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Detection of La Crosse Arbovirus Antigen in Mosquito Pools: Application of Chromogenic and Fluorogenic Enzyme Immunoassay Systems

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An enzyme immunoassay producing either a chromogenic or fluorogenic end product was developed and evaluated for detecting La Crosse viral antigen within mosquito pools. The enzyme immunoassay was found to be sensitive, detecting one infected mosquito within a pool of 100 mosquitoes, and specific, distinguishing between closely related California group viruses. Assays were completed within 5 h after the addition of test samples. La Crosse viral antigen could be readily detected in mosquito pools after seven freeze-thaw cycles.

Public health surveillance of arboviruses typically involves some combination of three standard methods: (i) detecting human (or sentinel) disease or infection, (ii) monitoring vector infection rates, and (iii) monitoring antibody prevalence rates or virus infection rates, or both, in an enzootic vertebrate host (1, 7, 9, 10). Vector surveillance provides estimates of population densities and viral infection rates which are necessary to predict the risk of subsequent human and animal disease. Unfortunately, current arbovirus surveillance methods limit the number of samples that can be processed in a time period appropriate to institute or assess control measures. One of the limiting factors is the time and cost for viral isolation and subsequent identification. With conventional surveillance bioassays (7, 9, 17), a minimum of 1 week from receipt of the field specimens is usually required for virus isolation and identification.

Enzyme immunoassay (EIA) systems have gained wide acceptance as tools for the rapid diagnosis of many infectious disease agents by the detection of specific antigens (11, 19). Most EIA systems use a solid-phase component which permits the separation of bound from free molecules as immunlogical components are added sequentially. EIAs may result in a visible, chromogenic reaction (EIA-C) or they can be modified to produce a fluorogenic (EIA-F) reaction (4, 20).

EIA systems have been used to detect arbovirus antigens within mosquito pools. Arthropods are commonly pooled for testing in the surveillance of vector-borne diseases because of the large numbers of specimens to be processed. Dengue virus (types 1, 2, and 4)-infected *Toxorhynchites amboinensis* mosquitoes were distinguished from yellow fever virus-infected mosquitoes by a dengue antigen-detecting EIA (13). EIA was sensitive enough to detect viral antigen in pools containing two infected mosquitoes, but specificity among the dengue viruses was poor. Guaroa virus (Bunyaviridae) antigen has been detected in infected mosquitoes by EIA (K. Obom, Master of Public Health essay, Yale University, New Haven, Conn., 1980). Guaroa antigen in a pool containing 10 infected mosquitoes and 40 noninfected mosquitoes was easily detected by EIA, whereas antigen in a pool containing 2 infected mosquitoes and 48 noninfected mosquitoes was only slightly distinguishable from the negative control.

We are currently examining the use of EIA-C and EIA-F systems to replace common virological assays used in the surveillance of arboviruses in mosquito populations. We report here studies with La Crosse (LAC) virus, a major cause of encephalitis in children in the United States (16), and its vector *Aedes triseriatus*.

MATERIALS AND METHODS

Virus stocks. The following virus stocks were prepared in BHK-21 cells and in suckling mice: La Crosse, Keystone, Jamestown canyon, snowshoe hare, trivittatus, dengue 2, western equine encephalitis, and Flanders viruses. The maintenance of cells and the preparation of virus stocks followed procedures previously outlined (17). Virus titers were determined by micrometabolic inhibition tests (12).

Antibodies. Swiss mice were immunized with a suckling mouse brain preparation of LAC virus by standard procedures for producing hyperimmune as-

citic fluids (17). After 35 days ascitic fluids were collected by paracentesis and stored at −20°C. New Zealand rabbits were immunized intravenously with two doses (5 weeks apart) of sucrose gradient-purified, BHK-21 cell culture-derived LAC virus (5). After 7 weeks, rabbits were bled and serum was stored at −20°C. Immunoglobulin fractions of ascitic fluids and sera were collected by ammonium sulfate precipitation (8). Immunoglobulin G (IgG) was further purified by affinity chromatography with a protein A Sepharose CL-4B (Pharmacia, Inc.) column (6). In preliminary studies, the IgG fraction was separated with a G-200 Sephadex column. Protein concentrations were determined by a spectrophotometric reading at 280 nm. Stocks of immunoglobulin and IgG were stored in phosphate-buffered saline (PBS; 0.02 M phosphates, 0.15 M NaCl; pH 7.5) and Tris-buffered saline (0.05 M Trizma, Sigma Chemical Co.; pH 7.5 at 25°C), respectively, with the inclusion of 0.1% sodium azide.

