# PREVALENCE RATES OF LACROSSE VIRUS (CALIFORNIA ENCEPHALITIS GROUP) IN LARVAE FROM OVERWINTERED EGGS OF *AEDES TRISERIATUS*[1]

M. A. LISITZA[2], G. R. DEFOLIART[2], T. M. YUILL[3] AND M. G. KARANDINOS[2]

University of Wisconsin, Madison, WI 53706

ABSTRACT. Prevalence levels of LaCrosse (LAC) virus in larvae from overwintered eggs of *Aedes triseriatus* were between .0029 and .0059 (calculated as minimum field infection rates) during a 2-year study of 2 localities in southwestern Wisconsin that have long been known as sites of endemic LAC virus activity. In several other localities that had not been previously studied but which had ample *Ae. triseriatus* populations overwintering virus prevalence did not exceed .0014. In a one-year comparison of a north slope with an opposing south slope that was drier, had less ground cover and fewer chipmunks, a significantly higher overwintering virus prevalence was detected on the north slope. It appeared that, within a given endemic locality, any treehole capable of supporting the development of *Ae. triseriatus* larvae is a potential overwintering site for LAC virus. The data provide baseline information on virus prevalence that will be useful in attempting to model the epidemiology of LAC virus.

LaCrosse virus (California encephalitis group) has been shown to be transmitted transovarially by the mosquito, *Aedes triseriatus* Say, and to survive the winter in the diapaused eggs of this mosquito (Watts et al. 1973, 1974). In future attempts to model the endemic maintenance of LAC virus and to quantify its prevalence in a given area we plan to use as our baseline measurement the prevalence of virus in the overwintered eggs. Our main objective in the study reported here was to determine the range of LAC virus overwintering prevalences in *Ae. triseriatus* at sites where the virus was known to have been continuously active for a number of years. These sites were considered to have all of the necessary requisites for continuous maintenance of the virus. Several other localities with ample *Ae. triseriatus* populations but with unknown virus history were studied for comparison. All sites except one were located in Iowa County, Wisconsin, which lies in the eastern part of the endemic zone comprised of southeastern Minnesota, southwestern Wisconsin and northeastern Iowa. The study was continued for 2 years in order to gain information on year-to-year as well as site-to-site variations in overwintering virus prevalence. Results of a similar 1-year study in the highly endemic LaCrosse, Wisconsin vicinity were reported previously by Beaty and Thompson (1975).

## MATERIALS AND METHODS

STUDY SITES. During 1974, 5 study sites were used, 4 of which were located in Iowa County which lies within the eastern part of the known LAC endemic zone. The endemic zone in Wisconsin corresponds roughly with the so-called Driftless Area or unglaciated area of the southwestern part of the State. The 5th site in 1974, the University of Wisconsin Arboretum, is in Dane County, within the city limits of Madison, and is considered to lie east of the endemic zone. In 2 of the areas, Hanson and Davis, LAC virus was known from previous studies to have persisted over a period of several years.

During 1975, studies at the University of Wisconsin Arboretum and the Kaser site were discontinued. To permit specific comparisons of existing sites with adjacent

[1] Research supported by the College of Agricultural and Life Sciences University of Wisconsin, Madison and by NIH Grant AI-07453. We thankC. E. Garry, P. R. Grimstad, W. R. Hansen and Mr. B. D. Nassif for assistance during this study.

[2] Department of Entomology.

[3] Department of Veterinary Science.

areas, however, 2 new sites, Hanson B and Burkholder N were established near the Hanson and Burkholder S sites, respectively. All of the study sites were second growth oak woods with the exception of the Kaser site which was a mature and undisturbed oak woods. Each year the sites were searched in early spring for all potential treeholes.

