ISOLATIONS OF LaCROSSE VIRUS OF THE CALIFORNIA GROUP FROM TABANIDAE IN WISCONSIN[1]

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Isolations of the Jamestown Canyon serotype of the California encephalitis virus (CEV) group were reported by DeFoliart *et al.* (1969) from members of the dipterous family Tabanidae (horse flies and deer flies). They also reported isolations of the virus from *Aedes* mosquitoes.

We report here isolations of a second member of the CEV group, LaCrosse virus, from Tabanidae, these isolations being from the large horse fly, *Hybomitra lasiophthalma* (Macq.). LaCrosse virus is the only member of the California group presently known to present a human health problem in Wisconsin, having been responsible for more than 50 cases of California encephalitis since 1960 (Thompson and Inhorn, 1967). All other isolations of LaCrosse virus from arthropods in Wisconsin were from mosquitoes, with by far the highest infection rates in nature in *Aedes triseriatus* (Say) (Thompson *et al.*).

MATERIALS AND METHODS. The tabanids and mosquitoes reported on here were collected in 1967 during a study of host preferences of hematophagous Diptera at the Sandhill Game Farm, Wood County, west central Wisconsin. All the tabanids and most of the mosquitoes that were pooled for attempted virus isolations were collected in large Saran screen cages (Chicopee Manufacturing Co., Cornelia, Georgia) in which a host animal had been exposed for a period of time. The cages and methods of exposing hosts were described by Wright and DeFoliart (1970). At the end of an exposure period the entrance of the cage was closed and the insects inside were collected by using a modified flashlight type aspirator equipped with removable collecting tubes. When sufficient numbers were in a tube it was removed and placed in a dry-ice chest. When dead all insects from a common collection were placed in a plastic box which was sealed, labeled and placed in a large dry-ice chest for transportation to the laboratory. There they were stored at —20° C. until removed for identification. They were thawed just enough to permit identification and sorting as to species, sex, and state of engorgement. They were then pooled and refrozen on dry-ice for a short period before attempts were made to isolate viruses.

Only *Anopheles earlei* Vargas and some *Mansonia perturbans* (Walker) were not collected in the Saran cages. They were collected instead by aspirator as they rested in deer box traps.

The procedures used to attempt to isolate viruses were as described by Anslow *et al.* (1969). Identification of each of the three suckling mouse lethal isolates depended upon the activity of that agent and its homologous antibody in agar gel immunodiffusion (Papadopoulos *et al.*, 1970), in complement fixation tests, and in neutralization tests in suspended cell culture.

For virus neutralization a modified metabolic inhibition test (MIT) was used, employing Vero cells in uniform suspension of 25,000 per ml. in growth medium (Medium 199 with Hank's balanced salt solution supplemented with minimum es-

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TABLE 1.—Tabanidae from Sandhill Game Preserve, Wood County, Wisconsin captured during June, 1967, and pooled for attempted isolations of arboviruses.

Species	Total individuals/ total pools
Hybomitra lasiophthalma	121/20*
Hybomitra illota	34/2
Hybomitra epistates	12/2
Hybomitra typhus	12/1
Hybomitra nuda	4/1
Chrysops excitans	12/1
Total	195/27

* Three pools, each containing five specimens, were positive for LaCrosse serotype of California encephalitis virus.

sential maintenance vitamins, 10 percent heat-inactivated fetal calf serum, and antibiotics). Of the above-described cell suspension, 0.75 ml. was dispensed into each well in clear plastic panels to be used in the planned test. The constant virus-varying serum method was used in this procedure. Each virus dose was calculated to contain 100 $TCID_{50}$. Test serum was used in 4-fold serial dilutions. Serum-virus mixtures were incubated at 37° C. for 60 minutes, then transferred to an ice bath until dispensed into the previously described MIT panels, 0.1 ml. serum-virus mixture per cup and 3 cups per serum dilution. In the MIT panel, the mixtures were incubated for 2 to 9 days at 37° C. Results were read colorimetrically.

RESULTS. Table 1 shows the number of individuals of each species of Tabanidae that were pooled and the total number of pools processed. For comparative purposes, Table 2 shows Culicidae from the same location that were pooled and inoculated into mice. There were no isolates from the 2,285 mosquitoes, but one of the six tabanid species, *Hybomitra lasiophthalma* (Macq.), yielded arbovirus isolates.

