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An Integrated Target Sequence and Signal Amplification Assay, Reverse Transcriptase–PCR–Enzyme-Linked Immunosorbent Assay, To Detect and Characterize Flaviviruses

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We previously described a reverse transcriptase-PCR using flavivirus genus-conserved and virus species-specific amplimers (D. W. Trent and G. J. Chang, p. 355–371, *in* Y. Becker and C. Darai; ed., Frontiers of Virology, vol. 1, 1992). Target amplification was improved by redesigning the amplimers, and a sensitive enzyme-linked immunosorbent assay (ELISA) technique has been developed to detect amplified digoxigenin (DIG)-modified DNA. A single biotin motif and multiple DIG motifs were incorporated into each amplicon, which permitted amplicon capture by a biotin-streptavidin interaction and detection with DIG-specific antiserum in a colorimetric ELISA. We evaluated the utility of this assay for detecting St. Louis encephalitis (SLE) viral RNA in infected mosquitoes and dengue viral RNA in human serum specimens. The reverse transcriptase-PCR-ELISA was as sensitive as isolation of SLE virus by cell culture in detecting SLE viral RNA in infected mosquitoes. The test was 89% specific and 95 to 100% sensitive for identification of dengue viral RNA in serum specimens compared with isolation of virus by *Aedes albopictus* C6/36 cell culture and identification by the indirect immunofluorescence assay.

Flaviviruses are arthropod-transmitted viruses that belong to the family *Flaviviridae* (1). Viruses in this family include the etiologic agents of dengue (DEN), yellow fever (YF), Japanese encephalitis (JE), West Nile encephalitis, Murray Valley encephalitis, St. Louis encephalitis (SLE), and tick-borne encephalitis (9). Conventional flavivirus diagnosis is based on isolation and identification of virus from clinical specimens or the presence of virus-specific antibody in the patient serum specimen (9). The exceptional sensitivity of the PCR permits a millionfold amplification of specific nucleotide sequences in vitro (14). This technique provides an attractive approach for rapid detection and identification of flaviviruses in mosquitoes and clinical specimens when virus cultivation is difficult or time consuming and when diagnosis impacts on clinical treatment and has implications for vaccination and mosquito control.

Several reverse transcriptase (RT)-PCRs using different pairs of primers (amplimers) for specific viruses have been developed for detecting flavivirus RNA. Identification of virus to species level has usually been accomplished by determining the size of amplified DNA by agarose gel electrophoresis (3, 6–8, 10) or by hybridization with labeled virus species-specific (VSS) probes (2, 6, 8). Agarose gel electrophoresis is more convenient but less sensitive than hybridization. A combination of nested PCR and agarose gel electrophoresis is more sensitive than hybridization for detecting and identifying the four serotypes of DEN virus (DEN1 through DEN4) (8). However, this technique is subject to amplicon contamination during the second cycle of amplification. We have described a simple strategy to detect medically important flaviviruses in a single-vessel RT-PCR (15). This assay consists of a flavivirus genus-conserved (FGC) amplimer pair (FUDJ9166 and CFDJ9977) and 11 individual VSS up amplimers that hybridize with specific sequences in the carboxyl-terminal one-third portion of the NS5 (nonstructural) gene. The FGC amplimer pair amplifies an amplicon of 830 bp in the NS5 gene of all flaviviruses tested (15). To determine the species of viral RNA, the FGC down amplimer (CFDJ9977) is used in combination with an individual VSS up amplimer. Amplicons of appropriate sizes are generated if the virus-specific up amplimer and viral RNA are homologous. This RT-PCR can also be used to identify previously unrecognized flaviviruses by nucleotide sequence analysis of the DNA amplified by the FGC amplimer pair (15).

To improve the sensitivity and specificity of the flavivirus RT-PCR, we have modified the FGC down amplimer, CFD2-4, and developed an integrated target and signal amplification assay. This assay employs RT-PCR in the presence of digoxigenin (DIG)-11-dUTP to amplify target sequences in the NS5 gene of the flavivirus RNA and uses the enzyme-linked immunosorbent assay (ELISA) to detect the DIG-modified amplicon DNA. Amplimers have been designed to detect and type the medically important flaviviruses in mosquitoes and serum specimens.

MATERIALS AND METHODS

Virus strains and specimens. Viruses used in this study were obtained from the reference collection at the Division of Vector-Borne Infectious Diseases, Centers for Disease

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Control and Prevention (CDC), Fort Collins, Colo. YF virus strain 17D and SLE virus strain MSI-7 were titrated in Vero cells (16).

