

SIMULTANEOUS DETECTION OF THREE MOSQUITO-BORNE ENCEPHALITIS VIRUSES (EASTERN EQUINE, LA CROSSE, AND ST. LOUIS) WITH A SINGLE-TUBE MULTIPLEX REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ASSAY

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ABSTRACT. Three mosquito-borne human encephalitis viruses (eastern equine encephalitis virus [EEE], St. Louis encephalitis virus [SLE], and La Crosse encephalitis virus [LAC]) are sympatric in the southeastern United States. However, little is known concerning the temporal and spatial pattern of the distribution of these viruses in this area. As part of surveillance activities to detect the transmission of these 3 viruses in the Tennessee Valley area, we developed a single-tube multiplex reverse transcriptase polymerase chain reaction (RT-PCR) assay capable of detecting these 3 mosquito-borne viruses in a single reaction. Three viruses were differentiated by size of amplified products. Sensitivities of the multiplex RT-PCR assay for SLE, EEE, and LAC were 1–3 log median tissue culture infective doses per pool, roughly comparable to the reported sensitivity of PCR detection assays for the individual viruses, and 1 log more sensitive than antigen-capture assays for SLE and EEE. The sensitivity of the multiplex PCR was not changed significantly when carried out in the presence of extracts prepared from 50 uninfected mosquitoes. The cost of the assay is estimated at \$2.98 per test, similar to the cost of other RT-PCR-based assays for viruses. However, adaptation of the RT-PCR to a multiplex format adds less than \$0.01 to the per-unit cost of an RT-PCR assay targeting a single virus species. Analysis of these data suggests that the single-tube multiplex RT-PCR assay represents a sensitive, specific, cost-effective, and rapid method for monitoring activities of the 3 endemic mosquito-borne human encephalitis viruses in mosquito populations in the southeastern United States.

KEY WORDS Reverse transcriptase polymerase chain reaction, St. Louis encephalitis, eastern equine encephalitis, La Crosse encephalitis, arbovirus

INTRODUCTION

Arboviral encephalitis viruses represent an important health threat to humans. In the eastern United States, 3 major encephalitis viruses are known to be transmitted. These include eastern equine encephalitis virus (EEE), St. Louis encephalitis virus (SLE), and La Crosse encephalitis virus (LAC). All 3 of these viruses have been documented in the southeastern United States (Morris 1988, Tsai and Mitchell 1989, Mancao et al. 1996). Although documented cases of human infections with these viruses are relatively rare, the severe morbidity and mortality associated with these viruses makes them potentially serious health threats. As a result of this threat, several states carry out surveillance programs whose goal is to provide early detection of outbreaks of these viruses.

One of the most efficient ways to detect transmission of the encephalitis viruses is to document the presence of the viruses in the vector mosquito population. However, present methods for the detection of these viruses in mosquitoes are time-consuming, which delays getting results to surveillance personnel and increases costs. Several different methods have been developed to detect the presence of these viruses in vector mosquitoes. These include direct viral isolation by tissue culture (Nas-

ci and Mitchell 1996), antigen-capture assays with monoclonal antibodies specific for each of the viruses (Hildreth et al. 1984, Tsai et al. 1987, Olson et al. 1991), and amplification of viral-specific DNA products from viral RNA by reverse transcriptase polymerase chain reaction (RT-PCR; Armstrong et al. 1995, Nawrocki et al. 1996). Each of these methods has its advantages and disadvantages. The tissue culture method, although the most sensitive method of viral identification, is costly and requires a well-equipped laboratory with BSL3 biohazard certification. The tissue culture method requires several days to obtain a result. The enzyme immunoassay (EIA) and RT-PCR-based assays are less expensive, more rapid than tissue culture (Nawrocki et al. 1996) and are highly specific for viral RNA. In some cases, particular mosquito species have been shown to play an important role in maintaining or transmitting more than 1 of the encephalomyelitis viruses (Morris 1988, Reisen and Monath 1989, Tsai and Mitchell 1989). In the case of these species, it may be necessary to assay mosquitoes for each of the viruses that may be transmitted. Thus, in areas such as the southeastern United States, where more than 1 virus is found, it can be necessary to run multiple tests on a single sample to ensure the detection of all viruses.

