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Rapid Detection and Typing of Dengue Viruses from Clinical Samples by Using Reverse Transcriptase-Polymerase Chain Reaction

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We report on the development and application of a rapid assay for detecting and typing dengue viruses. Oligonucleotide consensus primers were designed to anneal to any of the four dengue virus types and amplify a 511-bp product in a reverse transcriptase-polymerase chain reaction (PCR). First, we produced a cDNA copy of a portion of the viral genome in a reverse transcriptase reaction in the presence of primer D2 and then carried out a standard PCR (35 cycles of heat denaturation, annealing, and primer extension) with the addition of primer D1. The resulting double-stranded DNA product of the RT-PCR was typed by two methods: dot blot hybridization of the 511-bp amplified product to dengue virus type-specific probes or a second round of PCR amplification (nested PCR) with type-specific primers, yielding DNA products the unique sizes of which were diagnostic for each dengue virus serotype. The accumulated data demonstrated that dengue viruses can be accurately detected and typed from viremic human serum samples.

Dengue viruses (family *Flaviviridae*, genus *Flavivirus*) occur as four antigenically distinct serotypes. Infection with any of them generally leads to a mild, self-limiting febrile illness (dengue fever). However, a more severe form of the disease, involving vascular and hemostatic abnormalities (dengue hemorrhagic fever-dengue shock syndrome [DHF-DSS]), is responsible for a high mortality rate, primarily among children. Indeed, DHF-DSS is a leading cause of hospitalization and death among children in Southeast Asia, where more than one million cases were recorded between 1987 and 1989 (8). Over 30,000 deaths due to DHF-DSS in children have been reported worldwide since 1950 (7). Millions of human dengue infections occur each year, and over two billion people are at risk of infection.

These viruses are transmitted between humans primarily by *Aedes aegypti* and *Aedes albopictus* mosquitoes and are endemic in most areas in which the vectors occur (5). In dengue-endemic areas, dengue infections are recorded annually, with nonimmune children being the principal susceptible hosts. In addition, epidemics occur when a vector is introduced into previously dengue-free areas (8). The viruses replicate in cells of the macrophage-mononuclear cell lineage, and the severity of disease appears to be correlated with the ability of the viruses to infect these cells (7). Infection with one of the serotypes stimulates the production of neutralizing antibodies directed primarily against the envelope protein, conferring lifelong immunity to the serotype. The existence of waning neutralizing antibodies to one serotype may promote the enhancement of infection upon subsequent infection with another serotype. In this antibody-dependent enhancement model, severe disease is postulated to be the result of heterologous, nonneutralizing antibodies facilitating virus infection of mononuclear cells. Alternatively, it has been postulated that there exist viral and/or other host factors which may be primary risk factors in the production of more severe disease (5, 7).

Whether severe pathogenesis is caused by antibody-dependent enhancement or by some other mechanism, tools for rapid and specific laboratory diagnosis, including virus typing, are needed. Such diagnosis is necessary so that appropriate prevention, treatment, and control measures can be initiated and accurate epidemiologic data can be maintained. That one of the four dengue virus serotypes is responsible for a particular infection can be serologically deduced by traditional assays, including serum dilution-plaque reduction neutralization, complement fixation, or hemagglutination inhibition. The infecting serotype is inferred by measuring a fourfold or greater rise or fall in antibodies to the particular serotype. In practice, specific diagnosis often is not possible because of the extensive cross-reactivity of antibodies to flaviviruses, particularly between dengue viruses (10). Paired serum samples are needed; this requirement causes a delay in diagnosis, and results are rarely clear-cut.

Virus isolation from patient serum collected in the acute phase of illness or from arthropod vectors can be accomplished with cell cultures or mosquitoes. Currently, the most sensitive method of virus detection is inoculation of adult *A. aegypti* or *Toxorhynchites* species mosquitoes and fluorescent-antibody staining of mosquito brain tissues with dengue virus type-specific monoclonal antibodies (6). However, virus isolation takes from days to weeks and is not always successful because of small amounts of viable virus in the inocula, virus-antibody complexes, and inappropriate handling of samples. A clear need exists for an assay that can be performed rapidly and that is sufficiently sensitive and specific to be clinically and epidemiologically useful.

