ISOLATION OF LACROSSE AND OTHER ARBOVIRUSES FROM INDIANA MOSQUITOES, 1961–1982

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ABSTRACT. Eight isolations of three arboviruses were made from mosquitoes collected in Indiana during 1978–80. With the seven isolations made prior to 1966, the total number of virus isolates from mosquitoes in this state is now 15. Flanders virus has been isolated six times, St. Louis encephalitis and Trivittatus (TVT) viruses three times each, LaCrosse (LAC) virus twice and Bunyamwera group virus (probably Cache Valley virus) once. This is the first report of LAC and TVT virus isolations from mosquitoes in Indiana.

As in many areas of North America, a diversity of arboviruses occurs in Indiana. Unpublished evidence is available to document the periodic or continuous presence of 10 arboviruses in that state. From 1955 through 1982, 486 laboratory documented cases of St. Louis encephalitis (SLE) occurred in residents of 92 Indiana counties. In 1975 when SLE was epidemic throughout most of the upper Midwest and southeastern Canada, Indiana reported the third highest prevalence of cases, 323 with 17 deaths. St. Louis encephalitis occurred in persons residing in 61 counties. Since 1964, when surveillance for California (CAL) serogroup virus infections was begun in the Midwest, 58 confirmed cases (presumably caused by La Crosse (LAC) virus) have been reported in pediatric residents of Indiana (Indiana State Board of Health, unpublished data). Eastern equine encephalomyelitis virus has been isolated from equine brain tissue and serological evidence of western equine encephalomyelitis virus in horses in Indiana has been confirmed by the National Veterinary Services Laboratories at Ames, Iowa (James Pearson, NVSL, personal communication). In addition, serologic surveys of white-tailed deer in northern Indiana and of humans residing in 34 of 92 Indiana counties have provided evidence of infection of both species with Jamestown Canyon virus, a subtype of Melao virus of the CAL serogroup (Grimstad et al. 1982; R. D. Boromisa and P. R. Grimstad, unpublished data). Of 10,194 human sera, 0.23% had neutralizing antibodies to Trivittatus (TVT) virus of the CAL serogroup (Grimstad, unpublished data). The annual avian serological survey conducted in the state has shown that house sparrows from throughout Indiana also have antibody to Mermet virus (Simbu serogroup) (Calisher, Ahmann et al. 1981).

Despite this evidence of extensive arbovirus prevalence, there have been only two published reports of virus isolations from mosquitoes collected in Indiana (Newhouse and Siverly 1966, Kokernot, Hayes, Will et al. 1969). The purpose of this paper is to summarize all currently available information regarding viruses isolated from mosquitoes in Indiana.

The first published record of virus isolations from mosquitoes in Indiana was that of Newhouse and Siverly (1966). They reported three isolates of SLE virus from *Culex pipiens* complex mosquitoes collected on October 10–12, 1964. Two were from specimens collected in a flood tunnel and a culvert in Evansville, Vanderburgh County and one was

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from specimens collected in a sewer in Boonville, Warrick County.

This first published report is antedated by an unpublished record from the Centers for Disease Control which documents the isolation of a Bunyamwera serogroup virus, probably Cache Valley (CV), from three unfed *Psorophora* sp. mosquitoes. This collection was made by R. W. Chamberlain and D. W. Sudia in Ripley County on August 30, 1961.

The second published report was that of Kokernot, Hayes, Will et al. (1969). Three isolates of Flanders (FLA) virus were obtained from *Culex pipiens* complex mosquitoes collected in September 1965. Two isolates were from Osgood, Ripley County and one from Dupont, Jefferson County.

The isolates reported here were recovered from mosquitoes collected in the course of field studies conducted by Ball State University and Indiana State Board of Health personnel during the three summers, 1978–80.