As part of a surveillance effort to detect EEE, SLE, and LAC transmission in the Tennessee Valley, we have developed a multiplex RT-PCR capable of detecting viral RNA from the 3 viruses in a single reaction. This method employs a single-tube protocol, minimizing the manipulations needed to carry out the RT-PCR assay. The adaptation

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Table 1. Oligonucleotide primer sequences and multiplex reverse transcriptase polymerase chain reaction products.

Viruses ¹	Primer sequences	Expected size of amplicons (base pairs)
EEE	5' TACCCTACACTTAACCTACCCGC 3' 5' TGTCGTTTGCCTGGTTTAGGT 3'	298
SLE	5' CGATTGGATGGATGCTAGGTAG 3' 5' ACTCGGTAGCCTCCATCTTC 3'	240
LAC	5' TCAAGAGTGTGATGTCGGATTTGG 3' 5' GGAAGCCTGATGCCAAATTTCTG 3'	715

¹ EEE, eastern equine encephalitis; SLE, St. Louis encephalitis; LAC, La Crosse encephalitis.

of RT-PCR assays for each of the viruses to a single-tube format also means that any of the 3 viruses endemic to the southeastern United States may be detected in a single reaction, minimizing the number of tests needed to fully characterize each sample.

MATERIALS AND METHODS

Virus stocks: Eastern equine encephalomyelitis virus (NJ/60 strain, passage 6), SLE (TBH-28, unknown passage number), and LAC (Prototype) were kindly provided by the Centers for Disease Control and Prevention (Fort Collins, CO). These viruses were amplified in Vero cell culture and stored at -80°C . Titers of amplified viruses were determined by microtitration assay (Hierholzer and Killington 1996) and calculated as previously described (Reed and Munch 1938). The titers of stock viruses were 7.37 (LAC), 8.73 (EEE), and 8.03 (SLE) log median tissue culture infective doses (TCID₅₀)/ml.

Single-tube multiplex RT-PCR: The RNA extraction was performed with Trizol LS (Life Technologies, Grand Island, NY) according to manufacturer's instructions. Multiplex RT-PCR was performed with a single-tube RT-PCR kit (ProSTAR HF Single-Tube RT-PCR System, Stratagene, LaJolla, CA) according to the manufacturer's instructions. Reactions were carried out in a total volume of 40 μl , and contained 3 primer pairs (1 each for EEE, SLE, and LAC; Table 1). Oligonucleotide primers for SLE were selected from the membrane M protein and envelope protein genes. The EEE primer pairs were modified slightly from previously published primers for this virus (Armstrong et al. 1995). Similarly, the LAC primers were adapted from previously described sequences (Wasieloski et al. 1994). The primer pairs were designed so that the predicted melting points and optimal annealing temperatures for PCR were within 3.3°C of one another, as determined by the algorithms contained in the Oligo[®] program package (Rychlik 1992). Two hundred picomoles of each primer set were used in the reaction. The thermal profile consisted of 42°C for 15 min, 95°C for 1 min, and 40 cycles of 95°C for 30 sec, 58°C for 30 sec, and 68°C for 2 min, followed by 68°C for 10

min. The reaction products were separated on a 2.0% agarose gel (SeaKem LE, FMC Bioproducts, Rockland, ME) and detected by staining in 2 $\mu\text{g/l}$ liter ethidium bromide.

To test the specificity of the assay and its ability to detect mixed infections, RNA was extracted from 1 ml of 6–6.5 log TCID₅₀/ml of each of the 3 viral stocks. The purified RNA was resuspended in 10 μl of RNase-free water. One microliter of the purified RNA (corresponding to 5–5.5 TCID₅₀) from each virus was combined in each of the 8 possible combinations and used as a template in the RT-PCR.