The development of the polymerase chain reaction (PCR) (11) has facilitated the appearance of a number of diagnostic assays for detecting viruses, including several for dengue viruses (3, 4). We attempted to develop a PCR-based assay

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TABLE 1. Oligonucleotide primers used to amplify and type dengue viruses

Primer	Sequence	Genome position[a]	Size, in bp, of amplified DNA product (primers)[b]
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	134–161	511
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616–644	511
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	568–586	482 (D1 and TS1)
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	232–252	119 (D1 and TS2)
TS3	5'-TAACATCATCATGAGACAGAGC-3'	400–421	290 (D1 and TS3)
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	506–527	392 (D1 and TS4)

[a] The genome positions of D1 and D2 are given according to the dengue type virus 2 published sequence (2), and the map positions of the dengue virus type-specific primers (TS1, TS2, TS3, and TS4) are given according to their respective published sequences (2, 9, 12, 15).

[b] The size of the amplified product obtained with each of the type-specific primers (TS1 to TS4) was determined from the priming position of primer D1 within each respective genome. The priming position for D1 in each dengue virus genome was as follows: type 1, 105; type 2, 134; type 3, 132; and type 4, 136.

that would detect and correctly type dengue viruses in serum samples from humans with dengue fever or in mosquitoes infected with dengue viruses. The data presented in this paper demonstrate that this assay is sufficiently rapid and accurate to allow reliable case diagnoses and to be useful in epidemiologic studies.

MATERIALS AND METHODS

Virus strains. Virus seeds were obtained from the collection at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control, Fort Collins, Colo. Prototype dengue virus strains (dengue virus types 1 [Hawaii], 2 [New Guinea C], 3 [H-87], and 4 [H-241]) were titrated in Vero cells by a standard plaque assay.

RNA extraction. Viral RNA was isolated by a modified form of the procedure described by Chomczynski and Sacchi (1). In brief, human serum samples or supernatant fluid from virus-infected cells was mixed with an equal volume of guanidine isothiocyanate lysis buffer: 8 M guanidine isothiocyanate, 50 mM sodium citrate, 100 mM 2-mercaptoethanol, 1% Sarkosyl, and 1 μg of yeast tRNA per ml. For RNA extraction from infected cells, we used a half concentration of lysis buffer. The solution was sequentially mixed with the following (all added in relation to the final volume of sample plus lysis buffer): a 1/10 volume of 2 M sodium acetate (pH 4), an equal volume of water-equilibrated phenol, and a 2/10 volume of chloroform. The mixture was centrifuged at 16,000 × g for 15 min, and the aqueous phase was removed and combined with an equal volume of isopropanol to precipitate the RNA. After centrifugation, the resulting RNA pellet was washed with 75% ethanol and dissolved in water. Control RNA used in sensitivity studies was quantitated by spectrophotometric analysis at 260 nm, and the concentration was calculated as follows: one unit of optical density = 40 μg/ml.

Selection and synthesis of oligonucleotide primers. Dengue virus consensus primers D1 and D2 were designed from available published sequences with the aid of a sequence analysis computer program (2, 9, 12, 15). The following criteria were used in designing the primers: (i) maximum homology to the four serotypes, (ii) high melting temperature, and (iii) nonhomology to other regions of dengue virus genomes. Primers D1 and D2 fulfilled these criteria and are shown in Table 1, along with their genome positions and product sizes when used in enzymatic amplifications. The type-specific primers shown in Table 1 (TS1, TS2, TS3, and TS4) were designed to anneal specifically to each of their respective genomes. Oligonucleotides were synthesized by use of an Applied Biosystems (Foster City, Calif.) synthesizer and standard phosphoramidite chemistry and purified on a NENSORB (DuPont NEN, Boston, Mass.) column.