LARVAL MOSQUITO COLLECTIONS. Collections of larvae were made periodically from May 14 to June 14, 1974, and from May 15 to June 6, 1975. Collections were not made after the 1st pupae were observed in order to insure that all larvae collected were from overwintered eggs. An attempt was made to collect most of the larvae from within each treehole. The larvae were siphoned up with a turkey baster and brought back to the laboratory in sterile glass jars. The baster was washed with distilled water after sampling each treehole. Fourth instar *Ae. triseriatus* larvae were separated from *Ae. hendersoni,* then pooled in groups of up to 25 (1974) or 30 (1975) larvae in sterile 1-dram glass vials containing 6 glass beads. The pools were frozen at −60°C to await virus assay. Smaller larvae were reared in sterilized enamel pans using the original water plus tap water, fed ground TetraMinR (staple fish food) until 4th instar and then pooled as just described.

VIRUS ISOLATION: Larval pools were ground by putting the frozen sample on a vortex mixer for 1 minute, then adding 1 ml diluent (199 Hanks with 20% heat-inactivated fetal calf serum, 800 units of potassium penicillin G and 800 mg of streptomycin sulfate per ml, and 7.5% sodium bicarbonate to a pH of 8.0) and mixing on the vortex again for 15 sec. Five to six 1-to 3-day-old Swiss albino mice were inoculated intracerebrally with 0.02 ml per mouse. The mice were observed daily for 10 days following inoculation in 1974, for 7 days in 1975, and those dead or moribund were frozen at −60°C for virus identification.

VIRUS IDENTIFICATION. Mouse brain material was harvested, diluted to 20% with diluent and these suspensions were used in a microtissue culture neutralization test for virus identification, employing the constant serum-varying virus technique in Vero cells as described by Pantuwatana et al. (1972). Reference antisera, hyperimmune mouse ascitic fluids (MAF) were prepared against prototype strains of 4 California group viruses, La-Crosse (LAC), Snowshoe hare (SSH), Jamestown Canyon (JC) and trivittatus (TVT), all know to occur in Wisconsin. MAF was prepared by the technique of Brandt et al. (1967), with Sarcoma 180 cells (Tikasingh et al., 1966) using virus in the following suckling mouse brain passage: LAC in the 5th, SSH in the 23rd, JC in the 6th and TVT in the 17th. All samples were tested against all 4 antisera. A sample was regarded as LAC if homologous log neutralization indices were at least 2.5 logs more than heterologous indices. During 1975, 3 samples, because of low virus titers, showed cross reaction with SSH. However, since the neutralization pattern, as compared with the reference virus/antisera control, indicated that LAC was present, these samples were counted as positive.

DATA ANALYSIS. In arbovirus field studies, the mosquito infection rate is usually reported as the minimum field infection rate (MFIR) and is calculated by either dividing the number of positive pools by the total number of specimens assayed (the method we used) or by dividing the total number of specimens assayed by the number of positive pools. These methods yield only the minimum possible infection rate of a sample as there may be more than one infected individual in a positive pool. Further, it is obvious that the degree of probable error increases as the ratio of infected to uninfected pools increases. In samples from some treeholes in our study this ratio was as high as 1:4 to 1:2. This raised a question as to whether the mean infection rates in those treeholes were high enough to alter conclusions based on calculation of the minimum field infection rates. As a result, mean infection rates were estimated by the method described below. For comparisons between sites and

between years, the mean infection rates were then compared using a 1-tailed Z test.

METHOD OF ESTIMATING MEAN INFECTION RATE. Let a random sample of L mosquito larvae be taken from a large population. If $\ell$ is the (unknown) number of infected larvae in the sample, then $\ell$/L is the estimated proportion of infected larvae in the population. We randomly divide these L larvae into G groups with n larvae per group (n = L/G) and determine in the laboratory the number g of infected groups. The number X of infected larvae in a group has a binomial distribution, namely

$$b(X, e/L, n) = \left(\frac{n}{x}\right)\left(\frac{e}{L}\right)^{x}\left(1-\frac{e}{L}\right)^{n-x} \quad (1)$$
$$\text{for } X = 0, 1, 2, ---n$$

We know that if there is one or more infected larvae in a group (x = 1,2, . . . n), then the group will be positive. If x = 0, then the group will be negative. The probability of obtaining a negative group can be estimated from (1) for x = 0, namely

$$b(0,e/L, n) = \left(1-\frac{n}{L}\right) \quad (2)$$

We equate (2) with the observed proportion of negative groups, i.e.