To determine the sensitivity of the RT-PCR, serial dilutions of 3 stock viruses were prepared in cold BA-1 medium (199 medium containing 1% bovine serum albumin with antibiotics and adjusted to pH 7.6 with Tris and sodium bicarbonate; Tsai et al. 1987), and RNA was extracted. To assess the effect of mosquito extract on the sensitivity of the assay, the serial dilutions of the viral stocks were mixed with 50 *Aedes aegypti* (L.) in a final volume of 1 ml of medium. Homogenates were prepared from the virus mosquito mixture by vortexing the mixtures in the presence of 4 copper-plated steel beads (Copperhead[®] BBs, Crosman, East Bloomfield, NY). Homogenates were transferred to 1.5-ml centrifuge tubes and clarified by centrifugation at $13,000 \times g$ for 1 min. The RNA was extracted from the supernatants as described above, and used as a template in the RT-PCR assay. After RT-PCR, the products were treated with 5 units of RNase (United States Biochemical Corp., Cleveland, OH) for 30 min at 37°C to remove the mosquito RNA present in the reaction and to assist in the visualization of the PCR products.

Antigen-capture assays: Antigen-capture assays for EEE and SLE were carried out following previously published methods (Tsai et al. 1987, Olson et al. 1991). Antibodies to EEE and SLE and positive control antigens for both viruses were kindly provided by Roger Nasci of the Centers for Disease Control (Fort Collins, CO). Serial dilutions of the viral stocks were prepared as described above, and 100 μl of each dilution of each virus was tested in each well by EIA. Assays were carried out in quadruplicate. The average optical density (OD) value

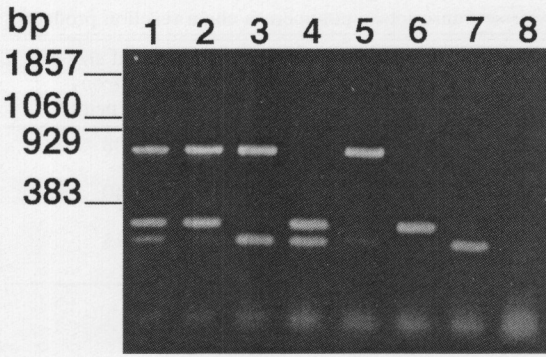


Fig 1. Specificity test of multiplex reverse transcriptase polymerase chain reaction (RT-PCR) for La Crosse encephalitis virus (LAC), eastern equine encephalitis virus (EEE), and St. Louis encephalitis virus (SLE). The RNA was extracted from 6–6.5 log median tissue culture infective doses of each virus. Different combinations of the 3 viruses were tested in each reaction. Lane 1, LAC, EEE, and SLE; lane 2, LAC and EEE; lane 3, LAC and SLE; lane 4, EEE and SLE; lane 5, LAC; lane 6, EEE; lane 7, SLE; lane 8, RT-PCR negative control.

of 4 wells was then calculated. If the average OD value was at least 2-fold higher than that of the negative control (BA-1), the sample was scored as positive.

RESULTS

To develop a multiplex PCR assay capable of detecting EEE, SLE, and LAC, primer sets were developed that were specific for each of the viruses. These primer sets were developed employing data from previous studies reporting PCR assays for these viruses (Wasieloski et al. 1994, Armstrong et al. 1995) and the published sequences of the complete genomes of these viruses as a starting point. The primer pairs were engineered to ensure that they were closely matched both for guanine and cytosine content and predicted annealing temperature, and chosen so that the resulting amplification products from each virus were easily discriminated on the basis of size. An optimized single-tube RT-PCR assay was then developed that utilized all 3 primer sets in a single reaction, as described in the Materials and Methods. As expected, each of the primer pairs produced a single diagnostic amplification product from each of the viruses tested (Fig. 1). All 3 viruses were easily differentiated on the basis of the size of the amplified products: 298 base pairs (bp) (EEE), 240 bp (SLE), and 715 bp (LAC) (Fig. 1). When the assay was tested with RNA isolated from individual viral stocks, only the single band diagnostic for that particular virus was obtained (Fig. 1), confirming the specificity of the primers for their particular virus. To confirm that the multiplex assay was capable of detecting multiple infections in a single reaction, mixing exper-

iments were carried out that consisted of all 3 possible pairs of viral RNA as well as reactions containing all 3 viral RNA samples. These experiments demonstrated that the assay was capable of detecting mixed infections containing any combination of viruses, as well as single infections (Fig. 1).