All *H. lasiophthalma* collected on 13 and 14 June, 1967, were sorted into 20 pools on 6 September. Each pool was inoculated into suckling mice on 25 September, 1967. Of the 20 pools, 3 yielded a suckling mouse-lethal agent, each of which was later shown to be closely related, if not identical, to LaCrosse virus. Each of the three isolates was recovered from a pool of *H. lasiophthalma* collected 13 June. Pools collected at the same site on 14 June yielded no virus nor did any of seven pools of other tabanids collected 14 June at the same site (Table 1).

Table 3 records the history, including successful reisolation from the original arthropod suspension, and certain characteristics of each virus. None of these is inconsistent with the assumption that each is an arbovirus.

The first evidence of identity came from application of the agar gel diffusion technique. Before antibody was produced, infected suckling mouse brain antigen was found to react in this test specifically with LaCrosse antibody (Papadopoulos *et al.*, 1970). Confirmatory evidence was supplied by the Zoonoses Research Labora-

TABLE 2.—Culicidae from Sandhill Game Preserve that were captured in 1967 and pooled for attempted isolations of arboviruses.

Species	Total indivdiuals/total pools			
	June	July	August	Totals
A. abserratus-punctor	48/1			48/1
A. canadensis	115/3	182/5		297/8
A. cinereus	231/6	235/6		466/12
A. communis gr.	30/1	212/5		242/6
A. stimulans gr.	153/3			153/3
A. vexans	340/6	353/8		693/14
M. perturbans	15/1	108/3	86/2	208/6
C. morsitans	73/2			73/2
An. earlei		32/2	48/2	80/4
C. restuans	24/1			24/1
Totals	1029/24	1122/29	134/4	2285/57

TABLE 3.—History and characteristics of isolates, Wisconsin strains, LaCrosse serotype of California encephalitis virus from the tabanid, *H. lasiophthalma,* collected at Sandhill.

A P No.	Number of insects	Date collected (1967)	Date mice inoculated (1967)	Incubation period, orig. passage	Date of second recovery	LD_{50}*	Ether** sensitivity
950B	5	13 June	25 Sept.	11 days	19 Oct. '67	$10^{-6.4}$	2.66
952A	5	13 June	25 Sept.	5–6 days	19 Oct. '67	$10^{-5.0}$	1.5
952B	5	13 June	25 Sept.	5 days	19 Oct. '67	$10^{-6.0}$	2.75

* Expression of infectivity of 4th suckling mouse brain passage of each isolate as determined from death pattern resulting from intracerebral inoculation of serial 10-fold dilutions of each virus into young adult mice.

** Log_{10} of infectivity lost during overnight exposure to absolute diethyl ether.

tory, in the form of comparative complement-fixation tests (CF). Table 4 presents results of CF tests using the unknown and reference antigens against antibody as serum from guinea pigs that received two doses of suckling mouse brain suspension. The CF tests were conducted in microtiter plates following the LBCF (Casey, 1965) methods. It was apparent that the three isolates were serologically related and similar to or identical with LaCrosse virus. The sucrose-acetone extracted suckling mouse brain antigen of each of the three isolates reacted with antibody to LaCrosse virus at dilutions of 1:32 or higher but reacted at 1:16 dilution or lower with snowshoe hare, *trivittatus,* or Jamestown Canyon antiserum.

Table 5 records results with virus neutralization in suspended cell culture.

The three isolates from the tabanids appeared clearly to be related exclusively to the LaCrosse virus, differing decidedly in antigenic specificity from the *trivittatus* and from the Jamestown Canyon viral strains.

DISCUSSION. There is evidence, not complete, that the vector of LaCrosse virus to man in Wisconsin is the tree-hole breeding mosquito, *Aedes triseriatus.* This mosquito species has shown a high infection rate from July to September in wooded areas of unglaciated southwestern Wisconsin where the virus is thought to be endemic (Thompson *et al.*). Other data of an ecological or epidemiological nature and the preliminary results of transmission experiments involving *Aedes triseriatus* tend to support the above hypothesis.

The isolation of LaCrosse virus from

TABLE 4.—Cross complement fixation of the three tabanid isolates and five reference California viruses.

	Anti-Serums								
	G. P. Serums								
Antigens	950/B	952/A	952/B	LaX	SSH	Triv	JTC	BFS	NSMB
950/B	5/7*	6	5	4	3	2	2	2	<2
952/A	6**	7/5	6	4	3	<2	2	<2	<2
952/B	5	6	5/6	4	3	2	2	2	<2
LaX	7	7	5	5/8	4	4	3	3	<2
SSH	<2	5	4	<2	5/7	2	<2	<2	<2
Triv	<2	3	4	<2	<2	7/6	<2	<2	<2
JTC	4	5	4	2	2	4	4/7	<2	<2
BFS	4	6	4	3	4	5	2	4/7	<2
NSMB	<2	<2	<2	<2	<2	<2	<2	<2	<8/5
Serum Control	<2	<2	<2	<2	<2	<2	<2	<2	<2

* Serum titer/antigen titer. All titers indicated by dilution number: 2=8, 2=1:8 with 8=512.