Insectary-maintained *Culex pipiens* mosquitoes were inoculated intrathoracically with 0.5 PFU of SLE virus per 350 nl. Since mosquitoes are more susceptible than Vero cells for flavivirus replication, the virus dose used is sufficient to infect all inoculated mosquitoes. Sixteen uninfected mosquitoes were frozen at zero hour as the normal mosquito control. Two to four infected mosquitoes were removed and frozen at −70°C at 4.5, 18.5, 48, 96, and 144 h post inoculation (p.i.). Individual mosquitoes were homogenized separately in 1 ml of Eagle's minimal essential medium containing 5% bovine serum albumin and frozen at −70°C. The infection status of each mosquito was verified by plaque titration in Vero cells (16).

Serum specimens from DEN virus patients were obtained from the San Juan Laboratories, Division of Vector-Borne Infectious Diseases, CDC, San Juan, P.R. To evaluate the sensitivity and specificity of the RT-PCR-ELISA, we analyzed 91 serum specimens previously shown to contain DEN virus as demonstrated by virus isolation in *Aedes albopictus* C6/36 cell culture and virus identification by indirect immunofluorescence assay (IFA) (5). Twenty-four specimens negative by C6/36 cell culture and IFA were included as controls. All of the specimens were assigned a random code prior to blind testing by RT-PCR-ELISA.

RNA extraction. Nucleic acids were extracted from virus seed stocks, homogenized mosquito suspensions, or human serum specimens by the method described by Lanciotti et al. (8). The extracted nucleic acid was resuspended in distilled water and stored at −70°C.

Capture of flaviviral RNA from mosquitoes before RT-PCR. To remove nonspecific inhibitors of the RT-PCR, viral RNA in infected mosquito extracts was captured by using a modification of the method described by Lanciotti et al. (8). Oligonucleotide primer Bio-CFDJ9977 or Bio-CFD2-4 served as the flaviviral RNA capture probe. Nucleic acid extracted from homogenized mosquitoes was mixed with 5 pmol of capture probe in wash buffer (0.1 M Tris-HCl, pH 5.5; 0.1 M NaCl), heated at 94°C for 2 min, and slowly cooled to room temperature. Biotinylated probe (Bio-probe)–viral RNA complexes were captured by the addition of 10 μl of prewashed streptavidin-coated magnetic beads (M-280 streptavidin, binding capacity of 2 pmol/μl; Dynal, Inc., Great Neck, N.Y.). Following incubation at room temperature for 30 min, Bio-probe–viral RNA–M-280 complexes were immobilized during all washing steps with a magnetic particle concentrator (Dynal MPC-E; Dynal, Inc.). Bio-probe–viral RNA–M-280 complexes were washed three times with wash buffer to remove unbound components, and the complexes were resuspended in 20 μl of distilled H_2O. Captured viral RNA was separated from the complex by heating at 80°C for 2 min and quick chilling at 4°C. RNA in the supernatant was separated from the magnetic beads at 4°C and collected.

Synthesis of oligonucleotide amplimers. The FGC and VSS amplimers used in the RT-PCR-ELISA, except CFD2-4 and DEN4-9580, have been previously described (Table 1) (15). FGC amplimer CFD2-4, whose 3′-terminal 21 residues are identical to the complete FGC amplimer CFDJ9977, provided more efficient amplification of DEN4 viral RNAs.

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer, model 380A, by using standard phosphoramidite chemistry (Applied Biosystems Inc., Foster City, Calif.). Biotin was attached to the 5′ end of the CFDJ9977 and CFD2-4 amplimers by using biotin-on phosphoramidite chemistry (Clontech Laboratories, Inc., Palo Alto, Calif.). Synthetic oligonucleotides were purified by using OPC columns (Applied Biosystems, Inc.).

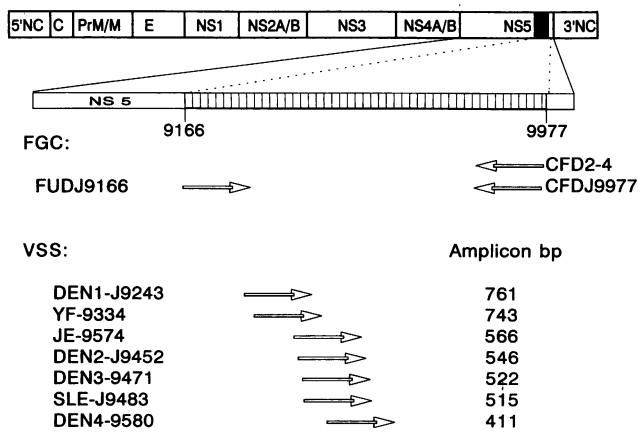

FIG. 1. Schematic diagram of the 11-kb flavivirus genome showing the relative locations of FGC and VSS amplimers. Arrows indicate the 5′-to-3′ direction of amplimers. Amplimers specific for DEN1, DEN2, DEN3, DEN4, JE, SLE, and YF viruses are designated DEN1-J9243, DEN2-J9452, DEN3-9471, DEN4-9580, JE-9574, SLE-J9483, and YF-9334, respectively. NC, noncoding region; C, nucleocapsid protein; PrM/M, premembrane and membrane protein; E, envelope protein; NS, nonstructural protein.

