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Reverse Transcription-PCR Detection of LaCrosse Virus in Mosquitoes and Comparison with Enzyme Immunoassay and Virus Isolation

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A reverse transcription-PCR (RT-PCR) assay was developed and compared with enzyme immunoassay (EIA) and virus isolation for detecting LaCrosse virus (LAC) in mosquito pools. All three techniques were able to detect a single LAC-infected mosquito in a pool of 99 negative mosquitoes. Virus isolation was the most sensitive of the three techniques; it was possible to isolate virus immediately following intrathoracic inoculation of mosquitoes. RT-PCR was second in sensitivity; LAC RNA was detected 1 day postinfection. EIA detected LAC antigen 2 days postinfection. Additionally, RT-PCR and EIA were able to detect LAC RNA and protein, respectively, from mosquito samples which were subjected to seven freeze-thaw cycles, and RT-PCR was able to detect LAC RNA from mosquito samples which remained at room temperature for up to 7 days.

Monitoring of vector populations for the prevalence of arboviruses is an important component of assessing disease risk to humans and animals. Surveillance for arboviruses includes the measurement of mosquito population densities and virus infection rates (13). Virus isolation (VI) in bioassays, such as suckling mice and cell cultures, and subsequent serologic identification of isolates have classically been used to determine field infection rates in vectors. These techniques are time-consuming and expensive and require special facilities. Modern biotechnological assays are capable of providing a rapid diagnosis of arbovirus prevalence rates in vectors. Enzyme immunoassay (EIA) is a sensitive, specific, and rapid technique for the detection of viral antigens in mosquito pools even in the absence of infectious virus (7–10, 14, 17). In addition, it requires little specialized equipment, making it a practical technique for use in the field. Detection of virus-specific nucleic acids is an alternative to virus isolation and/or antigen detection for arbovirus surveillance. The two principal approaches have been detection of analyte by hybridization and more recently by PCR. The latter is a more sensitive technique for the detection of arboviral nucleic acids in mosquitoes (12, 15).

We report here the development of a reverse transcription-PCR (RT-PCR) assay for the detection of LaCrosse virus (LAC) analyte in mosquitoes. RT-PCR, VI, and EIA were compared for their abilities to detect LAC and/or LAC analyte in pools of *Aedes triseriatus* mosquitoes.

MATERIALS AND METHODS

Virus stocks. The original strain of LAC bunyavirus, isolated in 1965 from the brain of a 4-year-old girl who died of encephalitis in LaCrosse, Wis. (16), was passaged four times in suckling mouse brains and twice in BHK-21 cells. Snowshoe hare bunyavirus, SSH 76-Y-316, isolated from pooled *Aedes hexodontus* adults collected at Fort Smith, Northwest Territories, Canada, in 1976, was passaged twice in BHK-21 cells. Tahyna bunyavirus, TAH prototype, obtained from Yale Arbovirus Research Unit, New Haven, Conn., was passaged four times in BHK-21 cells. The passage level of the flavivirus dengue-2, DEN 2 New Guinea C, was unknown, but the stock virus used in the present study was grown in C636 cells.

Mosquitoes. *A. triseriatus* and *Aedes aegypti* were maintained at 23 and 28°C, respectively, at 80% relative humidity and under a cycle of 16 h of light and 8 h of dark. Sugar cubes and water were provided ad libitum.

Mosquito infection and pool construction. *A. triseriatus* mosquitoes were infected by intrathoracic inoculation with between 10 and 50 50% tissue culture infective doses of LAC, SSH, or TAH stocks (determined by titration in BHK-21 cells). Similarly, *A. aegypti* mosquitoes were inoculated with approximately 100 PFU of DEN 2 virus (determined by plaque assay in BHK-21 cells). All mosquitoes were held for 14 days unless otherwise noted. Following the extrinsic incubation period, mosquitoes were cold anesthetized, their heads were severed, and they were squashed on slides and examined by immunofluorescence (2). Immunofluorescence-positive mosquitoes were used individually or were added to various numbers of negative mosquitoes to construct pools consisting of 1 virus-positive mosquito in a total of 10, 50, or 100 mosquitoes (see Table 1) and were frozen at −70°C prior to processing.

