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Source: Journal of Wildlife Diseases, 32(3) : 431-436

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-32.3.431

Journal of Wildlife Diseases, 32(3), 1996, pp. 431–436

ANTIBODIES TO ARTHROPOD-BORNE ENCEPHALITIS VIRUSES IN SMALL MAMMALS FROM SOUTHERN FLORIDA

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ABSTRACT: From 1987 through 1991, blood samples were collected from 10 species of small mammals in Indian River County, Florida (USA). Sera from 1,347 animals were analyzed for hemagglutination-inhibition (HI) antibody to St. Louis encephalitis (SLE) and eastern equine encephalitis (EEE) viruses. Of these, 75 (5.6%) were positive for HI antibody to SLE virus and 121 (9.0%) were positive for EEE antibody. Sera from five mammalian species were tested for neutralizing (NT) antibody to SLE, EEE, Highlands J (HJ, a member of the western equine encephalitis virus complex), or Everglades (EVE, a member of the Venezuelan equine encephalitis complex) viruses. By serum neutralization tests, 26 (46%) of 57 had SLE antibodies, 14 (24%) of 58 had EEE antibodies, two (3.2%) of 63 had HJ antibodies, and 9 (14%) of 63 had EVE antibodies. One *Sigmodon hispidus* and one *Peromyscus gossypinus* had NT antibodies both to EEE and HJ viruses. Blood samples from 512 mammals were tested for virus. Isolations of one EVE virus and two unidentified arenaviruses were made from *P. gossypinus*, and one EVE virus isolate was made from a *S. hispidus*.

Key words: Mosquito-borne arboviruses, St. Louis encephalitis, eastern equine encephalitis, small mammals, serosurvey, arenavirus.

INTRODUCTION

Rodents serve as important reservoir and amplification hosts for arboviruses in the Venezuelan equine encephalomyelitis (VEE) complex in Florida (Chamberlain et al., 1964), South America (Downs et al., 1962; Jonkers et al., 1968), and Central America (Scherer et al., 1985).

Eastern equine encephalitis (EEE) virus commonly infects birds, which serve as amplification hosts. In North America, the virus has rarely been isolated from small mammals in the field. However, forest dwelling rodents and marsupials are important reservoir and amplification hosts in South America (Scott and Weaver, 1989). In Florida (USA), Bigler et al. (1975, 1976) detected EEE hemagglutination-inhibition (HI) and neutralizing (NT) antibody in the opossum (*Didelphis marsupialis*), raccoon (*Procyon lotor*), gray fox (*Urocyon cinereoargenteus*), skunk (*Mephitis mephitis*), white-tailed deer (*Odocoileus virginianus*), and feral swine (*Sus scrofa*). In a comprehensive review of the EEE literature published before 1976, Horsfall (1976) listed isolations and serologic data from 25 small mammalian species collected throughout North America. Virus isolations were only reported from a raccoon and an opossum from Florida (Wellings et al., 1972).

St. Louis encephalitis (SLE) virus occurs throughout North, Central, and South America and in the Caribbean Basin (Chamberlain, 1980). The virus, as well as viral antibody, are detected most commonly in wild birds (McLean and Bowen, 1980). In South America, isolation of SLE virus from wild rodents and marsupials is evidence that these groups may serve as maintenance hosts (McLean and Bowen, 1980). In the western United States, ground squirrels are involved in the natural SLE virus cycle (McLean and Bowen, 1980). Small mammals positive for hem-

agglutination-inhibition (HI) or neutralizing (NT) antibody against SLE virus have been collected in the southern United States. Positive mammals from Florida included: cotton rats (*Sigmodon hispidus*), cotton mice (*Peromyscus gossypinus*), rice rats (*Oryzomys palustris*), and armadillos (*Dasypus novemcinctus*) (Henderson et al., 1962; Jennings, 1969; Day et al., 1995).