RT-PCR as a target sequence amplification system. A single-vessel RT-PCR was used to convert target viral RNA to cDNA with subsequent amplification of the cDNA to double-stranded DNA. Reactions were done in a 25-μl volume that contained the following components: resuspended viral RNA extract (1 to 10 μl); 50 mM KCl; 10 mM Tris-Cl, pH 8.5; 1.5 mM $MgCl_2$; 0.01% (wt/vol) gelatin; 200 μM (each) the four deoxynucleoside triphosphates (Promega, Madison, Wis.); 2.5 mM dithiothreitol; 250 nM (each) (unless otherwise specified) up and down amplimers; 2.5 U of RAV-2 RT (Amersham Corp., Arlington Heights, Ill.) per 100 μl; and 2.5 U of Amplitaq polymerase (The Perkin-Elmer Corp., Norwalk, Conn.) per 100 μl. Reaction mixtures were incubated in a model 9600 Thermocycler (The Perkin-Elmer Corp.) for 1 h at 55°C to facilitate the RT reaction and for 4 min at 94°C for denaturation, after which they were subjected to 25 cycles of denaturation (94°C for 1 min), amplimer annealing (55°C for 1 min), and amplimer extension (72°C for 3 min), followed by a 10-min incubation at 72°C.

To incorporate DIG-dUMP directly into the amplicon, reactions were performed as described above with the addition of 10 μM DIG-11-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in the reaction mixture. Although a higher concentration of DIG-11-dUTP in the reaction mixture resulted in increased optical density (OD) readings in ELISAs, test sensitivity was not significantly improved (data not shown).

ELISA amplification system. DIG-modified amplicons (DIG-amplicons) were characterized by agarose gel electrophoresis and ELISA. Five-microliter portions of reaction product were electrophoresed in 3% GTG agarose gels (FMC Bioproducts, Rockland, Maine), and the amplified DNA was stained with ethidium bromide. Because individual VSS amplimers primed at different positions in the NS5 gene of the viral RNA, the amplicon size for each flavivirus was different (Fig. 1 and Table 1).

TABLE 1. Amplimers used in RT-PCR-ELISA identification of flaviviruses

Amplimer type and designation	Oligonucleotide sequence (5′ to 3′)	Amplicon size (bp)
FGC amplimers		
Bio-CFD2-4[a]	5′-TTTGAGCATGTCTTCCGTCGTCATCC	
Bio-CFDJ9977[a]	5′- GCATGTCTTCCGTCGTCATCC	
FUDJ9166[b]	5′-GATGACACAGCAGGATGGGAC	838 or 832[c]
VSS up amplimers		
DEN1-J9243	5′-GCCTGAACATGCTCTATTGGCT	761
DEN2-J9452	5′-TCTTCAAAAGCATTCAGCACCT	546
DEN3-9471	5′-CCCATCCGCTAGAGAAGAAAATTACAC	522
DEN4-9580	5′-GGTTTGGCACTTCCCTCCTCTTCTTG	411
JE-9574	5′-GACCACAACACTTGGAACAGCTAC	566
SLE-J9483	5′-ACGATTGGCCAAAGCGGTTGAG	515
YF-9334	5′-ACAAGCAGTGATGGAAATGACA	743
Nested-PCR amplimers[d]		
D1	5′-TCAATATGCTGAAACGCGCGAGAAACCG	
D2	5′-TTGCACCAACAGTCAATGTCTTCAGGTTC	511
TS1	5′-CGTCTCAGTGATCCGGGGG	482
TS2	5′-CGCCACAAGGGCCATGAACAG	119
TS3	5′-TAACATCATCATGAGACAGAGC	290
TS4	5′-CTCTGTTGTCTTAAACAAGAGA	392

[a] Down amplimer.
[b] Up amplimer.
[c] Amplicon size obtained by using Bio-CFD2-4 or Bio-CFDJ9977, respectively.
[d] Reported by Lanciotti et al. (8).