Infection of mosquitoes. *Aedes triseriatus* were infected by intrathoracic inoculation with stock viruses (14); mosquitoes were held at 27°C for 15 days and then stored at −70°C. Before being pooled, each mosquito was examined for the presence of viral antigen by immunofluorescence. Infected mosquitoes were combined with noninoculated control mosquitoes to produce pools of known composition. Pools were triturated with a mortar and pestle in 1.0 ml of PBS supplemented with 10% heat-inactivated fetal bovine serum, 500 U of penicillin, and 50 μg of streptomycin. Pools were centrifuged at 800 × *g* at 4°C for 20 min, and the supernatant fluid was collected and stored at −70°C.

EIA. EIA procedures were similar to those of Yolken et al. (20) with some modifications. Briefly, the capture mouse immunoglobulin or IgG antibody was passively coated to the surface of 96-well, flat-bottom microtiter plates (MicroELISA substrate plate, Dynatech Laboratories, Inc.) for 20 to 40 h at 4°C with a diluent of 0.05 M carbonate-bicarbonate buffer (pH 9.6). After being coated wells were washed five times with PBS containing 0.05% Tween 20. Samples from mosquito pools were diluted in PBS containing 0.1% Tween 20 and 1.0% heat-inactivated fetal bovine serum and were then added to the wells and incubated for 1 h at 37°C. Plates were washed as noted above, and a detector antibody, rabbit immunoglobulin or IgG diluted in PBS with 0.1% Tween 20, 0.5% heat-inactivated fetal bovine serum, and 0.05% gelatin, was then added. After 1 h of incubation at 37°C, the plates were washed in PBS containing 0.05% Tween 20, and an IgG fraction of goat anti-rabbit IgG, which had been conjugated with alkaline phosphatase (18), was diluted in PBS with 0.1% Tween 20, added to the wells, and incubated for 1 hr at 37°C. The plates were then washed five times with 0.05 M Tris (pH 8.5), and a chromogenic or fluorogenic substrate solution was added. For EIA-C, *p*-nitrophenyl phosphate (1 mg of NPP per ml of diethanolamine buffer) was added to the wells and incubated for 20 to 30 min at 37°C. The results were read spectrophotometrically with a Titertek Multiskan plate reader (Flow Laboratories, Inc.) at 405 nm and scored qualitatively as −, +/−, +1, +2, +3, and +4. For EIA-F, the fluorogenic substrate, 4-methylumbelliferyl phosphate (MUP) (0.025 mg of MUP per ml of diethanolamine buffer), was added. After a 10- to 15-min incubation period at 37°C, the plates were scored qualitatively with a Blakray C-62 transilluminator (Ultra-Violet Products; 4).

Approximately 5 h was required for diagnosis after the addition of sample specimens to the microtiter plates. At each step 200 μl of each reagent was added per well. The concentration of capture and detector immunoglobulins was determined by checkerboard titrations. For the negative control, all test samples were routinely tested in wells coated with normal mouse immunoglobulin.

Freeze-thawing. Since mosquito pools frequently undergo multiple freeze-thaw (F-T) cycles during the process of collection and virus isolation, the effect of multiple F-T cycles upon EIA detection of LAC antigen was investigated. Samples were taken from three mosquito pools containing three infected mosquitoes and frozen. Selected samples were thawed and refrozen to produce samples varying in the number of passages through 0°C. In most virus laboratories, primary isolation specimens have passed through 0°C four times and upon reisolation six times. In these studies, the specimens had 4 to 14 such passages, which were designated as F-T cycles two through seven.