Mosquito processing. Mosquito pools containing virus-positive and -negative mosquitoes were triturated with a mortar and pestle in 2 ml of field diluent (0.01 M phosphate-buffered saline [PBS; pH 7.5], 10% heat-inactivated fetal bovine serum, 250 U of penicillin per ml, 250 μg of streptomycin per ml, and 2.5 μg of amphotericin B per ml). After centrifugation at 800 × *g* for 20 min at 4°C, the supernatants were immediately used for VI and were then stored at −70°C until needed for EIA. Mosquitoes designated for RT-PCR were stored at −70°C until RNA isolation.

Antibodies for EIA. (i) Capture antibody. BALB/c mice were primed intraperitoneally with 0.5 ml of Pristane (2,6,10, 14-tetramethylpentadecane). Hybridoma cells (10^7 cells [per

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ml] of clone 814-02, producing an immunoglobulin G2a monoclonal antibody against the nucleocapsid protein of TAH virus [6]) were inoculated intraperitoneally 3 weeks later, and ascitic fluid was harvested after 2 weeks.

(ii) Detector antibody. Antibodies to LAC were prepared in rabbits as described previously (5), and purified immunoglobulin G was obtained from serum by DEAE Affi-Gel Blue chromatography (Bio-Rad, Richmond, Calif.) (11).

Mosquito EIA. The mosquito EIA was similar in design to that reported previously (10). The optimum concentrations of the EIA reagents were determined by checkerboard titration. The capture antibody was diluted to 0.122 mg of protein per ml in 0.1 M carbonate-bicarbonate buffer (pH 9.25), and 100 μl was added to each of 95 wells of 96-well polystyrene enzyme-linked immunosorbent assay plates (catalog no. 25805-96; Corning). After coating for 18 to 24 h at 4°C, the plates were washed five times with washing buffer (PBS [pH 7.2] containing 0.1% Tween 20) and were incubated for 1 h at 37°C with 200 μl of 0.05 M *N*-acetyl cysteine per well. After removing the *N*-acetyl cysteine solution, 50-μl samples consisting of *Aedes* supernatants diluted 1:5 in 0.01 M PBS (pH 7.5)–0.1% Tween 20–1.0% fetal bovine serum (FBS) were added to the wells, and the plates were incubated for 2 h at 37°C. The plates were washed as described above, and 100 μl of 0.75 μg (per ml) of detector antibody (rabbit anti-LAC immunoglobulin G diluted in 0.01 M PBS–1% Tween 20–0.05% gelatin–0.5% FBS–1 mg of dextran sulfate per ml) was added to each well. After incubation for 1 h at 37°C, the plates were washed as described above and 100 μl of biotinylated donkey anti-rabbit antibody (Amersham, Arlington Heights, Ill.) diluted 1:1,000 in 0.01 M PBS–1% Tween 20–1 mg of dextran sulfate per ml was added to each well. After washing, 100 μl of streptavidin-peroxidase (Amersham) diluted 1:1,000 in 0.01 M PBS–0.1% Tween 20–1 mg of dextran sulfate per ml was added to each well. Plates were incubated for 1 h at 37°C, and a Pierce ImmunoPure Microwell Peroxidase Substrate Kit (Pierce, Rockford, Ill.) was used to detect peroxidase. Equal volumes of solution I (TMB [3′,3′,5′,5′-tetramethylbenzidine], 0.4 g/liter) and solution II (0.2% H_2O_2 in citric acid buffer) were mixed, and 100 μl was added to each well. The plate was developed in the dark for 10 min. The reaction was stopped with 100 μl of 1 M phosphoric acid per well, and the plates were read at 450 nm with a Bio-Rad microplate reader. Each sample was tested in triplicate, and the three absorbance values were averaged. The mean EIA absorbance value of the sample was considered positive if it exceeded the mean absorbance value plus 3 standard deviations of the corresponding negative control (uninfected mosquito pool).