A schematic representation of the ELISA detection protocol is shown in Fig. 2. DIG-amplicons were diluted in a fourfold dilution series with rinse buffer (phosphate-buffered saline with 0.05% Tween 20). Fifty-microliter aliquots of the diluted DIG-amplicons were added to each well of a 96-well ELISA plate (Immulon II; Dynatech Laboratories Inc., Chantilly, Va.) that had previously been coated with streptavidin (1 μg per well; Boehringer Mannheim Biochemicals) in 100 μl of coating buffer (Na_2CO_3-$NaHCO_3$, pH 9.6) and blocked with 100 μl of blocking buffer (3% normal goat serum in rinse buffer) per well. Biotinylated DIG-amplicons were bound to the microtiter plate through biotin-streptavidin interaction by incubation at 37°C for 1 h. The plates were washed three times with rinse buffer, followed by the addition to each well of 50 μl of rinse buffer containing 5 mU of horseradish peroxidase-conjugated sheep anti-DIG Fab fragment (Boehringer Mannheim Biochemicals), incubation at 37°C for 1 h, and three washes with rinse buffer. One hundred microliters of the enzyme substrate-color indicator (0.01% 3,3′,5,5′-tetramethyl benzidine, 0.005% H_2O_2 in 0.1 M citrate-acetate buffer, pH 6.0) was added to each well, and the plates were incubated at room temperature for 10 min. Reactions were stopped by the addition of 50 μl of 2 M H_2SO_4 per well, and the OD at 450 nm (OD_{450}) was measured in each well.

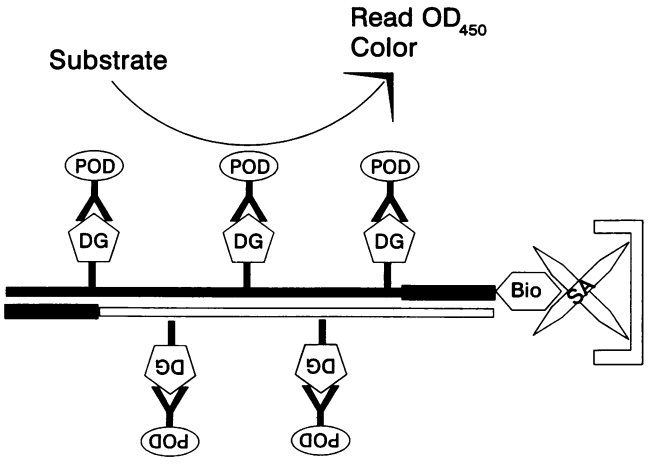

FIG. 2. Detection of DIG-dUMP (DG) incorporated DNA by a colorimetric ELISA technique. DIG-dUMPs were incorporated directly into the amplicon during target sequence amplification. Amplicons were captured by biotin (Bio)-streptavidin (SA) interaction and were detected by using horseradish peroxidase-conjugated sheep anti-DIG Fab fragment (POD) in a standard ELISA format. The biotin moiety was from biotinylated FGC amplimers, Bio-CFD2-4.

Development of testing algorithms for routine diagnosis of flavivirus infection. A panel of 115 randomly coded serum specimens, including 91 positive and 24 negative by virus isolation in C6/36 cells and IFA, was used in a blind test to determine the sensitivity and specificity of the RT-PCR-ELISA as a diagnostic procedure for DEN virus infection. Results were recorded as flavivirus negative, flavivirus positive (with DEN virus serotype), or virus species undetermined (Fig. 3). Specimens that gave disparate results were reevaluated by virus isolation in C6/36 cells and IFA (5) and nested PCR (8).

RESULTS

Specificity of the ELISA signal amplification system. Specificity of the ELISA in detecting flavivirus amplicons depends on the primary interaction of the target RNA sequence with amplimers used to prime synthesis of virus-specific amplicons during the RT-PCR. Although nonspecific target amplification can be decreased or eliminated by raising the amplimer annealing temperature from 50 to 55°C, some nonspecific amplimer-RNA interaction can occur at higher