Statistical analysis. Statistical analysis was performed with the computer program Statistical Analysis System (S.A.S.; S.A.S. Institute, Inc.). Absorbance values produced by the plate reader were analyzed both untransformed and transformed by $\log_{10}$. Qualitative scoring of EIA was converted to 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0. Agreement between qualitative scoring was estimated by the Kappa statistical test (3). For all analyses, statistical significance was reached at $P \leq 0.05$.

RESULTS

Preliminary studies to establish protocols. Preliminary studies with EIA-C were performed to construct a test protocol. Immunoglobulin and IgG were coated to the polystyrene with PBS (pH 7.0 to 8.0), Tris (pH 7.4 to 9.5), or carbonate-bicarbonate (pH 9.0 to 9.6) buffers. Since nonspecific reactions increased with lower pH, the use of carbonate buffer was standard. Coating with carbonate-bicarbonate buffer for 40 h at 4°C was superior to overnight (16- to 20-h) incubation. Both immunoglubulin (10 to 20 μg of protein per ml) and IgG (1 to 3 μg of protein per ml) were useable as capture or detector antibodies. However, nonspecific background levels were greater with immunoglobulin preparations. Blocking agents (fetal bovine serum or gelatin or both) reduced nonspecific reactions and were necessary for all EIA regardless of whether immunoglobulin or IgG was used. Mosquito pools were tested at a 1:10 dilution in a diluent containing 0.1% Tween 20, which was found to be essential for preventing false-positive reactions.

Nonspecific reactions. Nonspecific reactions associated with negative mosquito pools were examined to establish a minimum absorbance

TABLE 1. Distribution of absorbance values from negative controls[a]

Type	Composition of pool (+, −)	Total no. of mosquitoes in pool	No. of samples tested	Geometric mean	Range	Diagnostic criterion[b]
Pool	0+, 10−	10	24	0.052	0.039–0.060	0.081
	0+, 50−	50	24	0.072	0.060–0.097	0.105
	0+, 100−	100	24	0.082	0.064–0.110	0.128
Sample diluent			48	0.049	0.035–0.072	0.074

[a] Negative controls consisted of sample diluent and negative mosquito pools (denoted as n_1 infected (+) and n_2 noninfected (−); pool size = $n_1 + n_2$).

[b] Diagnostic criterion was the maximum absorbance value expected for a negative mosquito pool and was calculated by adding to the geometric mean 3 standard deviations.

value for considering a sample positive. A series of EIA-C and EIA-F were performed with only sample diluent and pools of 10, 50, or 100 noninfected mosquitoes. Three randomly selected 96-well MicroELISA plates were used to measure inter- and intraplate variations. Qualitatively, the test results were scored negative with both substrates. Quantitatively, the absorbance values of EIA-C did not follow a normal distribution but rather a log-normal distribution. Therefore, all statistical analyses were performed on $\log_{10}$ transformation of absorbance values. By analysis of variance, the absorbance values of the four negative controls (Table 1) differed significantly ($F_{3,114} = 94.31$; $P < 0.001$). Background absorbance values did increase with increased numbers of negative mosquitoes within a pool (Duncan multiple range test, $P < 0.05$). However, there was no detectable inter- or intraplate variation. A general diagnostic criterion was established with the pool size of 100 mosquitoes; three standard deviations were added to the geometric mean, which resulted in an absorbance value (0.13) above which samples were classified as positive.

Sensitivity of EIA. The minimum number of infected mosquitoes detectable by EIA was determined. Pools containing one or three infected mosquitoes and various numbers of negative mosquitoes were tested by EIA-C and EIA-F, and titers were determined to show the quantity of infectious virus present. Whether evaluated qualitatively or quantitatively, pools containing a single infected mosquito were detected by EIA (Table 2). Virus titrations of five pools containing only one infected mosquito yielded a geometric mean titer of 3.62 $\log_{10}$ 50% tissue culture infective dose units of LAC virus per ml, with a 95% confidence interval of 3.26 $\log_{10}$ to 3.98 $\log_{10}$. Thus, the EIA for detecting LAC antigen was estimated to be capable of detecting between 2 $\log_{10}$ and 3 $\log_{10}$ 50% tissue culture infective dose units per ml.