$\frac{G-g}{G}$, and solve for the unknown $\frac{e}{L}$

(method of moments).

Thus

$$\left(1-\frac{e}{L}\right)^{n} = \frac{G-g}{G} \quad (3)$$

Taking the logarithms in (3) and solving for $\ell$ we have

$$\frac{e}{L} = \left[\left(1 - \text{antilog}\frac{\log(G-g)-\log G}{n}\right)\right] \quad (4)$$

It can now be seen from (4) that for g = 0, $\ell$ = 0. As g increases approaching G, $\ell$ also increases. These two properties of (4) are consistent with common sense expectations.

Example #1: Hanson 1975, treehole #12 (Table 3).

L = 474, G = 16, g = 3
From (4) we find $\ell$ = 3.31

Example #2: Davis 1974, treehole #40.
L = 287, G = 13, g = 5
From (4) we find $\ell$ = 6.24

## RESULTS AND DISCUSSION

LAC virus was present in all areas sampled within the endemic zone (Table 1). Relatively high rates of virus prevalence in larvae from overwintered *Ae. triseriatus* eggs were found in both of the localities, Hanson and Davis farms, where LAC virus was known previously to have persisted continuously for several years. Within-year differences at the 2 sites and within-site differences from one year to the next at each site were not significant ($p>.05$) (Table 2).

The minimum overwintering prevalence rate of .0059 found at the Davis farm in the spring of 1974 matches the overwintering prevalence (.006) found in the vicinity of LaCrosse, Wisconsin by Beaty and Thompson (1975). The LaCrosse (city) area is considered to be in the heart of the LAC virus endemic zone that includes southeastern Minnesota, southwestern Wisconsin and northeastern Iowa. In one intense focus within their study area, Beaty and Thompson found a prevalence level of .012; this was largely the result, however, of one very "hot" treehole that yielded half of the isolates obtained. As a basis for future modeling of the epidemiology of LAC virus, our major aim in this study was to determine the range of overwintering virus prevalence that may be considered typical of highly endemic localities. Data from the 3 localities that have now been studied, i.e., Hanson, Davis and LaCrosse (city), suggest that the overwintering virus prevalence (calculated as

Table 1. Prevalence of LAC virus in *Aedes triseriatus* larvae from overwintered eggs in southwestern Wisconsin.

| Locality | Year | Treeholes | | | No. of larvae assayed | Total number of pools | No. of positive pools | Field infection rate[b] | |
|---|---|---|---|---|---|---|---|---|---|
| | | Total sampled | No. positive | % positive[a] | | | | minimum | mean |
| Hanson | 1974 | 17 | 6 | 50 | 2941 | 134 | 10 | .0034 | .0038 |
| | 1975 | 19 | 8 | 50 | 4409 | 164 | 13 | .0029 | .0032 |
| Davis | 1974 | 8 | 2 | 50 | 1194 | 55 | 7 | .0059 | .0072 |
| | 1975 | 9 | 2 | 50 | 1723 | 65 | 5 | .0029 | .0032 |
| Burkholder S | 1974 | 14 | 0 | 0 | 2254 | 108 | 0 | 0 | 0 |
| | 1975 | 15 | 1 | 13 | 3521 | 130 | 1 | .0003 | .0003 |
| Kaser | 1974 | 9 | 2 | 29 | 2187 | 120 | 3 | .0014 | .0014 |
| Hanson B | 1975 | 19 | 1 | 07 | 9693 | 334 | 2 | .0002 | .0002 |
| Burkholder N | 1975 | 12 | 5 | 56 | 4474 | 160 | 6 | .0013 | .0014 |
| UW Arboretum[c] | 1974 | 10 | 0 | 0 | 1544 | 73 | 0 | 0 | 0 |

[a] Based only on treeholes from which 100 or more larvae were collected.
[b] See text for explanation.
[c] In Dane County; considered to be east of the endemic zone.