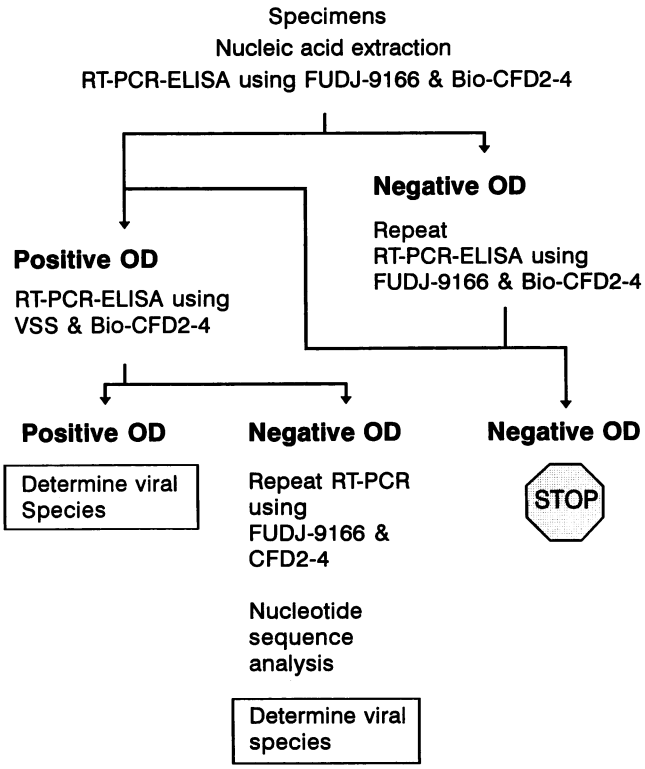

FIG. 3. Test algorithm for RT-PCR-ELISA detection of flavivirus RNA in clinical specimens.

amplimer concentrations and result in a higher nonspecific OD_{450}. To illustrate the effect of amplimer concentration on ELISA specificity, different concentrations (500, 250, 125, and 62.5 nM) of down FGC amplimer CFD2-4 and up VSS amplimer YF-9334, DEN1-J9243, DEN2-9452, DEN3-9471, DEN4-9580, or JE-9574 (Table 1) were used to detect a 6.2 $\log_{10}$ PFU equivalent of YF viral RNA (Fig. 4).

Down amplimer Bio-CFD2-4 paired with heterologous

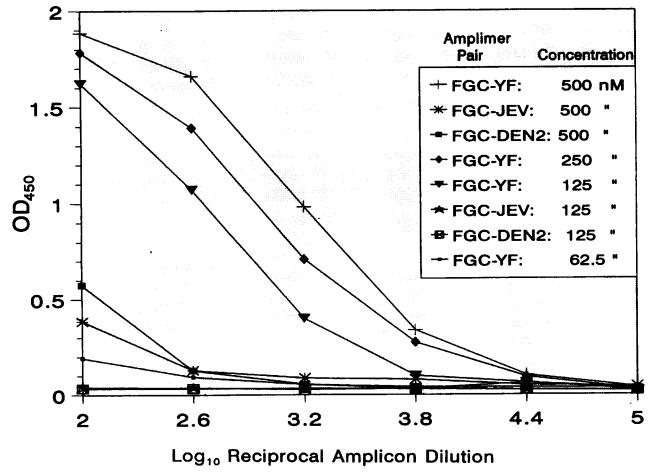

FIG. 4. Effect of amplimer concentration on the specificity of the RT-PCR-ELISA. Bio-CFD2-4 (FGC) was paired with different VSS amplimers, YF-9334 (YF), JE-9574 (JEV), and DEN2-J9452 (DEN2) in an RT-PCR-ELISA to detect YF viral RNA. Amplimer pairs were tested at 500, 250, 125, and 62.5 nM.

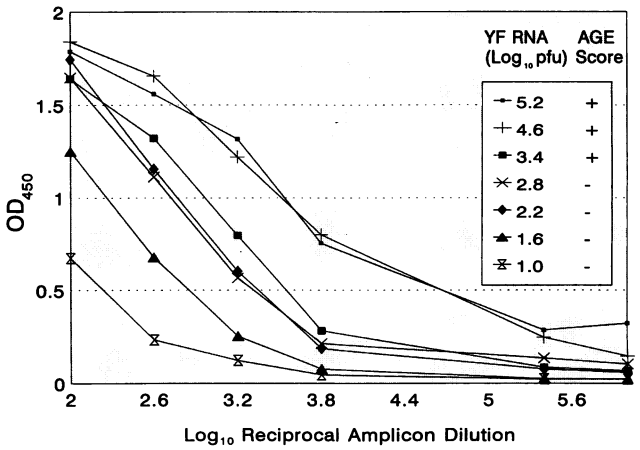

FIG. 5. Sensitivity of the RT-PCR-ELISA for detection of YF viral RNA. Assays were performed with amplimers Bio-CFD2-4 and YF-9334 at 250 nM for 25 PCR cycles. Colorimetric ELISA detection (graphs) of the amplicon is compared with visual detection of the ethidium bromide-stained amplicon analyzed by agarose gel electrophoresis (AGE score; + or −) following RT-PCR using a 1.0 to 5.2 $\log_{10}$ PFU equivalent of YF viral RNA.