VI. The titers of individual and pooled mosquitoes were determined by a microtitration assay in BHK-21 cells as described previously (9).

RNA extraction. Total mosquito RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method described by Chomczynski and Sacchi (4) and Olson et al. (15); this was followed by a 3 M sodium acetate (NaAC; pH 5.2) wash. Briefly, individual mosquitoes and pools containing up to 10 mosquitoes were triturated in 500 μl of solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate [pH 7], 0.5% Sarkosyl, 100 mM 2-mercaptoethanol). Fifty microliters of 2 M NaAc (pH 4.5), 500 μl of water-saturated phenol, and 100 μl of chloroform-isoamyl alcohol (49:1) were added sequentially, and the components were mixed after the addition of each component. Samples were incubated on ice for 15 min and were then centrifuged at 10,000 × *g* for 20 min at 4°C. The RNA in the aqueous layer was precipitated with isopropanol, washed with 800 μl of 3 M NaAc (pH 5.2), washed with 70% ethanol, dried at room temperature, and resuspended in 50 μl of diethylpyrocarbonate-treated H_2O. Pools of 50 or 100 mosquitoes were triturated in 2.0 ml of solution D, and the amounts of the other reagents were increased accordingly.

TABLE 1. Detection of LAC RNA, antigen, or virus in mosquito pools

Pool size (no. of mosquitoes)[a]	Assay	Samples[b]	Negative control	Diagnostic criterion[c]
1	RT-PCR	3/3	–	
	EIA	4/4 (0.397[d])	0.060	>0.125
	VI	4/4 (4.3[e])	–	
10	RT-PCR	3/3	–	
	EIA	4/4 (0.310[d])	0.032	>0.045
	VI	4/4 (3.7[e])	–	
50	RT-PCR	3/3	–	
	EIA	4/4 (0.263[d])	0.094	>0.108
	VI	4/4 (4.25[e])	–	
100	RT-PCR	3/3	–	
	EIA	4/4 (0.262[d])	0.086	>0.142
	VI	4/4 (4.5[e])	–	

[a] Pools consisted of one LAC-positive mosquito; the remaining mosquitoes were LAC negative.
[b] Number of samples positive/total number of samples.
[c] Values are mean absorbance plus 3 standard deviations of the respective uninfected control pool.
[d] Mean absorbance values for positive samples are indicated in parentheses.
[e] The mean $\log_{10}$ 50% tissue culture infective dose per milliliter for positive samples is indicated in parentheses.

RT-PCR primers. Primer 1 (5′-TCAAGAGTGTGATGTC GGATTTGG) is identical to nucleotides 71 to 94 of the LAC S-segment complementary sequence (1) and was used to prime cDNA synthesis from S-segment genomic RNA. Primer 2 (5′-GGAAGCCTGATGCCAAATTTCTG) is complementary to nucleotides 763 to 785 of the LAC S-segment complementary sequence (1) and was used with primer 1 for PCR amplification.

RT-PCR. RT of LAC S-segment RNA was accomplished in an RT reaction. One-tenth of the total RNA (5 μl) from each sample was incubated at 70°C for 10 min with 15 pmol of primer 1 in a total volume of 10 μl. After cooling to room temperature, the RNA was reverse transcribed with 67 U of SuperScript (Bethesda Research Laboratories, Gaithersburg, Md.) in 1× first-strand buffer (10 mM dithiothreitol, 10 μmol of each of the deoxynucleoside triphosphates, and 40 U of RNasin) in a 20-μl reaction mixture. Two microliters of cDNA was added to 48 μl of 1× PCR buffer (1.5 U of *Taq* polymerase [Promega, Madison, Wis.], 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 200 μM [each] deoxynucleoside triphosphates, and 50 pmol each of primers 1 and 2), and the mixture was thermocycled at 94°C for 1 min, 58°C for 1 min, and 70°C for 2 min through 25 cycles, and there was a final extension at 70°C for 7 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels.