Table 2. Statistical comparisons of mean infection rates between sites and between years.

| Locality and Year | | Z value* |
|---|---|---|
| Davis '74 | vs. Davis '75 | 1.54 |
| Hanson '74 | vs. Hanson '75 | 0.43 |
| Burkholder S '74 | vs. Burkholder S '75 | 1.00 |
| Davis '74 | vs. Hanson '74 | 1.42 |
| Davis '74 | vs. Kaser '74 | 2.76 |
| Hanson '74 | vs. Kaser'74 | 1.60 |
| Davis '75 | vs. Hanson '75 | — |
| Davis '75 | vs. Burkholder N '75 | 1.50 |
| Hanson '75 | vs. Burkholder N '75 | 1.80 |
| Hanson '75 | vs. Hanson B '75 | 4.89 |
| Burkholder N '75 | vs. Burkholder S '75 | 1.83 |

* $Z_{.05} = 1.64$; $Z_{.01} = 2.33$.

MFIR) will usually range from about .003 to .006. At other localities sampled in Iowa County during the 2-year period, overwintering virus prevalence did not exceed .0014 (Table 1).

Failure to isolate LAC virus from larvae collected at the Burkholder S site in 1974 prompted a comparative study of this site, a south-facing slope, with an opposing north-facing slope (Burkholder N) in 1975 (Table 1). Although no animal census or vegetational analysis was made, compared to the opposing north slope, the south slope is relatively dry, and ground cover and chipmunks are relatively scarce. Treeholes and *Ae. triseriatus* larvae are plentiful, however, in both areas. The 2 slopes are separated by a 0.3 km-wide band of pastures and cultivated crops on the valley floor, but the woods that cover the slopes are separated only by a gravel road 1.1 km up-valley from the study sites and are part of an extensive forested area covering many square km. One isolate was obtained from the Burkholder S (south slope) in 1975, indicating an overwintering prevalence of .0003 while 6 isolates were obtained from larvae on the north slope, giving a prevalence rate 4.3X greater than that on the south slope. This difference was significant at the 5% level and supports the finding of Gauld et al. (1974) that LAC virus activity is greater in areas where ground cover and chipmunks occur at greater densities. As a result, exposed, relatively dry, south-facing slopes, of which the Burkholder S site is typical of many in southwestern Wisconsin, are probably not important as sites of high LAC virus endemicity. Large populations of *Ae. triseriatus* are often found on such slopes, but they may utilize animals other than chipmunks as major sources of blood meals, thereby possibly reducing the probability of amplification when virus is introduced via infected mosquitoes flying in from endemic foci. At the Burkholder S site it was noted that a herd of cattle had access to the area both years, but the frequency with which they grazed there was not determined.

A highly significant difference ($p>.01$) was detected between the Hanson site and a site nearly adjacent to it that we have designated as Hanson B (Table 1). This is perplexing as the 2 sites occupy parallel south slopes within the same forest, only a narrow dirt road interrupting the canopy

Table 3. LAC virus isolation record for individual treeholes in Hanson woods, 1974–1975.

| Treehole number | Total *A. triseriatus* larvae assayed; (number of isolations in parentheses) 1974 | 1975 | Both |
|---|---|---|---|
| 9 | 1,111 | 169 | 1,280 |
| 19 | 583 (4) | 532 (1) | 1,115 (5) |
| 12 | 221 | 474 (3) | 695(3) |
| 5 | 181 | 472 (4) | 653 (4) |
| 6 | 134 (1) | 436 (1) | 570 (2) |
| 16 | 197 (2) | 338 | 535 (2) |
| 18 | 128 | 200 (1) | 328 (1) |
| 14 | 92 (1) | 209 | 301 (1) |
| 3 | 0 | 259 (1) | 259 (1) |
| 8 | 29 (1) | 215 | 244 (1) |
| 7 | 7 | 234 (1) | 241 (1) |
| 15 | 30 | 187 | 217 |
| 10 | 0 | 183 (1) | 183 (1) |
| 17 | 38 | 135 | 173 |
| 2 | 36 | 136 | 172 |
| 11 | 7 | 148 | 155 |
| 21 | 104 (1) | 0 | 104 (1) |
| 13 | 5 | 22 | 27 |
| 4 | 1 | 4 | 5 |
| 20 | 0 | 2 | 2 |