VSS up amplimers DEN2-J9452 and JE-9574 at a concentration of 500 nM gave unacceptably high OD_{450}s of 0.58 and 0.38, respectively, at an amplicon dilution of 1:100 (Fig. 4). Background ODs were below 0.05 when 250, 125, or 62.5 nM heterologous VSS amplimers were used in RT-PCRs (Fig. 4). The homologous Bio-CFD2-4–YF-9334 amplimer pair at concentrations of 500, 250, and 125 nM resulted in similar OD_{450}s of 1.62 to 1.88 at the 1:100 dilution of DNA amplified from YF viral RNA (Fig. 4). Amplicon yields were nearly equal when this amplimer pair was used at concentrations of 500 and 250 nM but decreased by about 80% at the 125 nM concentration (data not shown). At an amplimer concentration of 62.5 nM, amplicons were undetectable by agarose gel electrophoresis (not shown) but were weakly positive by ELISA (Fig. 4). Experiments using heterologous and homologous amplimers with DEN1, DEN2, DEN3, DEN4, or JE viral RNAs gave similar results (data not shown). On the basis of high amplicon yield and low nonspecific reactivity, an amplimer concentration of 250 nM was selected for use throughout the remainder of this study.

Sensitivity of RT-PCR-ELISA for detecting viral RNA. YF viral RNA was used to illustrate the sensitivity of the RT-PCR-ELISA as an integrated amplification and detection system. Various amounts of virus RNA, equivalent to 5.2, 4.6, 3.4, 2.8, 2.2, 1.6, or 1.0 $\log_{10}$ PFU of YF virus, and 250 nM amplimer pair Bio-CFD2-4–YF-9334 were used. The relative sensitivities of the RT-PCR-ELISA and the agarose gel electrophoresis-ethidium bromide staining methods were compared for detection of DIG-amplicons amplified from various amounts of YF viral RNA (Fig. 5). The RT-PCR-ELISA enabled detection of a 10-PFU equivalent of YF viral RNA (Fig. 5), in contrast to agarose gel electrophoresis, with which a specific amplicon band was visible only when greater than a 640 (or 2.8 $\log_{10}$) PFU equivalent of YF viral RNA was used (Fig. 5). Thus, ELISA was at least 64-fold more sensitive than agarose gel electrophoresis-ethidium bromide staining for detection of DIG-amplicons.

Viral RNA detection in mosquitoes infected with SLE virus. Application of the RT-PCR-ELISA to detect viral RNA in flavivirus-infected mosquitoes was investigated by analyzing

TABLE 2. Comparison between virus isolation and RT-PCR-ELISA in detection of SLE virus-infected mosquitoes

Time (h) p.i.	No. of specimens tested	Result by:		
		Virus isolation[a]	RT-PCR-ELISA[b]	
			Uncaptured RNA[c]	Captured RNA[d]
4.5	2	−, −	−, −	−, −
18.5	4	−, −, −, −	−, −, −, −	−, 2.6, −, 2.6
48.0	3	1.7, 2.4, 2.2	−, −, 2.6	2.6, 2.6, 2.6
96.0	3	3.4, 4.8, 5.4	3.2, 3.8, >4.4	3.2, 3.8, >4.4
144.0	4	5.7, 4.6, 5.6, 5.9	>4.4, 3.8, >4.4, >4.4	>4.4, 3.8, >4.4, >4.4
0 (uninfected controls)	16	− (all)	<2.0 (all)[e]	<2.0 (all)

[a] −, negative result. Numbers indicate $\log_{10}$ PFU/ml measured by plaque titration in Vero cells.
[b] −, negative result. Numbers indicate $\log_{10}$ reciprocal endpoint titers determined by ELISA.
[c] Nucleic acid extracted from mosquito homogenates was used directly in RT-PCR-ELISA.
[d] Virus-specific RNA purified from nucleic acid extracts by Bio-probe–streptavidin capture.
[e] All uninfected control mosquitoes had OD_{450}s of 0.1 to 0.25 at a 1:100 amplicon dilution.

mosquitoes inoculated with SLE virus. Mosquitoes were assayed for infectious virus by plaque titration in Vero cell monolayers and for viral RNA by RT-PCR-ELISA at various times p.i. SLE virus could be isolated from mosquitoes 48 to 144 h p.i. (Table 2). Only one of the three mosquitoes tested at 48 h p.i. was positive by RT-PCR-ELISA when total nucleic acid was used in the test without purification of the viral RNA. However, following capture and elution from probe-magnetic bead complexes, viral RNA was detected by RT-PCR-ELISA in all of the mosquitoes tested at 48 to 144 h p.i., as well as in two of four mosquitoes tested at 18.5 h p.i. (Table 2). Although detection of SLE viral RNA in mosquitoes by RT-PCR-ELISA without RNA capture was less sensitive than virus detection by isolation of virus in cell culture, RT-PCR-ELISA of captured SLE viral RNA was at least as sensitive as virus isolation (Table 2).