RESULTS

Detection of LAC in parenterally infected mosquitoes. The ability of RT-PCR, EIA, and VI to detect a single LAC-infected mosquito in pools of 1, 10, 50, and 100 total mosquitoes was examined (Table 1). RT-PCR of mosquito pools resulted in a specific 715-bp product in the LAC-positive pools

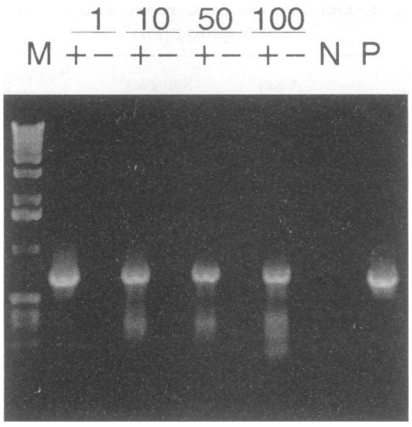

FIG. 1. RNAs extracted from pools of 1, 10, 50, and 100 mosquitoes were used for RT-PCR. One-tenth of the RNA extracted from mosquitoes was reverse transcribed with primer 1. One-tenth of the cDNA was used in a PCR that included primers 1 and 2 for the amplification of a 715-bp PCR product. One-sixth of the PCR mixtures was electrophoresed in a 1.5% agarose gel. Lane M, a 1-kb DNA ladder; lanes +, one LAC-infected mosquito within the pool; lanes −, pools of uninfected mosquitoes; lanes N and P, negative (uninfected mosquito RNA) and positive (LAC S-segment cDNA) controls, respectively.

and no specific product in the LAC-negative controls (Fig. 1). All three techniques detected LAC in every pool size, with no false-positive results.

Sensitivities of RT-PCR, VI, and EIA. To determine the sensitivities of RT-PCR, EIA, and VI, mosquitoes were parenterally infected with LAC and were assayed at hours 0 and 10 and days 1, 2, 3, and 7 postinfection (Table 2). VI was the most sensitive technique; virus was isolated from four of four LAC-infected mosquitoes immediately following inoculation. RT-PCR was second in sensitivity and detected LAC RNA in three of three LAC-infected mosquitoes 1 day postinoculation. EIA was the least sensitive and detected four of four LAC-infected mosquitoes 2 days postinoculation.

Specificity of RT-PCR. The specificity of the LAC RT-PCR was investigated by analyzing individual mosquitoes infected with serologically related and unrelated viruses. RNAs extracted from three LAC-, SSH-, and TAH-inoculated *A. triseriatus* mosquitoes and three DEN 2-inoculated *A. aegypti* mosquitoes were reverse transcribed and PCR amplified as described above (data not shown). All LAC-, SSH-, and TAH-infected mosquitoes yielded a diagnostic 715-bp PCR product, as expected from LAC, while the 715-bp product was absent from the DEN 2-inoculated *A. aegypti* samples.

TABLE 3. Effects of freezing-thawing on detection of LAC in mosquitoes by RT-PCR, EIA, and VI

Sample[a]	Assay results after the following no. of freeze-thaw cycles:								
	3			5			7		
	RT-PCR[b]	EIA[c]	VI[d]	RT-PCR	EIA	VI	RT-PCR	EIA	VI
1	+	0.12	2.80	+	0.18	1.55	+	0.13	−
2	+	0.208	3.05	+	0.11	−	+	0.11	−
3	+	0.173	3.05	+	0.24	−	+	0.20	−

[a] See footnote *a* of Table 2.
[b] See footnote *b* of Table 2.
[c] Absorbance values given as the arithmetic means of three tests. The EIA diagnostic criterion was >0.054.
[d] See footnote *d* of Table 2.