Our objective in this study was to determine the prevalence of mosquito-borne viral agents and antibody in small mammals captured in Indian River County, Florida, and to determine whether small mammals can serve as natural sentinels to measure the frequency and magnitude of arbovirus transmission in the field.

MATERIALS AND METHODS

All mammals for this study were captured in Indian River County, Florida (27°45′N, 80°45′W), between January 1987 and July 1991 (University of Florida Animal Use Approval Number 8427; Epidemiology of arboviruses in southern Florida). For rodents, 10 peanut butter-baited, folding-style Sherman® live traps (8 × 9 × 23 cm) (Forestry Supply, Jackson, Mississippi, USA) were set along 50, 100-m-transects. Traps were baited, set overnight, and checked early in the morning. Forty traps were distributed along four transects at one time. Traps remained at the same position along a transect for three consecutive trap nights, after which they were moved to a new transect. Transects were located in mesic hammock, pine flatwood, and hardwood swamp habitats (Kale et al., 1990). Larger mammals, such as gray squirrels (*Sciurus carolinensis*) and opossums, were caught in live traps (18 × 18 × 61 cm) (Havahart®; Forestry Supply) set on the ground near four bird feeders. Two were located in mesic hammocks, one was located in a pine flatwood habitat, and one was located in an urban habitat.

Trapped mammals were released into a heavy mesh insect net (Forestry Supply) to facilitate handling. A 0.5 ml blood sample was collected from unanesthetized cotton rats and gray squirrels via cardiac puncture. A 0.3 ml sample was taken from unanesthetized mice in the same manner and mixed with 0.7 ml of biological field diluent (BFD) composed of 90% minimum essential medium with Hank's salts (Sigma Chemical Company, St. Louis, Missouri, USA), 10% fetal bovine serum (FBS) (Intergen Co., Purchase, New York, USA), 200 U/ml penicillin, 200 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml kanamycin (all antibiotics were from Sigma Chemical Company). After the blood sample was collected, animals were aged, sexed, marked with a numbered ear tag (National Band and Tag Company, Newport, Kentucky, USA), and released at the capture site.

One drop of blood (0.006 ml) was used for virus isolation attempts after mixing it in 0.7 ml of BFD. The blood and BFD was mixed in a cryopreservation vial (Fisher Scientific, Orlando, Florida) and placed on wet ice for approximately 2 hr in the field, and during transport to the laboratory, where samples were frozen at −70 C until shipment on dry ice to the Tampa Branch Laboratory, Tampa, Florida.

The remaining blood was allowed to clot for 24 hr at 24 C and then was centrifuged at 3400 × *G* for 30 min. The resulting serum was tested for SLE antibody. A micro-adaptation of the HI antibody test of Beaty et al. (1989) was used with a hemagglutinin (HA) prepared from a Florida human SLE virus isolate (TBH-28) (Centers for Disease Control and Prevention, Ft. Collins, Colorado, USA). All sera also were tested in the same manner for HI antibody against EEE virus using an HA prepared from a Florida human EEE virus isolate (D64-837).

Briefly, HA antigens were titrated at optimal pH to an endpoint allowing the addition of 4 to 8 HA units in a 0.025 ml volume to each aliquot of diluted serum. Two-fold serial dilutions of acetone-treated sera starting at 1:10 in 0.4% bovalbumin-borate-saline (Beaty et al., 1989) at pH 9.0 were prepared in 96-well disposable microtiter U plates with 0.025 ml loops (Dynatech Co., Chantilly, Virginia, USA). Control wells were provided for serum hemagglutinins, antigen, and diluent. Four to eight units of antigen were added to the serum dilutions and the mixture was incubated overnight at 4 C. Following incubation, 0.05 ml of a standardized goose erythrocyte suspension (Beaty et al., 1989) was added to the serum dilutions and to control wells of test serum, known positive and negative sera, antigen, and goose erythrocytes. Incubation proceeded at 22 to 24 C. When there were 4 to 8 units of HA present, based on the antigen titration, the test findings were recorded. A titer of ≥1:10 was regarded as diagnostic of infection with an agent antigenically similar to that used in the test.