RT-PCR-ELISA for routine detection and typing of DEN viruses from viremic human serum. To determine the sensitivity and specificity of the RT-PCR-ELISA as a diagnostic test, a panel of 115 randomly coded human serum specimens was analyzed. Ninety-one of the serum specimens had previously been shown to contain DEN virus by virus isolation and IFA in C6/36 cell cultures (5). By the testing algorithm shown in Fig. 3, 97 of the serum specimens were positive for DEN viral RNA by RT-PCR-ELISA. A comparison of the PCR results with those obtained by virus isolation and IFA revealed discordance for 14 specimens, as shown in Table 3. These 14 specimens were again tested by virus isolation and IFA in C6/36 cell culture. By virus isolation and IFA, specimens 17, 30, 93, 107, 124, 163, 187, and 200, which were initially reported as negative, were positive for DEN2 virus on retrial (Table 3). The remaining six discordant specimens (8, 15, 48, 91, 135, and 180) were retested by RT-PCR-ELISA as well as by the nested-PCR technique (8). Specimens 91 and 135, which were identified by virus isolation and IFA as positive for DEN3 and DEN4 virus, tested positive for DEN2 and DEN3 viral RNA, respectively, by RT-PCR-ELISA and nested RT-PCR. These two specimens were categorized as indeterminate and not analyzed further. Specimens 8 and 180 were classified as negative by IFA but as DEN2 positive by RT-PCR-ELISA and nested PCR. Specimens 15 and 48 were DEN1 positive and DEN2 positive by IFA but were both negative by RT-PCR-ELISA and nested PCR. Specimens 8 and 180 were recorded as false negative by IFA, whereas specimens 15 and 48 were false negative by RT-PCR-ELISA.

Two-way comparisons were made between the RT-PCR-ELISA results and those obtained by virus isolation and IFA (Table 4). Both methods provided 89% overall specificity and 98% overall sensitivity in the detection of specimens containing DEN virus or viral RNA. Both methods showed 95 to 100% sensitivity in the identification of human serum specimens infected with each of the four serotypes of DEN virus (Table 4). Identification of DEN virus or viral RNA in these clinical specimens by RT-PCR-ELISA was comparable to the virus isolation-IFA method in terms of specificity and sensitivity. However, RT-PCR-ELISA offers the advantage of speed in diagnostic testing.

DISCUSSION

The epidemiology and clinical diagnosis of flaviviruses, which are important causes of human disease, are major public health concerns (9). The RT-PCR amplification of viral RNA provides a rapid and specific technique for the detection and identification of flaviviruses and is as sensitive as virus isolation in cell cultures and in mice (2, 3, 6–8, 10). Because several flaviviruses may be transmitted by the same

TABLE 3. Repeat testing of 14 human serum specimens for the presence of DEN virus (C6/36 cell culture and IFA) or DEN viral RNA (RT-PCR)

Specimen no.	Serotype identified by:			
	C6/36 cell culture and IFA		RT-PCR	
	Preliminary	Retrial	ELISA	Nested[a]
8	N[b]	N	2	2
15	1	1	N	N
17	N	2	2	ND[c]
30	N	2	2	ND
48	2	N	N	N
91	3	N	2	2
93	N	2	2	ND
107	N	2	2	ND
124	N	2	2	ND
135	4	N	3	3
163	N	2	2	ND
180	N	N	2	2
187	N	2	2	ND
200	N	2	2	ND

[a] The nested RT-PCR was performed as described by Lanciotti et al. (8).
[b] N, negative specimen.
[c] ND, not done.

TABLE 4. Specificity and sensitivity of IFA or RT-PCR-ELISA to detect DEN virus or viral RNA from human serum specimens

Result and/or serotype	No. identified by IFA/no. identified by PCR[a]	No. identified by PCR/no. identified by IFA[b]	Specificity[c]		Sensitivity[d]	
			IFA	PCR	IFA	PCR
Positive						
DEN	97/95	97/95	89	89	98	98
DEN1	26/25	25/25			96	96
DEN2	36/35	37/35			97	95
DEN3	10/10	10/10			100	100
DEN4	25/25	25/25			100	100
Negative	16/14	16/14				
False positive	2 (IFA)	2 (PCR)				
False negative	2 (IFA)	2 (PCR)				