Specificity of EIA. The specificity of the LAC-EIA system was investigated by using mosquito pools containing three individuals infected with serologically related and unrelated viruses. Only California group viruses (2) reacted in EIA systems; LAC, snowshoe hare and Jamestown canyon viruses reacted most strongly, whereas Keystone and trivittatus viruses were classified as negative (Table 3). Specificity between LAC, snowshoe hare and Jamestown canyon viruses seemed to be improved with EIA-F (Table 3). The increased specificity was confirmed in three additional replicates conducted in parallel.

Effect of freeze-thawing upon antigen detection. The number of F-T cycles did not affect the qualitative or quantitative diagnostic result; a positive sample scored +1 or greater or produced an absorbance value greater than 0.13, respectively (Table 4). However, a closer examination by analysis of variance revealed that pool absorbance values varied individually in response to the number of F-T cycles. In general, there was a slight decline in absorbance values for pools two and three and a slight increase for pool one.

Correlation of qualitative and quantitative results. Qualitative results, whether chromogenic or fluorogenic, correlated closely with the quantitative results obtained with the spectrophotometric plate reader. Using the Spearman and Kendall correlation tests, we demonstrated the absorbance values to be significantly associated with the qualitative results of EIA-C ($r = 0.935$, $P < 0.001$) and EIA-F ($r = 0.921$, $P < 0.001$). When the qualitative scoring was categorized as negative (0 and 0.5), indeterminate (1.0 and 1.5), and positive (2.0 and greater), the agreement between EIA-C and EIA-F was 82.5%, (Kappa = 0.825, $P < 0.0001$; Table 5). The six discordant samples were mosquito pools containing snowshoe hare virus- and Jamestown canyon virus-infected mosquitoes.

DISCUSSION

This study supports the general assessment that EIA is suitable for the detection of an arboviral antigen in infected arthropods. It is sensitive enough to detect easily a single LAC-infected mosquito among a pool of 100 mosqui-

TABLE 2. Detection of LAC viral antigen in mosquito pools containing infected mosquitoes[a]

Mosquito Pools		EIA-C and EIA-F results[b] with			
Composition of pool (+, −)	Total in pool	NPP as substrate		MUP as substrate	Virus isolation[c]
		Mean absorbance	Qualitative score	Qualitative score	
1+, 0−	1	0.420	+2	+3	+
1+, 9−	10	0.405	+2	+3	+
1+, 49−	50	0.392	+2	+3	+
1+, 99−	100	0.413	+2	+3	+
3+, 0−	3	0.947	+3	+4	+
3+, 7−	10	0.941	+3	+4	+
3+, 47−	50	0.869	+3	+4	NT
3+, 97−	100	0.985	+3	+4	+
0+, 0−	0	0.065	−	−	−
0+, 3−	3	0.097	−	−	−
0+, 10−	10	0.078	−	−	−
0+, 50−	50	0.088	−	−	−
0+, 100−	100	0.090	−	−	−
LAC virus tissue cultures fluid, positive control		0.445	+2	+3	+

[a] All pools were tested at a 1:10 dilution and the positive control was tested at a 1:100 dilution. EIA systems consisted of a visual enzymatic product (EIA-C) from the substrate NPP and a fluorogenic enzymatic product (EIA-F) from the substrate MUP.

[b] All EIA results are an average of three tests; absorbances are expressed as geometric means and qualitative scoring ranges from − to +4.

[c] Virus isolation performed with BHK-21 cells. NT, Not tested.

TABLE 3. Specificity of LAC viral antigen-detecting EIA systems[a]

Mosquito pools		EIA-C and EIA-F results[b] with		
Virus	Composition of pools (+, −)	NPP as substrate		MUP as substrate
		Mean absorbance	Qualitative score	Qualitative score
Control	0+, 3−	0.047	−	−
LAC	3+, 0−	0.812	+3–+4	+4
Snowshoe hare	3+, 0−	0.429	+2–+3	+1–+2
Jamestown canyon	3+, 0−	0.176	+1	+/−
Keystone	3+, 0−	0.093	−	−
Trivittatus	3+, 0−	0.054	−	−
Western equine encephalitos	3+, 0−	0.050	−	−
Dengue 2	3+, 0−	0.055	−	−
Flanders	3+, 0−	0.043	−	−
LAC virus tissue culture fluid as positive control		0.728	+3	+4
Sample diluent as negative control		0.047	−	−

[a] All pools were tested at a 1:10 dilution and the positive control was tested at a 1:100 dilution. EIA systems consisted of a visual enzymatic product (EIA-C) from the substrate NPP and a fluorogenic enzymatic product (EIA-F) from the substrate MUP.