** Serum titer *vs.* optimal dilution of antigen.

TABLE 5.—Cross-neutralization by the metabolic-inhibition test of three tabanid isolates and three reference California-group viruses.

Virus	Serum* LaX	Triv	JTC
952A	1260*	20	<10
952B	920	30	<10
950	3020	20	<10
LaCrosse	890	<10	<10
Trivittatus	<10	1260	<10
Jamestown Canyon	30	30	85

* 50% Serum neutralization end point calculated by the method of Reed & Muench.

the tabanid, *H. lasiophthalma,* presents a situation quite different from that which we usually associate with this virus. Not only were the isolations from an unexpected insect source, and made at an unusual time, mid-June, but were from specimens from a geographic area where the virus has been found on only one other occasion. Later in the same year, LaCrosse virus was isolated from a pool of *Aedes communis* group mosquitoes captured about 10 miles south of Sandhill. An important question concerning these isolations is whether the tabanids were mechanically or biologically infected. None of the specimens pooled contained even a partial blood meal which suggests that they had not fed recently unless an attempt was made with no blood being imbibed. The fact that at least three specimens yielded virus suggests the source was probably near to the site of collection.

Now that *H. lasiophthalma* has been associated with two serotypes of CEV, Jamestown Canyon (DeFoliart *et al.,* 1969) and LaCrosse, it is interesting to speculate on the possible source of infection for these large horse flies. Perhaps as suggested by the above authors these insects, which are mainly regarded as large mammal feeders, feed more often than we think on small mammals which we associate with the CEV group, or viruses of this group may be associated with large mammals to a greater extent than we now suspect. Greater knowledge of the feeding associations of these tabanids will help eventually to determine whether they are potential mechanical or biological vectors of some of the arboviruses.

SUMMARY. LaCrosse virus was isolated from three pools consisting of unengorged specimens of the horse fly, *Hybomitra lasiophthalma* (Macq.), captured during June, 1967, in west-central Wisconsin. Identification of each of the three suckling mouse lethal isolates depended upon the activity of that agent and its homologous antibody in agar gel immunodiffusion, in complement fixation, and in neutralization tests in suspended cell culture. These isolations indicate only that tabanids can become infected in nature; they do not necessarily provide evidence that tabanids can transmit the virus or that they have a role in its maintenance in nature.

References

ANSLOW, R. O., THOMPSON, W. H., THOMPSON, P. H., DEFOLIART, G. R., PAPADOPOULOS, O. AND HANSON, R. P. 1969. Isolation of Bunyamwera—group viruses from Wisconsin mosquitoes. Amer. J. Trop. Med. and Hyg. 18(4):599–608.

CASEY, H. L. 1965. Adaptation of LBCF method of micro technique. *In* Standard Diagnostic Complement-Fixation Method and Adaptation to Micro Test. Public Health Monogr. No. 74, pp. 17–28.

DEFOLIART, G. R., ANSLOW, R. O., HANSON, R. P., MORRIS, C. D., PAPADOPOULOS, O. AND SATHER, GLADYS E. 1969. Isolation of Jamestown Canyon serotype of California encephalitis virus from naturally infected *Aedes* mosquitoes and tabanids. Amer. J. Trop. Med. and Hyg. 18(3): 440–447.

PAPADOPOULOS, O., ANSLOW, R. O. AND HANSON, R. P. 1970. Application of the immunodiffusion procedure to rapid identification of arboviruses and to detection of specific antibody in sentinel animals. Amer. J. Epidemiol. 92(2):145–150.

THOMPSON, W. H. AND INHORN, S. L. 1967. Arthropod-borne California group viral encephalitis in Wisconsin. Wis. Med. J. 66:250–253.

THOMPSON, W. H., ANSLOW, R. O., HANSON, R. P. AND DEFOLIART, G. R. 1970. LaCrosse virus isolations from Wisconsin mosquitoes, 1964–68. (In preparation.)

WRIGHT, R. E. AND DEFOLIART, G. R. 1970. Associations of Wisconsin mosquitoes and woodland vertebrate hosts. Ann. Ent. Soc. Amer. 63: (3):777–786.