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ANTIBODIES TO ST. LOUIS ENCEPHALITIS VIRUS IN ARMADILLOS FROM SOUTHERN FLORIDA

Jonathan F. Day,[1] Eleanor E. Storrs,[2] Lillian M. Stark,[3] Arthur L. Lewis,[3,4] and Sharon Williams[2]

[1] Florida Medical Entomology Laboratory, Institute of Food and Agricultural Sciences, University of Florida, 200 9th Street SE, Vero Beach, Florida 32962, USA
[2] Florida Institute of Technology, Department of Biological Sciences, 150 West University Boulevard, Melbourne, Florida 33901-6988, USA
[3] Tampa Branch Laboratory—Virology Section, Florida Department of Health and Rehabilitative Services, 3952 West M. L. King, Jr. Boulevard, Tampa, Florida 33614, USA
[4] Present address: 13510 Gibbons Pass, Tampa, Florida 33613, USA

ABSTRACT: From January 1990 through March 1991, 189 armadillos (*Dasypus novemcinctus*) were collected from Brevard and Glades Counties in southern Florida (USA). The sera were analyzed for hemagglutination-inhibition (HAI) antibodies against St. Louis encephalitis (SLE) and eastern equine encephalomyelitis (EEE) viruses. None of the armadillos had detectable HAI antibody to EEE virus, but 59 (31%) had antibodies against SLE virus. Sera from 31 of the HAI-positive armadillos contained significant levels of neutralizing (NT) antibody to SLE virus. Armadillos captured during the 1990 SLE human epidemic in south Florida had a greater prevalence of HAI and NT antibody to SLE virus than did animals captured before the start of the epidemic. This is evidence that armadillos were fed on by mosquitoes infected with SLE virus. We propose that armadillos may be involved in the SLE amplification and transmission cycles in Florida.

Key words: Armadillo, St. Louis encephalitis, *Dasypus novemcinctus*.

INTRODUCTION

The nine-banded armadillo (*Dasypus novemcinctus* L.) was introduced into Florida (USA) in Brevard County in 1922 through escape from a local zoo and again from a migration from Texas (USA) in 1972. The spread of armadillos within the state from this source was well documented (Humphrey, 1974).

St. Louis encephalitis (SLE) virus is a mosquito-borne arbovirus found in North, Central, and South America. Human epidemics of SLE virus were reported in 1959, 1961, and 1962 on Florida's southwest coast (Chamberlain, 1980), and in 1977 and 1990 in the south central section of the state (Day and Curtis, 1993). Despite the known presence of SLE virus in the New World tropics, large-scale human epidemics have not been reported (Spence, 1980). The biotic and abiotic factors that interact together to result in human SLE epidemics remain poorly understood.

Eastern equine encephalomyelitis (EEE) virus is a second economically important mosquito-borne arbovirus found in Florida. The virus is enzootic to fresh water swamps throughout the central part of the state and is responsible for equine and human cases during the spring and summer months (Bigler et al., 1976).

The large-scale involvement of mammalian species in the maintenance and amplification of SLE and EEE viruses has not been documented. However, because SLE virus, SLE hemagglutination-inhibition (HAI) antibody, and SLE neutralizing (NT) antibodies have been detected in a number of wild mammal species, the SLE amplification and maintenance cycles may involve more than just wild birds and mosquitoes (McLean and Bowen, 1980).

There has been little evidence for the involvement of armadillos in SLE or EEE maintenance and amplification. Extensive wild vertebrate surveillance during an interepidemic period (1965 to 1967) in Florida resulted in positive HAI and NT titers to SLE virus from one armadillo captured in Orange County in 1965 (Nichols and Bigler, 1969).

Our objective was to determine the prevalence of SLE and EEE antibodies in armadillos. We also evaluated their possible involvement in the pre-epidemic and

epidemic cycling of SLE virus in southern and central Florida from January 1990 through March 1991.

MATERIALS AND METHODS

The armadillos for this study were captured in southern Florida in Brevard and Glades counties. Animals from southern Brevard County were captured in open grassy areas along canal banks (27°50'N, 80°30'W). Armadillo burrows were located along the upper portions of the canal banks dominated by live oak (*Quercus virginiana*) and saw-palmetto (*Serenoa repens*) stands. The lower portion of the canal bank consisted of open grassy areas where the armadillos foraged for food.

The Glades County collection site was located on a cattle ranch near Fisheating Creek (27°14'N, 81°27'W). Armadillos there were captured as they foraged in open pastures.

At both sites, foraging armadillos were captured with long-handled, heavy duty bird nets. Armadillos were captured between 1 January 1990 and 10 March 1991.

After capture, armadillos were placed immediately into a 43 × 32 × 20 cm Doskocil Cabin Kennel (Doskocil Mfg. Co., Inc., Arlington, Texas, USA) for short-term transportation (<24 hr) or into a 48 × 30 × 25 cm Doskocil Kennel Cab II (small) for long-term transportation (>24 hr) from the field. In the laboratory, armadillos were housed individually in an 81 × 56 × 58 cm Doskocil Vari-kennel (large) and maintained on a high quality, commercial cat chow (Purina Mills, Inc., St. Louis, Missouri, USA) softened with tap water. The animal rooms were air-conditioned, indoor, and mosquito-proof, precluding any infection with the SLE virus after the armadillos arrived.