Effect of freezing-thawing on LAC detection. Since mosquito pools frequently undergo multiple freeze-thaw cycles during the process of collection, mosquito identification, and VI, the effects of multiple freeze-thaw cycles upon RT-PCR, VI, and EIA detection of LAC were investigated. Individual positive mosquitoes which had been demonstrated by immunofluorescence to be infected with LAC were subjected to seven cycles of freezing to −70°C and were then incubated at room temperature for 1 h. Samples were obtained from freeze-thaw cycles 3, 5, and 7 (Table 3). Virus was isolated in one of three mosquitoes after five freeze-thaw cycles, but could not be isolated after seven freeze-thaw cycles. Both RT-PCR and EIA detected LAC analyte after seven freeze-thaw cycles.

Stabilities of RT-PCR samples. To determine the stability of AGPC-extracted mosquito RNA, individual LAC-infected mosquitoes were ground in 500 μl of solution D and were left at room temperature. Three mosquito triturates were transferred to −20°C after 1, 3, and 8 days posttrituration. RT-PCR amplification resulted in LAC-specific product in all of the samples (Fig. 2).

DISCUSSION

Our studies show that RT-PCR compares favorably to VI and EIA for the detection of LAC analyte in *A. triseriatus* mosquitoes. All three techniques were sensitive enough to reliably detect a single LAC-infected mosquito in pool sizes as large as 100 mosquitoes, with no false-positive results.

VI proved to be the most sensitive of the techniques. Virus was isolated immediately following inoculation, while RT-PCR required 1 day and EIA required 2 days postinoculation for

TABLE 2. Sensitivity of RT-PCR, VI, and EIA for detection of LAC following intrathoracic inoculation of mosquitoes

Sample[a]	Assay results at the following times postinfection:																				
	0 h			10 h			1 day			2 days			3 days			7 days			Negative control		
	RT-PCR[b]	EIA[c]	VI[d]	RT-PCR	EIA	VI	RT-PCR	EIA	VI	RT-PCR	EIA	VI	RT-PCR	EIA	VI	RT-PCR	EIA	VI	RT-PCR	EIA	VI
1	−	0.019	2.1	−	0.026	2.1	+	0.067	4.1	+	0.318	4.8	+	0.281	4.3	+	0.459	4.6	−	0.031	−
2	−	0.026	1.3	−	0.026	2.1	+	0.082	4.1	+	0.181	4.8	+	0.379	3.8	+	0.226	3.3			
3	−	0.060	1.3	−	0.060	2.1	+	0.099	4.3	+	0.207	4.8	+	0.596	5.3	+	0.360	4.8			
4	NT	0.050	2.3	NT	0.050	1.6	NT	0.103	4.3	NT	0.204	5.1	NT	0.259	5.8	NT	0.681	5.3			

[a] Each samples contained one inoculated mosquito.
[b] +, LAC-specific PCR product; −, absence of LAC-specific PCR product; NT, not tested.
[c] Absorbance values given as the arithmetic means of three tests. The EIA diagnostic criterion was >0.105.
[d] Log_{10} 50% tissue culture infective dose per milliliter.

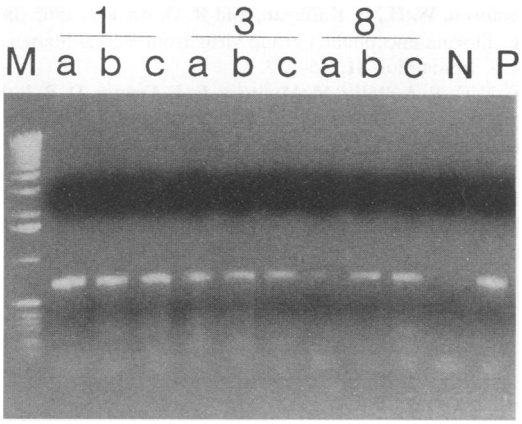

FIG. 2. Mosquitoes were triturated in 500 μl of solution D and were left at room temperature for 1, 3, and 8 days. RNA was extracted from three samples (lanes a, b, and c) for each time point. One-tenth of the RNA from each sample was assayed by RT-PCR. One-sixth of the PCR mixtures was electrophoresed in a 1.5% agarose gel. Lane M, a 1-kb DNA base pair ladder; lanes N and P, negative (uninfected mosquito RNA) and positive (LAC S-segment cDNA) controls, respectively.

