was a significant difference between these results ($P = 0.01^{-9}$).

Affected alligators had similar histopathologic lesions in the skin and superficial dermis. The superficial dermis, immediately beneath the epidermis, contained multiple round to ovoid lesions composed of large numbers of lymphocytes and macrophages (Figure 2). These foci were relatively sharply demarcated, contained variable numbers of tangible body macrophages, and sometimes surrounded small blood vessels. Within these accumulations of inflammatory cells, there was a

marked loss of the thick collagenous stroma that makes up the normal dermis. There was a mild compression of collagen surrounding the lesions. The collagen extended a short way into the lesions and then abruptly ended. Small, scattered areas of collagenolysis were noted around the margins of some of the lesions. The overlying epidermis was usually intact, but was sometimes mildly attenuated. Occasionally, lymphocytes extended into the epidermis immediately overlying the lesions. In these areas, the overlying scale occasionally contained foci of disorganized keratin above the epidermal lymphocytic infiltration. No organisms were seen within the skin or the superficial dermal lymphoid accumulation. Histopathology confirmed the presence and absence of LPSA lesions in the treatment (40/40; 100%) and control (41/41; 95% CI: 0–7.3%) groups, respectively. Immunohistochemical results for WNV in the skin were inconclusive.

DISCUSSION

The results of this study suggest a strong association between the presence of LPSA and WNV. To obtain definitive confirmation that WNV is responsible for the lesions, an experimental study fulfilling Koch's postulates would be required. Unfortunately, conducting such a study at this time is difficult due to the housing and biosafety requirements. To conduct such a study would require a BL-3 laboratory. Because of the large size and demands of these animals, this is not possible at this time; however, plans to perform such a study are in progress. Nonetheless, without the privilege of having performed an experimental infection, the data clearly show a very strong association and strongly suggest that WNV is highly correlated to the presence of LPSA lesions.

A disadvantage of the RT-PCR procedure is its inability to differentiate between viable (live) and dead virus. The isolation of WNV from some of the skin lesions was attempted, but was not successful. This may be explained by the chronicity of the disease leading to a lower amount of virus present in the tissue, or to the virus no longer being viable. One interesting finding that helps provide some insight into this matter is the fact that only 67.5% of the liver-brain pools tested positive in the treatment group, as compared to 97.5% of the skin samples. This finding is consistent with an expected reduction in tissue levels with chronicity of the disease. Perhaps earlier sampling after the initial WNV outbreak would yield different results, e.g., having a higher proportion of tissues that test positive for WNV and being able to culture the virus from the skin. The few positive results in TxB may be explained by the fact that LPSA lesions are sometimes difficult to visualize and, in a positive skin, any given section has the possibility of housing a lesion. An interesting finding is the fact that the skin lesions appear to be more predictive for the diagnosis of WNV than the liver-brain tissue. This is evident when comparing the results of the RT-PCR between the skin lesions (TxA) (95% CI: 93–100%) and the liver-brain pool (Tx L/B) (95% CI: 53–82%) (Table 1). The inconclusive immunohistochemistry results were somewhat unexpected, as immunohistochemistry has been used to identify WNV in alligator tissues, although not skin, in the past.[14] However, those instances in which immunohistochemistry was used were also acute WNV infections, as opposed to what we believe is a chronic infection, by the time that LPSA lesions are present on the skin. As with the negative culture results, the amount of virus present within the lesions may be too small to be detected via immunohistochemistry. In addition, there may be some differences in the techniques used when performing this test on alligator skin versus other tissues or even skin from other species. Regardless of the reason, it appears that immunohistochemistry is not a useful tool for diagnosis of WNV in the skin of alligators affected with LPSA.

Skin manifestations of WNV have been described in humans, although histopathologic evaluation of the lesions has been limited. Nonetheless, there are reports of exanthema and rash associated with WNV infection dating back to 1956.[1–5,8,10,11,15,16] There is also a report of WNV identification in the skin of two goshawks (*Accipiter gentilis*) via immunohistochemistry.[18] These reports support our theory of LPSA as a skin manifestation of WNV in American alligators. This type of inflammation in the skin of alligators may be prompted by an aggressive immune response secondary to chronic exposure to WNV, but further studies are required to understand the immune response to the virus in alligators.

Acknowledgments: The authors would like to thank the Louisiana Department of Wildlife and Fisheries, the Louisiana Fur and Alligator Advisory Council, the Louisiana Alligator Farmers and Ranchers Association, Noel Kinler, Ruth Elsey, and the United States Department of Agriculture, Animal and Plant Health Inspection Services (Federal Grant 05-9722-1575-CA) for their support and funding of this research.

LITERATURE CITED

1. Anderson, R. C., K. B. Horn, M. P. Hoang, E. Gottlieb, and B. Bennin. 2004. Punctate exanthem of West

Nile virus infection: report of 3 cases. J. Am. Acad. Dermatol. 51: 820–823.