Once in the laboratory, the sex of each armadillo was determined and armadillos were separated by weight into three age categories: young (<2.5 kg), young adult (2.6–3.0 kg), and adult (>3.1 kg).

To obtain blood, armadillos were anesthetized with a mixture of Ketaset (ketamine hydrochloride equivalent to 100 mg/ml; Ayerst Laboratories, Inc., New York, New York, USA) and PromAce (acepromazine maleate equivalent to 10 mg/ml; Ayerst Laboratories, Inc., New York, New York). The dosage used was 33 mg/kg of Ketaset and 1.1 mg/kg of PromAce administered by intramuscular injection.

Blood was collected from the right rear saphenous vein. One drop of blood was mixed in 0.7 ml of laboratory prepared Biological Field Diluent (BFD) for viral isolation attempts. The BFD was composed of 90% Minimum Essential Medium with Hank's salts (Sigma Chemical Company, St. Louis, Missouri), 10% fetal bovine serum (Intergen Co., Purchase, New York), 200 U/ml penicillin (Sigma), 200 μg/ml streptomycin (Sigma), 2.5 μg/ml amphotericin B (Sigma), and 50 μg/ml kanamycin (Sigma). The remaining blood was allowed to clot, centrifuged, and the serum was used for SLE hemagglutination-inhibition (HAI) and neutralizing (NT) antibody analysis.

A micro-adaption of the HAI antibody test of Beaty et al. (1989) was used with a hemagglutinin (HA) prepared from a Florida human SLE isolate (TBH-28). Additionally, all sera were examined in the same manner for the presence of HAI antibody against EEE virus using an HA prepared from a Florida human EEE isolate (D64-837).

Briefly, HA antigens were titrated at their optimal pH's 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-extracted sera starting at 1:10 in 0.4% bovalbumin-borate-saline, 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 4.0 C. Following the overnight incubation, 0.05 ml of a standardized goose erythrocyte suspension (Beaty et al., 1989) was added to the serum dilutions and to the control wells consisting of test serum controls, known positive and negative serum controls, antigen controls, and goose erythrocyte controls. Incubation proceeded at 22 to 24 C. When the antigen back titration indicated 4 to 8 units of HA to be present, the test findings were recorded.

A titer of 1:10 or greater was regarded as diagnostic of infection with an agent antigenically similar or related to that used in the test.

Confirmation of the SLE-HAI findings was attempted by examining sera for the presence of specific NT antibody for SLE. To that end, a sub-sample of each positive group of armadillos from each field collection was examined for NT antibody to SLE virus. Neutralizing antibodies may be measured by an in vivo test procedure when available. A common in vivo basic applied test, serial virus dilution with undiluted serum, was used (Beaty et al., 1989). The challenge virus, SLE P15, represented a geographic Florida isolate obtained from a pool of *Culex nigripalpus* Theobald mosquitoes.

Prior to initiation of the test, serum was inactivated for 30 min at 56 C. Equal amounts of inactivated serum were challenged with appropriate dilutions of the challenge virus. Serum-

TABLE 1. Hemagglutination (HAI) and neutralizing (NT) antibodies, and St. Louis encephalitis (SLE) and eastern equine encephalitis (EEE) viral isolation attempts from field-collected armadillos captured between January 1990 and March 1991 in Brevard and Glades Counties, Florida.

Capture date	Number bled	Number of armadillos positive for SLE HAI antibody	Percent HAI positive	Number of armadillos positive for SLE NT antibody/ number tested	Number of arboviral (EEE and SLE) isolations made/ number tested
January to February 1990	49	6	12[a]	3/3	0/0
July to August 1990	17	4	24[b]	4/4	0/6
September to October 1990	40	15	37[b]	6/6	0/40
November to December 1990	34	15	44[b]	3/3	0/34
February to March 1991	49	19	39[b]	15/15	0/49
Total	189	59	31	31/31	0/129

[a,b] Means in this column followed by different letters differed significantly ($P < 0.05$) in the G-test.

virus mixtures were incubated for 2 hr at 37 C and were then transferred to an ice bath for immediate inoculation. Each mixture was inoculated, 0.03 ml intracerebrally, into four to six 3- to 4-wk-old mice. Observation of the 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 or 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. Levels of less than 1.0 were considered negative, 1.0 to 1.6 equivocal, and 1.7 or greater as positive. The neutralization test for arboviruses is almost always the most specific tending to confirm HAI test findings (Beaty et al., 1989).

One- to three-day-old mice were used for viral isolation attempts with armadillo blood mixed 1:7 in BFD and stored at −70 C. One litter of eight suckling mice was inoculated with each undiluted thawed inoculum. Injections were 0.015 ml by the intracerebral and 0.03 ml by the intraperitoneal routes. Observation was conducted, post-inoculation, daily for at least 14 days. A 1:10 suspension of brain material from animals noted to be ill or found dead was passed to additional litters by the same routes to confirm the presence of an arboviral agent.