If there was a sufficient amount of serum, NT antibody tests for SLE, EEE, HJ, and EVE viruses were done. Two NT techniques were used and none of the sera were tested with both techniques. Many of the sera tested for NT antibody had first tested positive for HI antibody to either SLE or EEE viruses. The first

technique used to test for the presence of NT antibody was serial virus dilution with undiluted serum (Beaty et al., 1989). The challenge viruses were Florida isolates SLE (strain P15) and EEE (strain D64-837).

Before initiation of the serial virus dilution test, serum was heated for 30 min at 56 C. Equal amounts of serum were mixed with appropriate dilutions of virus. Serum-virus mixtures were incubated for 2 hr at 37 C and were then transferred to an ice bath for immediate intracerebral inoculation (0.03 ml of each serum-virus mixture) into each of four to six 3-to-4 wk old mice. Observation of inoculated animals continued for 14 days. Deaths were recorded and a lethal dose causing mortality in 50% of the test animals (LD_{50}) was determined by the method of Reed and Muench (1938).

The LD_{50} virus dilutions for each series of serum-virus mixtures, along with that of the control, were determined to a single decimal point. A logarithmic LD_{50} was expressed as the exponent of the reciprocal of the endpoint dilution. The log neutralization index of each serum was obtained by subtracting its LD_{50} from that of the control. Indices of <1.0 were considered negative, of 1.0 to 1.6 were equivocal, and of ≥ 1.7 were positive.

Our second method of analyzing serum aliquots for NT antibody was the plaque reduction neutralization test (PRNT), as described by Olson et al. (1991). In brief, NT titers were determined by a constant-virus serum-dilution procedure using African green monkey kidney (Vero) cells (Dr. G. V. Ludwig, Department of Cell Culture and Hybridoma Production, U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, USA) contained in 6-well plates. Sera were incubated with approximately 100 plaque-forming units for 1 hr at 37 C. The virus-serum solution was then incubated on Vero cells for 1 hr at 37 C, after which an agar overlay without neutral red was added. A second agar overlay, 2% of which was a commercially prepared stock solution of neutral red (3.333 g/liter of water) (Gibco, Grand Island, New York) was added 2 days later for EEE, EVE, and HJ viruses and 3 days later for SLE virus. Beginning with a 1:10 dilution, 10-fold dilutions of serum that caused an $\geq 80\%$ reduction in the number of plaques as compared to negative controls were considered antibody positive. Virus stocks used for the PRNT were as follows: EEE virus strain ME-77132, EVE virus (VEE virus complex, subtype II) strain Fe3-7c, HJ virus strain Ct An-B8-74, and SLE virus strain TNM4-212. Negative control sera were from chickens previously inoculated with diluent and which did not have detectable antibody when tested by HI or PRNT. Positive controls were from chickens previously inoculated with the respective virus and which had detectable antibody titers to the appropriate virus by HI and PRNT.

Newborn mice (1 to 3 days of age) were used for virus isolation attempts with mammalian blood mixed 1:7 in BFD and stored at −70 C. One litter of eight suckling mice was inoculated with each thawed inoculum. Injections in each mouse were 0.015 ml by the intracerebral and 0.03 ml by the intraperitoneal routes. After inoculation, mice were observed daily for at least 14 days. A 1:10 suspension of brain material from sick or dead mice was homogenized in BFD. The homogenate was centrifuged twice, first at $750 \times G$ for 20 min to remove large debris, then at $3700 \times G$ for 1 hr. The resulting supernatant was passed through a 0.45 μm syringe filter of sterile mixed esters of cellulose that was pre-treated with FBS to prevent virus adsorption to the filter, and passed to a second litter of suckling mice. In the event of sickness or death in the second passage mice, brain material was harvested and the NT antibody tests described above were used to confirm the identity of the isolated viral agent.