2. Asnis, D. S., R. Conetta, A. A. Teixeira, G. Waldman, and B. A. Sampson. 2000. The West Nile virus outbreak of 1999 in New York: the Flushing Hospital experience. Clin. Infect. Dis. 30: 413–418.

3. Cernescu, C., N.-I. Nedelcu, G. Tarei, S. Ruta, and T. F. Tsai. 2000. Continued transmission of West Nile virus to humans in southeastern Romania, 1997–1998. J. Infect. Dis. 181: 710–712.

4. Estival, J. L., F. Skowron, M. Dupin, and P. Combemale. 2001. Primo-infection a virus West-Nile (French). Ann. Dermatol. Venereol. 128: 656–658.

5. Ferguson, D. D., K. Gershman, A. LeBailly, and L. R. Petersen. 2005. Characteristics of the rash associated with West Nile virus fever. Clin. Infect. Dis. 41: 1204–1207.

6. Jacobson, E. R., P. E. Ginn, J. M. Troutman, L. Farina, L. Stark, K. Klenk, K. L. Burkhalter, and N. Komar. 2005. West Nile virus infection in farmed American alligators (*Alligator mississippiensis*) in Florida. J. Wildl. Dis. 41: 96–106.

7. Jacobson, E. R., A. J. Johnson, J. A. Hernandez, S. J. Tucker, A. P. Dupuis II, R. Stevens, D. Carbonneau, and L. Stark. 2005. 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. J. Wildl. Dis. 41: 107–114.

8. Klein, C., I. Kimiagar, L. Pollak, R. Gangelman-Marton, A. Itzhaki, R. Milo, and J. M. Rabey. 2002. Neurological features of West Nile virus infection during the 2000 outbreak in a regional hospital in Israel. J. Neurol. Sci. 200: 63–66.

9. Klenk, K., J. Snow, K. Morgan, R. Bowen, M. Stephens, F. Foster, P. Gordy, S. Beckett, N. Komar, D. Gubler, and M. Bunning. 2004. Alligators as West Nile virus amplifiers. Emerg. Infect. Dis. 10: 2150–2154.

10. Marberg, K., N. Goldblum, V. V. Sterk, W. Jasinska-Klingberg, and M. A. Klingberg. 1956. The natural history of West Nile fever. Clinical observations during an epidemic in Israel. Am. J. Hyg. 64: 259–269.

11. Nash, D., F. Mostashari, A. Fine, J. Miller, D. O'Leary, K. Murray, A. Huang, A. Rosenberg, A. Greenberg, M. Sherman, S. Wong, and M. Layton. 2001. The outbreak of West Nile virus infection in the New York City area in 1999. N. Engl. J. Med. 344: 1807–1814.

12. Nevarez, J. G., M. A. Mitchell, and M. M. Garner. 2003. Characterizing lymphohistiocytic proliferative syndrome (''PIX'') in captive-reared American alligators (*Alligator mississippiensis*) from Louisiana. Final Report to the Louisiana Department of Wildlife and Fisheries Fur and Refuge Division, New Iberia, Louisiana. 15 pp.

13. Nevarez, J. G., M. A. Mitchell, A. J. Johnson, and N. Kinler. 2007. Establishing an association between West Nile virus exposure and the development of lymphohistiocytic proliferative syndrome of American alligators. J. Herp. Med. Surg. 17: 4–7.

14. Nevarez, J. G., M. A. Mitchell, D. Y. Kim, R. Poston, and H. Lampinen. 2005. West Nile virus in alligator, *Alligator mississippiensis*, ranches from Louisiana. J. Herp. Med. Surg. 5: 4–9.

15. Pealer, L. N., A. A. Marfin, L. R. Petersen, R. S. Lanciotti, P. L. Page, S. L. Stramer, M. G. Stobierski, K. Signs, B. Newman, H. Kapoor, J. L. Goodman, and M. E. Chamberland. 2003. Transmission of West Nile virus through blood transfusion in the United States in 2002. N. Engl. J. Med. 349: 1236–1245.

16. Sejvar, J. J., M. B. Jaddad, B. C. Tierney, G. L. Campbell, A. A. Marfin, J. A. VanGerpen, A. Fleischauer, A. A. Leis, D. S. Stokic, and L. R. Petersen. 2003. Neurologic manifestations and outcome of West Nile virus infection. JAMA 290: 511–515.

17. Van Belle, G. 2002. Statistical Rules of Thumb. John Wiley & Sons, New York, New York. Pp. 29–51.

18. Wünschmann, A., J. Shivers, J. Bender, L. Carroll, S. Fuller, M. Saggese, A. van Wettere, and P. Redig. 2005. Pathologic and immunohistochemical findings in goshawks (*Accipiter gentiles*) and great horned owls (*Bubo virginianus*) naturally infected with West Nile virus. Avian Dis. 49: 252–259.

Received for publication 22 February 2008