The sensitivity of the multiplex PCR for each of the viruses was determined by assays carried out on log dilutions of viral stocks with a known titer. In these experiments, RNA was prepared from dilutions of the viral stocks, and from dilutions of the viral stocks mixed with 50 mosquitoes and subsequently homogenized as described in the Materials and Methods. In the assays employing the purified viral stocks, the multiplex RT-PCR detected EEE and SLE RNAs extracted from 2–3 log TCID₅₀ and LAC RNA from as low as 1–3 log TCID₅₀ (Fig. 2A). The detection sensitivity was found to be slightly lower (<1 log) when the assay was used to test homogenates prepared from mixtures of the viral stocks and 50 *Ae. aegypti* mosquitoes (Fig. 2B).

Sensitive and specific antigen-capture assays have been developed for both EEE and SLE (Hildreth et al. 1984, Tsai et al. 1987, Olson et al. 1991). To compare the relative sensitivity of the multiplex PCR to that of the antigen-capture assays, RT-PCR and antigen-capture assays were carried out in parallel on log dilutions of the viral stocks, prepared as described above. The multiplex RT-PCR assay was found to be approximately 1 log more sensitive than the antigen-capture assays for the detection of both EEE and SLE (Table 2).

Polymorphisms in the viral sequences might introduce mismatches between the primer and target sequences, resulting in the inability of the assay to detect certain viral isolates. To investigate this possibility, the GenBank nucleotide sequence database was searched with each primer sequence to identify polymorphisms in the sequences used to design the primers. No polymorphisms were detected in the sequences from which the primers were designed in either of the LAC primers, or in the SLE 5' or EEE 3' primer. In contrast, polymorphisms were detected in the EEE 5' primer and in the SLE 3' primer. In the case of the EEE 5' primer, 21 of 24 (87.5%) of the sequences in the database matched the sequence of the primer exactly, whereas 12.5% of the sequences contained a single polymorphism. In contrast, polymorphisms in the area used for the SLE 3' primer were more common. A total of 13% (11 of 83) of the sequences in the database contained a single polymorphism at position 7 and 43% (36 of 83) had 2 polymorphisms, at positions 5 and 8. To determine if these polymorphisms would interfere with the ability of the RT-PCR assay to detect virus isolates containing these polymorphisms, viral RNA isolated from viral strains containing these polymorphisms was employed as templates in the multiplex PCR. As shown in Fig. 3, both viral RNA samples were detected by the PCR.