STUDY AREAS

Mosquitoes from which viruses were isolated came from one site in Marion County (Indianapolis) and three sites in Delaware County (Muncie). The former site is the Indianapolis Livestock Market located within the city boundaries; the three Delaware County sites are properties owned by Ball State University. The Ball State Wildlife Preserve (BSWP) is located 4.4 km west of the center of Muncie and comprises 6.6 ha. The preserve is a remnant of White River bottomland which was isolated by the channeling of the river and subsequent levee construction. The old channel within the preserve is a shallow oxbow pond. This bottomland contains numerous ground depressions and a forest dominated by American sycamore (*Platanus occidentalis* L.), eastern cottonwood (*Populus deltoides* Bartr.), hackberry (*Celtis occidentalis* L.) and silver maple (*Acer saccharinum* L.). A second site, the Esther L. and Robert H. Cooper Memorial Woodland Area (CMWA), is located approximately 6.4 km northwest of the center of Muncie. This 12.75 ha tract comprises 5.5 ha of open land and 7.25 ha of woodland. There are two levels of canopy in the wooded area due to the removal of grazing animals in 1950. The upper story includes northern red oak (*Quercus rubra* L.), white oak (*Q. alba* L.), bur oak (*Q. macrocarpa* Michx.), shagbark hickory, (*Carya ovata* (Mill.) K. Koch.), and bitternut hickory (*C. cordiformis* (Wang.) K. Koch.). The lower story is dominated by white ash (*Fraxinus americana* L.), green ash (*F. pennsylvanica* Marsh.), sugar maple (*Acer saccharum* Marsh.) and slippery elm (*Ulmus rubra* Mühl.). The third site, Ginn Woods (GW), is located approximately 17.7 km north of Muncie. Comprising 65.2 ha, the woods is almost exclusively composed of American beech (*Fagus grandifolia* Ehrh.) and sugar maple (*A. saccharum*), and is the only beech-maple forest of comparable size remaining in east-central Indiana.

MATERIALS AND METHODS

ISOLATION OF VIRUSES FROM FIELD-COLLECTED MOSQUITOES. In 1978 and 1979 mosquitoes were collected with battery operated, dry-ice baited CDC miniature light traps (Hausherr's Machine Works, Tom's River, NJ) and by aspiration from human bait, culverts and other resting places. The standard techniques of Sudia and Chamberlain (1967) were used in handling and processing the collected mosquitoes. These were identified to species at Ball State University (Muncie) or the Indiana State Board of Health (Indianapolis), pooled by species, site and date and stored at −70°C in mechanical freezers. All pools were shipped frozen on dry-ice to the University of Notre Dame where they were processed for attempted virus isolation.

Mosquitoes were thawed, then sorted on a chill table and pooled into groups of 50 or fewer. Pooled mosquitoes were triturated in 1 dram screw-cap glass vials containing 8–9 glass beads (4 mm diam.) and 1 to 2 ml of diluent. Diluent consisted of Medium 199 supplemented with 20% heat inactivated fetal bovine serum (FBS) and antibiotics (500 units potassium penicillin and 500 g streptomycin sulfate) and 5 g of fungizone per ml. Vials containing mosquitoes, beads and diluent were mixed (Vortex Genie Mixer, Scientific Industries, Inc., Bohemia, NY) at high speed for 5–10 sec., then plunged into an ice bath. The grinding action of the glass beads readily disintegrated all mosquito tissues. Each pool vial was mixed 2–3 times in short bursts then cooled in the ice bath to minimize heat buildup from friction of the glass beads; approximately 20–25 pools at a time were processed in this manner. By the time trituration of the last pool was completed most of the debris had settled in the vials processed first. A 0.75 ml aliquot of the crude supernatant fluid was drawn off and centrifuged at 15,000 × g for 15 min at 4°C.

After centrifugation, and depending on which mosquito species constituted the pool, supernatant fluids were inoculated in one or more of 3 ways: a) directly (0.2 ml) onto monolayers of a continuous line of African green monkey kidney (Vero) cells, b) in a similar manner onto monolayers of primary Pekin

duck embryo (DE) cells; both types of cell cultures were grown in 6 well plastic trays (Linbro Division, Flow Laboratories, Inc.) and double overlayed in the manner described by Hayes et al. (1976) or, c) intracranially (0.03 ml) into individual 2- to 3 day old suckling mice (HARLAN/ICR strain; Harlan Industries, Inc., Indianapolis, IN). Remaining supernatant was frozen at −70°C in a mechanical freezer. Cell cultures were observed for a minimum of 10 days for plaque formation. When plaques appeared, virus was established by removing the agar overlay and suspending the remaining cells in 2 ml of diluent. These suspensions were inoculated into 2- to 3-day old ICR suckling mice; all passage and reisolation was performed in suckling mice.