between them. Here again, a possible, but untested, explanation is that there was greater use of hosts other than chipmunks in the Hanson B area. Deer made more use of this area for bedding down and there appeared to be fewer chipmunks than at the Hanson site. We plan to test this hypothesis by use of precipitin tests to identify bloodmeal sources of mosquitoes in these 2 areas and in the Burkholder areas discussed above. Despite the low overwintering virus prevalence at the Hanson B site, each of 4 sentinal chipmunks located there seroconverted to LAC virus during August, 1975 (Ksiazek and Yuill 1977).

In Table 3 the number of *Ae. triseriatus* larvae assayed in 1974 and 1975 and the number of isolates are given for individual treeholes. For the 2 years together, 12 of 17 treeholes from which at least 100 larvae were collected yielded isolates, a positive value of 71%. In general, it seems that the greater the number of larvae in a treehole the greater the number of isolates. The only pronounced exception was treehole No. 9. This treehole, however yielded 2 isolates in the spring of 1973 (Watts et al. 1974).

*Literature Cited*

Beaty, B. J. and W. H. Thompson. 1975. Emergence of LaCrosse virus from endemic foci. Fluorescent antibody studies of overwintered *Aedes triseriatus*. Am. J. Trop. Med. Hyg. 24: 685–691.

Brandt, W. E., E. L. Buescher and F. M. Hetrick. 1967. Production and characterization of arbovirus antibody in mouse ascitic fluid. Am. J. Trop. Med. Hyg. 16: 339–347.

Gauld, L. W., R. P. Hanson, W. H. Thompson and S. K. Sinha. 1974. Observations on a natural cycle of LaCrosse virus (California group) in southwestern Wisconsin. Am. J. Trop. Med. Hyg. 23: 983–992.

Ksiazek, T. G. and T. M. Yuill. 1977. Viremia and antibody response to LaCrosse virus in sentinal grey squirrels (*Sciurus carolinensis*) and chipmunks (*Tamais striatus*). Am. J. Trop. Med. Hyg. 26: 815–821.

Pantuwatana, S., W. H. Thompson, D. M. Watts and R. P. Hanson. 1972. Experimental infection of chipmunks and squirrels with LaCrosse and Trivittatus viruses and biological transmission of LaCrosse virus by *Aedes triseriatus*. Am. J. Trop. Med. Hyg. 21: 476–481.

Tikasingh, E., L. Spence and W. G. Downs. 1966. The use of adjuvant and Sarcoma 180 cells in the production of mouse hyperimmune ascitic fluids to arboviruses. Am. J. Trop. Med. Hyg. 15: 219–226.

Watts, D. M., S. Pantuwatana, G. R. DeFoliart, T. M. Yuill and W. H. Thompson. 1973. Transovarial transmission of LaCrosse virus (California encephalitus group) in the mosquito, *Aedes triseriatus*. Science 182: 1140–1141.

Watts, D. M., W. H. Thompson, T. M. Yuill, G. R. DeFoliart and R. P. Hanson. 1974. Overwintering of LaCrosse virus in *Aedes triseriatus*. Am. J. Trop. Med. Hyg. 23: 694–700.

## CHICAGO IN 1978

The annual meeting of the AMCA is scheduled for April 17 to 20, 1978. The hotel is the Pick-Congress. The chairman of the Local Arrangements Committee is Mr. E. E. Fetzer, Desplaines Valley Mosquito Abatement District, 8130 Ogden Ave., Lyons, Illinois 60534.