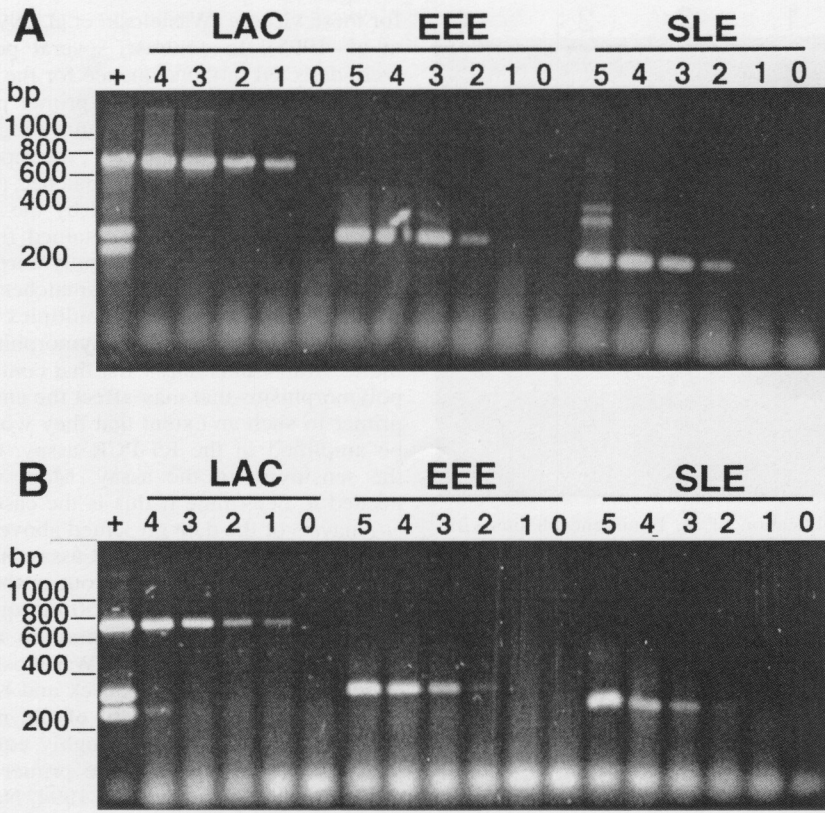


Fig. 2. Sensitivity test of multiplex reverse transcriptase polymerase chain reaction for La Crosse encephalitis virus, eastern equine encephalitis virus, and St. Louis encephalitis virus. The RNA was extracted from serial dilutions of each viral stock, and from serial dilutions mixed with 50 *Aedes aegypti* mosquitoes, as described in the text. A. Sensitivity of the assay in the absence of mosquitoes. B. Sensitivity of the assay in the presence of 50 *Ae. aegypti* mosquitoes. Labels of the lanes indicate virus titers (log median tissue culture infective doses) used in each reaction.

DISCUSSION

Arboviral surveillance programs are an important activity supported by the Departments of Health in many states. These programs have been instrumental in providing early warning of arboviral activity for many years. Historically, these programs have applied a multifaceted approach to their surveillance activities. These surveillance activities have

included case reports from physicians and veterinarians, detection of viral transmission by seroconversion of sentinel animals (primarily birds) and detection of virus in vector mosquito populations.

In detecting viral activity in vector mosquito populations, speed, specificity, sensitivity, and economy all are important factors in choosing a viral detection method. In this regard, RT-PCR-based methods of viral detection offer some important advantages. For example, in previous studies comparing viral culture, RT-PCR, and antigen-capture assays for the detection of SLE, the conclusion was made that the RT-PCR assay was more sensitive than the antigen-capture assay, and roughly as sensitive as the Vero cell culture assay. However, the RT-PCR assay was faster, less cumbersome, and less expensive than the vero cell culture assay (Narowski et al. 1996). The single-tube multiplex RT-PCR assay described above represents an improvement over the individual RT-PCR assays as a primary screening tool to detect viral activity in mosquito cultures. First, the multiplex format means that it is possible to use a single assay mix-

Table 2. Comparison of sensitivities between multiplex reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) for eastern equine encephalitis virus (EEE) and St. Louis encephalitis virus (SLE).

	Virus concentrations (log TCID ₅₀ /ml) ¹							
	EEE				SLE			
	5	4	3	2	5	4	3	2
ELISA	+	+	-	-	+	+	-	-
Multiplex RT-PCR	+	+	+	-	+	+	+	-

¹ TCID₅₀, median tissue culture infective doses.

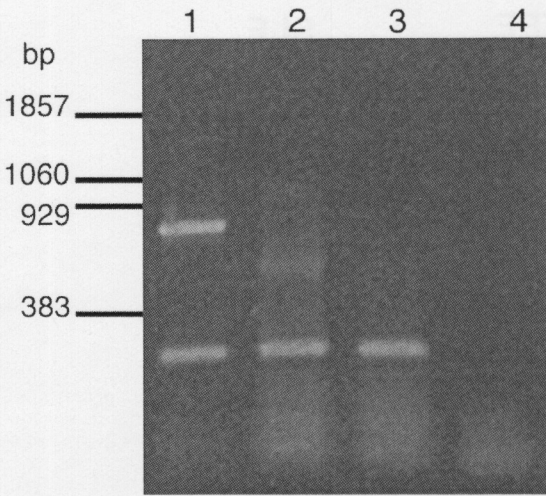


Fig. 3. Amplification of St. Louis encephalitis virus (SLE) isolates with polymorphisms in the 3' primer domain. The RNA was prepared from 2 viral isolates that exhibited either 1 or 2 mismatches to the 3' SLE primer used in the multiplex polymerase chain reaction (PCR). The viral RNA samples were used as a template in the multiplex PCR as described in the Materials and Methods. Lane 1, positive control (mixed La Crosse encephalitis virus and SLE RNA samples); lane 2, RNA prepared from strain Parton (1 mismatch with respect to the primer sequence); lane 3, RNA prepared from strain GML902612 (2 mismatches with respect to the primer sequence); lane 4, negative control.