IDENTIFICATION OF VIRUSES. For preliminary identifications of viruses, brains of moribund mice were tested as alkaline extracts in complement-fixation tests as described previously (Calisher and Maness 1975). These crude antigens were then tested against a battery of mouse hyperimmune ascitic fluids prepared with North American arboviruses (Tikasingh et al. 1966). For confirmation, neutralization tests in Vero cells were performed using the serum dilution-plaque reduction (SDPRN) technique of Lindsey et al. (1976). Subtyping of CAL serogroup viruses was by the same method (Lindsey et al. 1976; Calisher, Monath et al. 1981).

RESULTS AND DISCUSSION

A total of 9350 adult mosquitoes comprising 24 species was collected in Indiana from 1978 to 1982 (Table 1). *Aedes vexans* (Meigen) represented 30% of the specimens tested for virus, *Ae. trivittatus* (Coquillett) (18%), *Ae. triseriatus* (Say) (17%), *Coquillettidia perturbans* (Walker) (12%), and *Culex pipiens/restuans* (9%).

During this period, seven virus isolates were recovered from mosquitoes collected in Delaware County and one from mosquitoes collected in Indianapolis, Marion County (Table 2). From mosquitoes collected in 1978, three strains of FLA virus and one each of TVT and LAC viruses were isolated. One strain of FLA virus was isolated from a pool of *Cx. pipiens* mosquitoes aspirated from human bait near the center of Marion County. The other two FLA virus isolates were from Delaware County, the first from a pool of 28 *Cx. pipiens* complex mosquitoes collected on July 27 in a CDC light trap located at CMWA, the second from a pool of six *Cx. pipiens* complex mosquitoes aspirated from a culvert at the BSWP on August 9. All three FLA isolates were confirmed by SDPRN tests using Hart Park and FLA prototype viruses. Trivittatus virus was isolated from a pool of 25 *Ae. trivittatus* collected in a CDC light trap at CMWA on July 27. The 1978 LAC virus isolate was recovered from a pool of four *Ae. triseriatus* females collected in a CDC light trap located at GW on August 23.

In 1979, two isolates of TVT virus were made from *Ae. trivittatus* mosquitoes collected in Delaware County, one from Ginn Woods on August 10 and one from BSWP on August 11. Both isolations came from pools of 25 *Ae. trivittatus* mosquitoes collected with human bait. All isolates in 1978 and 1979 were from unfed female mosquitoes.

Table 1. Adult mosquitoes collected for virus isolation in Indiana, 1978–82.

Mosquito species	Year					Total
	1978	1979	1980	1981	1982	
Aedes hendersoni	220	3	0	0	0	223
Ae. sticticus	17	136	0	1	0	154
Ae. stimulans	8	94	0	0	0	102
Ae. triseriatus	1554[a]	46	0	0	0	1600
Ae. trivittatus	439[b]	1250[b]	1	1	0	1691
Ae. vexans	314	246	2117	100	0	2777
Anopheles punctipennis	105	18	3	2	3	131
Coquillettidia perturbans	0	1104	0	0	0	1104
Culex pipiens	90[c]	6	0	0	0	96
Cx. pipiens/restuans	354[c]	61	0	0	0	415
Cx. salinarus	20	267	0	0	0	287
Cx. sp.	309	70	4	3	0	386
Culiseta melanura	0	0	0	0	40	40
Psorophora columbiae	0	0	208	9	0	217
Other species	48	68	1	1	9	127
Total	3478	3569	2334	117	52	9350

[a] La Crosse virus isolated.
[b] Trivittatus virus isolated.
[c] Flanders virus isolated.

Table 2. Arbovirus isolations from mosquitoes in Indiana 1961–80.