[a] Number of specimens identified by IFA/number of the same specimens confirmed by PCR.
[b] Number of specimens identified by PCR/number of the same specimens confirmed by IFA.
[c] Specificity of assay = no. of negative specimens/(no. of negative specimens + no. of false-positive specimens) × 100.
[d] Sensitivity of assay = no. of positive specimens/(no. of positive specimens + no. of false-negative specimens) × 100.

species of mosquito in the same geographic area at the same time, the isolation of more than one virus from mosquitoes or clinical specimens is a possibility. By FGC- and VSS-amplimer-mediated RT-PCR (15), the RNA species of 11 medically important flaviviruses can be determined by the specific size of amplified DNA. This technique is also capable of identifying a previously uncharacterized or newly emerged flavivirus by use of the FGC amplimer pair and direct nucleotide sequence analysis of the amplified DNA (15).

Oligonucleotide probe hybridization (2, 6, 8) and reamplification with internal amplimers (8) utilize labeled probes or reamplification to increase the sensitivity and specificity of detection. These methods are more sensitive than agarose gel electrophoresis but are time-consuming and technically demanding. Detection and characterization of flaviviral RNA by the integrated target sequence (RT-PCR) and signal (ELISA) amplification system are accomplished by using the previously defined FGC and VSS amplimer sequences with some modifications (Table 1) (15). The Bio-CFD2-4 amplimer and DIG-11-dUTP used in the RT-PCR incorporate a biotin molecule and multiple DIG residues into the amplicon. This technique uses biotin-streptavidin as a separation system and DIG-anti-DIG as an indicator system to achieve a high level of sensitivity and technical simplicity. We detected as little as a 10-PFU equivalent of YF viral RNA, indicating this method is as sensitive as the nested-PCR method of Lanciotti et al. (8).

As with PCR detection of other RNA viruses, sensitivity is largely determined by the efficiency of reverse transcription of the flavivirus genomic RNA in producing cDNA prior to PCR (13). False-negative RT-PCR results were obtained when DEN viral RNA isolated from DEN virus-infected mosquitoes was used in the PCR (8). Lanciotti et al. (8) speculated that an inhibitory component present in the mosquito extract inhibited the PCR. We have developed a method that facilitates capture of flaviviral RNA prior to RT-PCR and improves the quality of isolated viral RNA. Using Bio-CFDJ9977 to capture viral RNA, we detected SLE viral RNA in experimentally infected mosquitoes by RT-PCR prior to virus isolation (Table 2). This suggests that the RNA capture procedure does remove nonspecific inhibitors and concentrates the viral RNA.

The RT-PCR-ELISA was equivalent to the standard virus isolation and IFA protocol in terms of test specificity (89%) and sensitivity (95 to 100%) in identifying DEN virus or viral RNA in clinical human serum specimens (Table 4). Six discordant specimens (Table 3) gave identical results when they were retested by RT-PCR-ELISA and nested PCR (8) (Table 3). Specimen 15, from which DEN1 virus was reisolated, was the only sample classified by the two PCRs as negative. Specimen 48, initially reported to be positive, was negative by the two PCRs and virus reisolation, suggesting that the virus had been inactivated and the RNA had been degraded. Results obtained by two different RT-PCR techniques for two indeterminate specimens, 91 and 135, differed from the initial results obtained by virus isolation and IFA. These discrepancies probably resulted from misinterpretation of the initial IFA. Unfortunately, we could not reisolate virus from these two specimens. Specimens 8 and 180 were positive by two different PCR techniques but negative by virus isolation, suggesting that inactive virus was present in these sera. Virus-antibody complexes or inactivation of viral infectivity during manipulation of the specimen may contribute to failure to isolate virus.

The RT-PCR for detection of flavivirus RNA is approximately 100 times more sensitive than an antigen capture assay (16). A major advantage of RT-PCR is that it can detect multiple flavivirus species in a single test, whereas antibody capture assays are virus specific. The technique we have developed has several advantages over other flavivirus PCR techniques and conventional methods. The entire method can be completed within 24 h, in contrast to the several days required to culture and perform immunologic identification. A single amplification with FGC amplimers is required to detect flavivirus RNA. A second test uses VSS amplimers to identify virus. Unlike amplimers that are located in virus structural genes, which are subject to more intense selection pressures, amplimers designed for this study hybridize with the highly conserved NS5 gene (11), which encodes a postulated viral RNA polymerase (14). Nucleotide sequence analysis of amplicons generated by the FGC amplimers can be used to identify previously unrecognized flaviviruses and to construct new VSS amplimers to facilitate virus identification in RT-PCR. The ELISA format for detection of amplicons offers increased sensitivity and is more reliable than the hybridization or nested-PCR methods.

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