RESULTS

Blood samples were collected from 1,347 mammals representing 10 species. Hemagglutination-inhibition antibody was identified in 196 (15%) of these, 75 (6%) were positive for SLE antibody and 121 (9%) were positive for EEE antibody (Table 1).

In virus NT tests, 26 (46%) of 57 sera were SLE-antibody positive, 14 (24%) of 58 were EEE-antibody positive, two (3%) of 63 were HJ-antibody positive, and nine (14%) of 63 were positive for EVE antibody.

Four virus isolations (all from 1988) were made from 512 blood samples collected between 1988 and 1991. Annual blood sample totals for virus isolation were: 323 (1988), 119 (1989), 64 (1990), and six (1991). Three isolates were from *P. gossypinus*; one was identified as EVE virus and two were unidentified arenaviruses. The remaining isolate was identified as EVE virus and was from the blood of a *S. hispidus*.

Serum NT tests were conducted on

TABLE 1. Hemagglutination inhibition (HI) and neutralizing (NT) antibody and viral isolations from mammals captured in Indian River County, Florida, 1987 through 1991.

Species	Number tested	Number HI-positive		Number NT-positive/Number tested				Virus isolations/ Number tested
		SLE[a]	EEE	SLE	EEE	HJ	EVE	
Didelphis marsupialis	28	5	6	8/9	2/7	0/7	0/7	0/14
Peromyscus gossypinus	492	7	66	0/2	8/21	1/20	4/20	3/189[b]
Sciurus carolinensis	170	6	3	9/14	NT[c]	NT	NT	0/111
Sigmodon hispidus	621	56	46	8/31	4/30	1/36	5/36	1/186[d]
Other[e]	36	1[f]	0	1/1[f]	NT	NT	NT	0/12
Totals for 10 species	1,347	75	121	26/57	14/58	2/63	9/63	4/512

[a] SLE = St. Louis encephalitis virus, EEE = eastern equine encephalitis virus, HJ = Highlands J virus, and EVE = Everglades virus.

[b] 1 Everglades virus and 2 unidentified arenavirus isolates.

[c] NT = none tested.

[d] 1 Everglades virus isolate.

[e] *Glaucomys volans* (n = 2), *Neotoma floridana* (n = 14), *Oryzomys palustris* (n = 15), *Procyon lotor* (n = 1), *Rattus rattus* (n = 3), and *Reithrodontomys humulis* (n = 1).

[f] One *Oryzomys palustris* had antibodies.

both unknown arenavirus isolates using three arenavirus antisera: lymphocytic choriomeningitis (LCM) virus, Tacaribe (TAC) virus, and Tamiami (TAM) virus immune sera. The unknown agents were not neutralized by either LCM or TAM antisera. Immune sera against TAC neutralized 1.2 $\log_{10}$ (LD_{50}) of the agent, an equivocal finding. Because TAC virus is an arenavirus, the equivocal finding against our unidentified isolates may represent antigenic similarity with the South American bat virus. We were unable to further characterize the isolates.

The effect of capture, handling, and blood collection on the subsequent long-term survivorship of small mammals collected for our study is not known. There was some mortality associated with the collection of blood. However, many animals were captured and bled more than once. Some antibody-positive animals were maintained in the laboratory where monthly blood samples were collected. One *S. hispidus* was bled 14 times over a 17-mo period, and then released at the original capture site. This animal suffered no apparent ill effects. Likewise, a *P. gossypinus* was captured and bled twice in the field. After the second blood sample was taken in the field, the mouse was returned to the laboratory and maintained there for 3 mo, with a blood sample collected each month. The mouse was released at the original capture site 1 mo after the last blood sample was collected.