Year	Virus	County of Location	Mosquito species	Collection method
1961	Bunyamwera prob. Cache Valley[1]	Ripley	*Psorophora sp.*	unknown
1964	St. Louis encephalitis[2]	Vanderburgh (Evansville)	*Culex pipens* complex	aspiration culvert
			Culex pipiens complex	aspiration flood tunnel
1964	St. Louis encephalitis[2]	Warrick (Boonville)	*Culex pipiens* complex	aspiration sewer
1965	Flanders[3, 4]	Ripley (Osgood)	*Culex pipiens* complex	aspiration probably storm sewer
1965	Flanders[3]	Jefferson (Dupont)	*Culex pipiens* complex	aspiration probably storm sewer
1978	Flanders	Marion (Indianapolis)	*Culex pipiens*	human bait
1978	Trivittatus	Delaware (CMWA)	*Aedes trivittatus*	CDC light trap
1978	Flanders	Delaware (CMWA)	*Culex pipiens* complex	CDC light trap
1978	Flanders	Delaware (BSWP)	*Culex pipiens* complex	aspiration culvert
1978	La Crosse	Delaware (GW)	*Aedes triseriatus*	CDC light trap
1979	Trivittatus	Delaware (GW)	*Aedes trivittatus*	human bait
1979	Trivittatus	Delaware (BSWP)	*Aedes trivittatus*	human bait
1980	La Crosse	Delaware (GW)	*Aedes triseriatus* (pupae)	oviposition trap

[1] Unpublished CDC records.
[2] Newhouse and Siverly (1966).
[3] Kokernot, Hays, Chan et al. (1969).
[4] Two isolations.

The 1978 LAC virus isolate has been characterized by Klimas et al. (1981) using the procedure of oligonucleotide fingerprinting. In terms of RNA, this isolate has been categorized as an "A" type LAC virus, with more oligonucleotides in common with the prototype LAC virus than with other ("B") isolates.

The recovery of this first LAC virus isolation from Indiana mosquitoes led to the deployment of 40 artificial oviposition sites (Loor and Defoliart 1969) in the GW study area in 1980. A total of 6265 larvae and pupae reared from eggs deposited in these oviposition traps were tested for virus. LAC virus was recovered from a pool of 15 pupae reared from eggs deposited during July 13–27 (Thomas and Pinger 1982). This isolation from pupae indicates that LAC virus is probably maintained at GW by transovarial transmission, the phenomenon first reported by Watts et al. (1973).

The isolation of TVT virus in Indiana was not unexpected. Neighboring states of Illinois and Ohio had reported isolations of TVT virus as early as 1969 and 1971 respectively (Kokernot, Hayes, Chan et al. 1969; Masterson et al. 1971). Furthermore, earlier serological studies of mammalian sera from the BSWP and CMWA indicated the presence of TVT virus activity; antibodies to TVT virus were detected in 8/13 red squirrels, 2/8 fox squirrels, and 4/13 eastern cottontail rabbits (Pinger et al. 1979).

Despite the high incidence of human SLE cases in Indiana in 1975, no isolations of SLE virus were made during the 1978–80 period. However, with the exception of 65 mosquitoes tested from central Marion County, no effort was made to collect in urban areas where the likelihood of SLE virus isolations would be increased. *Aedes* and *Coquillettidia* mosquitoes predominated in all collections (82% of the mosquitoes tested).

In summary, there have been 15 isolations of five viruses from Indiana, seven isolations before 1978 and eight isolations during the period 1978–80. Flanders virus was isolated six times, SLE and TVT viruses three each, LAC virus twice and Bunyamwera group virus (probably CV virus) once. This is the first report of LAC and TVT virus isolations from mosquitoes in Indiana.

Investigations of the ecology and epidemiology of arboviruses in Indiana are continuing and we anticipate isolations of other agents in the near future. The Indiana State Board of Health's mosquito-borne encephalitis surveillance program, which was initiated in late 1976 in response to the 1975 SLE epidemic, continues to focus on eastern, western and SLE viruses in a cooperative effort with the University of Notre Dame's Laboratory for Arbovirus Research and Surveillance. The major research emphasis of that laboratory is on CAL

sero-group viruses, and in particular, Jamestown Canyon virus. This report serves to establish a background for present and future investigations.

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