ture and a single PCR protocol to detect any of the 3 encephalomyelitis viruses that are common to the southeastern United States. Second, the use of a multiplex format makes it possible to detect any combination of viruses that may be present in a mosquito pool. This eliminates the need to carry out separate assays for each virus on mosquito pools containing vectors capable of transmitting more than 1 virus. Finally, the assay described above does not require separate reactions for the RT and PCR steps. This minimizes the number of manipulations needed for each sample, increasing the speed that each sample can be processed and decreasing the chances of cross-contamination among samples.

The sensitivity of PCR-based assays such as those described here may be adversely affected by polymorphisms in the sequences used to prepare the primers. To investigate this possibility, sequences in the GenBank database were examined for the presence of polymorphisms in the primer sites. No polymorphisms were identified in the LAC sequences, whereas only a minimal degree of polymorphism (1 nucleotide change in 12.5% of the sequences in a single primer) was noted in the EEE sequences. This is not surprising, given that these primers were modified from those used in previously developed and characterized RT-PCR assays

for these viruses (Wasieloski et al. 1994, Armstrong et al. 1995). In contrast, several polymorphisms were detected in the sequence for the 3' SLE primer. Fortunately, because this primer primes the reverse transcription reaction, annealing of this primer to its target occurs at 42°C, a temperature that is low enough to permit a mismatched primer to bind efficiently. This is supported by the fact that successful amplification was obtained from viral isolates incorporating up to 2 polymorphisms. However, the presence of such mismatches may increase the limit of detection of the multiplex PCR for viral strains containing these polymorphisms. Furthermore, viral strains may exist that contain additional polymorphisms that may affect the annealing of the primer to such an extent that they would no longer be amplified in the RT-PCR assay, thus reducing the sensitivity of the assay. More work will be needed to determine if this is the case.

Analysis of the data presented above suggests that the sensitivity of the RT-PCR assay is only marginally decreased when carried out in the presence of homogenates prepared from 50 mosquitoes. A pool size of ≤ 50 is commonly used in arboviral surveillance (Howe et al. 1992, Wasieloski et al. 1994, Nawrocki et al. 1996, Janousek and Kramer 1998). Furthermore, the sensitivity of the multiplex RT-PCR assays seems to be roughly equal to single-step assays employing single primer pairs (Howe et al. 1992, Wasieloski et al. 1994, Nawrocki et al. 1996), all of which report a sensitivity in the range of 2–3 log of virus.

If the multiplex PCR is to be used as a routine tool for arboviral surveillance, it must be cost effective relative to other methods of detecting the viruses in question. The cost of RT-PCR assays for encephalitis virus screening has been addressed by others, and found to be intermediate between that of immunological and tissue culture-based methods (Nawrocki et al. 1996). In this work, the cost of an RT-PCR assay to detect EEE was calculated and was found to fall between that of the immunological assays (EIA and enzyme-linked immunosorbent assay; ca \$1.00 per sample) and that of the assays involving tissue culture (ca \$7.00; Nawrocki et al. 1996). The RT-PCR-based assays are more rapid than the techniques relying on tissue culture and do not require the extensive biohazard containment facilities necessary to carry out these techniques. The single-tube multiplex RT-PCR assay described here has an estimated cost of reagents and disposable supplies of roughly \$2.98 per assay. The specialized equipment needed to carry out the assay will cost roughly \$5,000. Interestingly, the costs of carrying out the multiplex procedure are essentially the same as those of carrying out an RT-PCR assay to detect a single virus species, because the cost of the 2 additional primer pairs is only 0.6 cent per reaction. Therefore, the multiplex RT-PCR assay may be a useful and cost-effective technique to monitor mosquito-borne viruses in the areas

where little information on the viruses is available and where multiple arboviruses are circulating in hosts and mosquito populations, such as in the southeastern United States.

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