DISCUSSION

Isolation of EEE virus from a house mouse (*Mus musculus*) and an unidentified species of squirrel (*Sciurus* spp.) in New Jersey (USA) (Goldfield et al., 1968) represents the only published report of isolation of this virus from wild small rodents in the United States. Antibody against EEE virus has been detected in sera from *Peromyscus leucopus* and *Microtus pennsylvanicus* (Karabatsos, 1985). Detection of EEE antibody from *P. gossypinus* and *S. hispidus* in our study is the first in these rodent species and the first evidence that EEE virus infects Florida rodents.

Highlands J virus was originally isolated from a blue jay (*Cyanocitta cristata*) in Florida (Henderson et al., 1962). The cotton mouse and cotton rat that had NT antibody to HJ virus reported in our study are the first reported from field-collected rodents.

Everglades virus is common in wild rodents from southern Florida (Chamberlain

et al., 1969). The EVE virus isolated from a *P. gossypinus* collected in Indian River County is evidence that a VEE complex virus occurs further north in the state than previously suspected (Young and Johnson, 1969). Viruses belonging to the VEE complex may cross-react with EEE virus in HI tests (Karabatsos, 1985). Therefore, it is possible that the mammals in our study positive for HI antibody to EEE virus but negative for NT antibody against EEE virus were actually infected with viruses belonging to the VEE complex.

In our study, samples positive for HI antibody to SLE virus often did not have corresponding NT antibody titers. The HI test for flaviviruses cross reacts more than complement fixation and NT assays (Beaty et al., 1989). Therefore, interpretation of HI serologic data for wild mammals must be made with caution. A rodent-associated flavivirus, Cowbone Ridge (CR) virus (Calisher et al., 1969), occurs in south Florida, has an antigenic relationship to SLE virus, and should be included in future serologic surveys. Additional field work is necessary to determine the importance of small mammals in the natural SLE virus cycle in southern Florida.

All antibody-positive gray squirrels in our study were collected in 1990 and 1991, following a widespread human epidemic of SLE (Day et al., 1995). It is not known whether gray squirrels are an important link in the SLE virus amplification and transmission cycles in Florida. However, it is clear that they are fed on by infected vectors and mount an antibody response following exposure to the virus.

Enzootic and epizootic transmission of EEE and HJ viruses to wild birds is most commonly through the bite of infected *Culiseta melanura* (Coquillett), a mosquito species that feeds primarily on birds. Because of the occurrence of these viruses in small mammals in Florida, it is likely that other mosquito vectors are involved in the natural transmission cycles (Henderson et al., 1962). In Florida, EVE virus is transmitted by *Culex* (*Melanoconion*) *opistho-pus* Komp (Chamberlain et al., 1964, 1969). In addition, many EEE virus isolates have been made from *Culex nigripalpus* Theobald (Wellings et al., 1972), a species that feeds on the blood of a variety of vertebrates, including small mammals (Edman, 1974).

Even though rodents had HI antibody to EEE and SLE viruses, their value as natural sentinels to these viruses is limited because of the non-specificity of the HI test. The possible cross reaction of antibodies against EEE virus with HJ or EVE viruses, and antibodies against SLE virus with CR virus, renders interpretation of positive HI results questionable when testing small mammals collected in the field. At the present time, sentinel chickens that are not susceptible to infection by CR or EVE viruses provide the most reliable surveillance system for monitoring SLE and EEE virus transmission in the field (Day and Lewis, 1992).

ACKNOWLEDGMENTS

This work was supported by grants No. AI-20983, AI-26787, and AI 22119 from the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, and by contract No. LCNN9 from the Florida Department of Health and Rehabilitative Services. Our thanks to personnel from Indian River Mosquito Control District for their help with this project. We acknowledge the work of Dr. Arthur L. Lewis who aided with the serologic analysis of samples collected in 1987 and 1988. University of Florida Experiment Stations Journal Series R-04472.

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Received for publication 11 April 1995.