Statistical differences in the number of SLE-infected armadillos, their age and sex, and the location of capture were tested by using Unplanned Tests of the Homogeneity of Replicates Tested for Goodness of Fit (G-statistic) (Sokal and Rohlf, 1981).

RESULTS

Fifty-nine (31%) of 189 field-collected armadillos captured between January 1990 and March 1991 tested positive for HAI antibody to SLE virus. Significantly ($P < 0.05$) more armadillos collected between September 1990 and March 1991, during and immediately following the 1990 SLE epidemic, had SLE antibodies than those captured in January and February 1990 prior to the start of the epidemic transmission (Table 1).

Of the 31 HAI-positive armadillos tested, all were positive for NT antibody to SLE virus. No arboviruses were isolated from the blood of 129 armadillos collected between January 1990 and March 1991 (Table 1).

Most (140 of 189) armadillos were collected in Glades County. Of these, 47 (34%) were HAI-positive for SLE antibody. Twelve of the 49 (24%) armadillos collected in Brevard County contained HAI antibody to SLE virus. There was no significant difference in the number of HAI-positive armadillos collected in Brevard and Glades Counties.

Seventeen (24%) of 70 male and 42 (35%) of 119 female armadillos were HAI-positive for SLE antibody. This difference was not statistically significant when analyzed by the G-statistic. The young, young adult, and adult age groups were represented by 12, 15, and 162 animals respectively. Three

of 12 young armadillos were positive for SLE antibody, three of 15 young adults and 53 (33%) of 162 adults were HAI-positive. None of these differences were statistically significant when tested by the G-statistic.

No HAI antibody to EEE virus was detected in any of the 189 armadillos tested. No EEE virus was isolated from the blood of any of the 129 animals tested (Table 1).

DISCUSSION

Many (31%) armadillos collected in southern Florida during a known period of SLE transmission had HAI antibodies to SLE virus. In the only previous work done, one of 11 armadillos tested between 1965 and 1974 was HAI positive for SLE (Bigler et al., 1975).

The HAI test for flavivirus antibody is regarded as having the widest antigenic overlap among the HAI, complement fixation, and neutralization tests (Beaty et al., 1989). Therefore, interpretation of HAI serologic data for wild mammals must be made with caution. A rodent-associated flavivirus, Cowbone Ridge virus (Calisher et al., 1969), occurring in south Florida bears a distant relationship with SLE and should be considered in future serologic surveys. However, of the vertebrates caught in the south Florida Everglades, only the cotton rat (*Sigmodon hispidus*) has had evidence of widespread occurrence of Cowbone Ridge virus infection (Calisher et al., 1969).

Brevard County and Glades County both were involved in the 1990 SLE epidemic. The SLE seropositive rate of armadillos collected in Brevard and Glades Counties increased during the epidemic (Table 1); we propose that armadillos were likely bitten by SLE-infected mosquitoes in the field and that they mounted an immune response to the viral challenge.

The main vector of SLE virus in south Florida is *Culex nigripalpus* (Shroyer, 1991). This is a night-active species whose flight and host location behavior allow it access to nocturnally foraging armadillos. Edman (1974) reported blood feeding by *C. nigripalpus* on armadillos in the field. In fact, many mosquito species feed on armadillos (Edman, 1971, 1979).

A confirmed SLE virus isolation was made from an armadillo captured in Brazil (Vasconcelos et al., 1991). However, in our study no arboviral agents were isolated from the blood of 129 field-collected armadillos. This may be because blood samples were drawn in the laboratory weeks and even months after the armadillos were captured in the field. Viremias of SLE in wild birds are known to be extremely short, usually less than 1 wk (McLean and Bowen, 1980), and it is possible that viruses already had cleared from the armadillos' blood when the samples were drawn in the laboratory. However, because SLE virus has been isolated from a field-collected armadillo, and because armadillos maintain a low body temperature (30 C to 35 C) that depresses metabolic activity and cellular immunity (Purtilo et al., 1974), it is possible that armadillos may run viremias sufficient to infect susceptible mosquitoes. These possibilities should be investigated in the laboratory to determine the susceptibility of armadillos to SLE infection, the duration of SLE viremias in infected armadillos, and the ability of susceptible mosquito vectors to become infected with virus by feeding on SLE-infected armadillos.

It is not surprising that EEE antibody and virus were not detected in the armadillos we tested. While EEE virus is sometimes found in southern Florida, it is much more common in the northern half of the state. Furthermore, armadillos spend most of their time foraging in dry, upland habitats; areas where EEE virus is seldom found.

While it is unlikely that armadillos are more important than passerine birds as amplifying hosts for SLE virus, it is possible that armadillos contribute to the maintenance of SLE virus during interepidemic periods and that they are involved in viral amplification during pre-epidemic

and epidemic periods. We propose that armadillos were fed on by mosquitoes infected with SLE virus and that armadillos may be involved in the SLE amplification and transmission cycles in Florida.

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