LAC detection. RT-PCR detects positive mosquitoes a full day earlier in the extrinsic incubation period than EIA and gives more rapid results than VI. The greater sensitivity of RT-PCR over that of EIA is of unknown practical significance. Newly blood-fed mosquitoes are unlikely to be trapped during surveillance; therefore, very early detection of virus may not be important. On the other hand, in nature, low-titer blood meals, low ambient temperatures, or incompetent vectors could extend the extrinsic incubation period sufficiently such that a more sensitive assay such as RT-PCR might provide a better estimate of the population infection rate. Furthermore, the sensitivity of the RT-PCR might be enhanced by using more RNA during RT, using more cDNA during PCR, or increasing the number of amplification cycles. The combination of very high sensitivity and rapid results from RT-PCR may alert public health officials to epidemic conditions earlier than EIA and allow more time for mosquito abatement.

Both the RT-PCR and EIA techniques used in the present study are group-specific assays. VI is not a specific assay unless serological tests are performed to identify a virus. The specificity of an RT-PCR, by design, can be varied. In the present study primers were selected from the nucleocapsid sequence of the S segment. The result was an assay that amplified nucleic acids from all the California group viruses (LAC, SSH, and TAH) tested but not the heterologous flavivirus (DEN 2). These findings correlate with serological methods, such as complement fixation, for which the nucleocapsid protein is the principal antigen, and has historically been used as a bunyavirus group-specific test (3). Indeed, the EIA used a monoclonal antibody to the nucleocapsid protein of TAH as the capture antibody, making this another group-specific assay. The specificity of the RT-PCR could be virus and complex specific by selecting primers to a more variable region such as portions of the genome encoding the G1 glycoprotein. This might be done in a nested RT-PCR in which group-specific primers are used to reverse transcribe and then amplify viral RNA and a second set of primers is used to identify the virus (18). However, nested PCR strategies have the potential for increasing problems with cross-contamination.

RT-PCR and EIA are both sensitive techniques for the detection of LAC in mosquitoes. Both yield rapid results and both are suited for use with samples collected in the field, which are subject to temperature variations during transport and storage. RT-PCR and EIA detected analyte in samples passed through seven freeze-thaw cycles, and RT-PCR also amplified viral RNA from mosquitoes that were ground in solution D and left at room temperature for 1 week. This demonstrated that preservation of RNA from mosquitoes collected in the field is practical even in the absence of a cold chain; however, RT-PCR requires a laboratory setting for access to thermocyclers and electrophoresis equipment. The EIA can be completed in the field for immediate results.

RT-PCR shares many of the attributes of EIA; the test is sensitive, specific, and capable of providing diagnostic results within hours rather than days. However, while the practicality and cost-effectiveness of EIA for routine surveillance is well established, they are unknown for the RT-PCR. Hence, the diagnostic efficacy of RT-PCR in clinical and field applications remains to be determined.