[b] All EIA results are an average of three tests; absorbances are expressed as geometric means and qualitative scoring ranges from − to +4.

toes. The specificity of EIA systems seems to be comparable to or slightly better than that of the hemagglutination inhibition assays (2). Discrimination between closely related California group viruses appears to be improved with the substitution of fluorogenic for the chromogenic substrates. One possible explanation is that the enzymatic degradation of MUP is kinetically faster than that of NPP (15). Given the spectral sensitivity of human eyes, a homologous antibody-antigen reaction may produce the maximum detectable fluorescent product sooner than

TABLE 4. Effect of freezing and thawing in the detection of LAC viral antigen within mosquito pools[a]

F-T cycle no.[b]	Mean adsorbance values from EIA by pool and F-T cycle[c] in:		
	Pool 1[d]	Pool 2[d]	Pool 3[d]
2	0.599 (0.545–0.658)	0.476 (0.458–0.496)	0.549 (0.527–0.572)
3	0.559 (0.521–0.598)	0.461 (0.395–0.539)	0.579 (0.515–0.649)
4	0.750 (0.608–0.925)	0.454 (0.422–0.488)	0.544 (0.454–0.529)
5	0.690 (0.645–0.739)	0.416 (0.396–0.436)	0.490 (0.454–0.529)
6	0.700 (0.646–0.759)	0.430 (0.409–0.452)	0.590 (0.478–0.543)
7	0.528 (0.496–0.561)	0.502 (0.468–0.539)	0.479 (0.454–0.504)

[a] Absorbance values given as geometric mean of three tests. All samples were qualitatively scored +3 with EIA-C; EIA-F was not done.

[b] F-T 1 of mosquito tissues occurs during the procedure of collection, identification, storage (−70°C), and subsequent triturating of pools.

[c] By analysis of variance, geometric means of absorbance values for F-T cycles were significantly different for pool 1 ($F_{5,12} = 6.23$, $P = 0.0045$) and pool 3 ($F_{5,12} = 3.66$, $P = 0.0305$), although only border-line significant for pool 2 ($F_{5,12} = 2.70$, $P = 0.0737$).

[d] 95% confidence interval indicated in parentheses.

TABLE 5. Concordance of LAC viral antigen detection in mosquito pools by qualitative EIA-C and EIA-F[a]

EIA-F[b]	EIA-C		
	Negative pools	Indeterminate pools	Positive pools
Negative pools	30	3	0
Indeterminate pools	0	0	3
Positive pools	0	0	27

[a] Qualitative scores were grouped as negative = 0 and 0.5, indeterminate = 1.0 and 1.5, and positive ≥2.0.

[b] EIA-F was performed on the same plate after EIA-C; therefore, results were not obtained in parallel.

does a heterologous reaction. Confirmation of the apparent increase in specificity with fluorogenic substrates will require contrasting qualitative EIA-F results to spectrofluorometrical analyses.

EIA systems offer the advantage of speed of diagnosis. In the LAC model, viral antigen can be detected and identified within 5 h after the addition of the test sample. These systems will greatly increase the total number of specimens that can be assayed and should result in a more cost-efficient diagnostic procedure. It is important that repeated freezing and thawing of the specimens did not alter the diagnostic evaluation. Furthermore, both EIA-C and EIA-F may be evaluated qualitatively. A major limitation to the EIA technique for arbovirus surveillance is that only predetermined viruses are detected; new or unknown viruses in the population are undetected.

The estimation of infection rates among surveyed arthropods has always been hindered by the expense and difficulty associated with conventional virus assay techniques. This is not only true for human pathogenic viruses, but also for parasites and plant vectored pathogens. EIA holds great promise as a routine surveillance tool, permitting both rapid and inexpensive monitoring of infectious agents within a vector population.

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