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REFERENCES

1. **Akashi, H., and D. H. L. Bishop.** 1983. Comparison of the sequences and coding of La Crosse and snowshoe hare bunyavirus S RNA species. J. Virol. **45:**1155–1158.
2. **Beaty, B. J., and W. H. Thompson.** 1975. Emergence of La Crosse virus from endemic foci. Fluorescent antibody studies of overwintered Aedes triseriatus. Am. J. Trop. Med. Hyg. **24:**685–691.
3. **Calisher, C. H.** 1979. Antigenic relationships of the arboviruses: an ecological and evolutionary approach, p. 117–152. *In* New aspects in ecology of arboviruses. Institute of Virology, Slovak Academy of Sciences, Prague.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
5. **Dykers, T. I., K. L. Brown, C. B. Gundersen, and B. J. Beaty.** 1985. Rapid diagnosis of La Crosse encephalitis: detection of specific immunoglobulin M in cerebrospinal fluid. J. Clin. Microbiol. **22:**740–744.
6. **Gonzalez-Scarano, F., R. E. Shope, C. E. Calisher, and N. Nathanson.** 1982. Characterization of monoclonal antibodies against the G1 and N proteins of LaCrosse and Tahyna, two California serogroup bunyaviruses. Virology **120:**42–53.
7. **Hildreth, S. W., and B. J. Beaty.** 1984. Detection of eastern equine encephalomyelitis virus and Highlands J virus antigens within mosquito pools by enzyme immunoassay (EIA). I. A laboratory study. Am. J. Trop. Med. Hyg. **33:**965–972.
8. **Hildreth, S. W., and B. J. Beaty.** 1987. Economic comparison of enzyme immunoassay and virus isolation procedures for surveillance of arboviruses in mosquito populations. J. Clin. Microbiol. **25:**976–981.
9. **Hildreth, S. W., B. J. Beaty, H. K. Mayfield, R. F. Gilfillan, and B. J. Rosenau.** 1984. Detection of eastern equine encephalomyelitis virus and Highlands J virus antigens within mosquito pools by enzyme immunoassay (EIA). II. Retrospective field test of the EIA. Am. J. Trop. Med. Hyg. **33:**973–980.
10. **Hildreth, S. W., B. J. Beaty, J. M. Meegan, C. L. Frazier, and R. E. Shope.** 1982. Detection of LaCrosse arbovirus antigen in mosquito pools: application of chromogenic and fluorogenic enzyme immunoassay systems. J. Clin. Microbiol. **15:**879–884.
11. **Jamnback, T. L., B. J. Beaty, S. W. Hildreth, K. L. Brown, and C. B. Gundersen.** 1982. Capture immunoglobulin M system for rapid diagnosis of La Crosse (California encephalitis) virus infections. J. Clin. Microbiol. **16:**577–580.

12. **Lanciotti, R. S., C. H. Calisher, D. J. Gubler, G.-J. Chang, and A. V. Vorndam.** 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase polymerase chain reaction. J. Clin. Microbiol. **30:**545–551.
13. **Monath, T. P.** 1980. St. Louis encephalitis. American Public Health Associations Washington, D.C.
14. **Niklasson, B. S., and T. P. Gargan.** 1985. Enzyme-linked immunosorbent assay for detection of Rift Valley fever virus antigen in mosquitoes. Am. J. Trop. Med. Hyg. **34:**400–405.
15. **Olson, K. E., C. D. Blair, and B. J. Beaty.** 1990. Detection of dengue viral RNA in mosquito vectors by mixed phase and solution hybridization. Mol. Cell. Probes **4:**307–320.
16. **Thompson, W. H., B. Kalfayan, and R. O. Anslow.** 1965. Isolation of California encephalitis group virus from a fatal human illness. Am. J. Epidemiol. **81:**245–253.
17. **Tsai, T. F., R. A. Bolin, M. Montoya, R. E. Francy, D. B. Bailey, M. Jozan, and J. T. Roehrig.** 1987. Detection of St. Louis encephalitis virus antigen in mosquitoes by capture enzyme immunoassay. J. Clin. Microbiol. **25:**370–376.
18. **Wilson, W. C., and C. C. L. Chase.** 1993. Nested and multiplex polymerase chain reactions for the identification of bluetongue virus nfection in the biting midge, *Culicoides variipennis*. J. Virol. Methods **45:**39–48.