Amplification of dengue virus RNA. Target viral RNA was converted to a DNA copy (cDNA) prior to enzymatic DNA amplification by use of reverse transcriptase (RT) and the dengue virus downstream consensus primer (D2), homologous to the genomic RNA of the four serotypes. Subsequent *Taq* polymerase amplification was performed on the resulting cDNA with the upstream dengue virus consensus primer (D1). All relevant aspects of the RT-PCR ($MgCl_2$, primers, RT, *Taq* polymerase, number of cycles, and annealing temperature) were initially optimized by use of quantitated purified dengue virus RNA to achieve a maximum level of sensitivity. Of particular interest was the observation that rav-2 recombinant RT (Amersham, Arlington Heights, Ill.) consistently yielded at least 10-fold more amplified product than did Moloney murine leukemia virus RT obtained from a number of manufacturers (data not shown). The amplification reaction was routinely performed by combining the reverse transcription of viral RNA and the subsequent *Taq* polymerase amplification in a single reaction vessel. This method consistently yielded an equal or a greater level of double-stranded DNA product as separate RT reactions and PCRs. Target RNA was amplified in 100-μl volumes containing the following components: 50 mM KCl, 10 mM Tris (pH 8.5), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each of the four deoxynucleotide triphosphates, 5 mM dithiothreitol, 50 pmol each of primers 1 and 2, 2.5 U of rav-2 RT, and 2.5 U of Amplitaq polymerase (Perkin Elmer, Norwalk, Conn.). The reactions were allowed to proceed in an Ericomp (San Diego, Calif.) thermocycler programmed to incubate for 1 h at 42°C and then to proceed with 35 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 1 min), and primer extension (72°C, 2 min).

Dengue virus typing by dot blot filter hybridization of the amplified product. A 10-μl portion of the RT-PCR mixture was denatured in 0.3 M NaOH at 65°C for 30 min and then immobilized on four separate Duralon membranes (Stratagene, La Jolla, Calif.) by use of a 96-well vacuum manifold. The DNA was fixed to the membranes by UV irradiation for 15 s with a UV Stratalinker 2400 (Stratagene) and then stored until tested by hybridization. Oligonucleotides for type-specific hybridization were 3' end labeled with digoxigenin (DIG)-UTP (Boehringer Mannheim, Indianapolis, Ind.) by combining the oligonucleotide (10 μM) with DIG-UTP (100 μM) and 15 U of terminal deoxynucleotidyl transferase (Life Technologies, Inc., Gaithersburg, Md.) in the buffer supplied by the manufacturer and incubating the mixture for 1 h at 37°C. Terminal biotin labeling was less efficient in incor-

poration and hence less sensitive in hybridization reactions (data not shown). Each membrane was hybridized with one of the four dengue virus type-specific oligonucleotides in hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% blocking reagent [Boehringer Mannheim], 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate [SDS]) containing 100 ng of labeled oligonucleotide per ml. Hybridization reactions were performed for 16 to 20 h at 42°C. Membranes were washed twice for 20 min each time in 2× SSC–0.2% SDS and twice in 0.2× SSC–0.2% SDS. The bound probes were detected by incubation with alkaline phosphatase-labeled antibody to DIG and Lumi-Phos (Boehringer Mannheim) in accordance with the manufacturer's protocol. Visualization of bound probes was accomplished by exposing Kodak XAR film to the membranes for 3 to 15 min.

Dengue virus typing by second-round amplification with type-specific primers (nested PCR). A second amplification reaction was initiated with 10 μl of diluted material (1:100 in sterile distilled water) from the initial amplification reaction. The reaction mixture contained all the components described for the initial amplification reaction with the following exceptions: primer D2 was replaced with the dengue virus type-specific primers TS1, TS2, TS3, and TS4, and dithiothreitol and RT were eliminated. The samples were subjected to 20 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 1 min), and primer extension (72°C, 2 min). A 15-μl portion of the reaction product was electrophoresed on a 4% composite agarose gel (NuSieve 3:1; FMC BioProducts, Rockland, Maine) in 0.4 M Tris–0.05 M sodium acetate–0.01 M EDTA buffer. Because of the position of priming with each of the dengue virus type-specific primers (Table 1), the size of the resulting DNA band was characteristic for each dengue virus type.

Infection of mosquitoes and verification of infection. Insectary-maintained *A. aegypti* mosquitoes were infected by intrathoracic inoculation with undiluted human serum that had been shown in other assays to contain dengue type 2 virus. Mosquitoes were incubated at 30°C and 60 to 75% relative humidity. Pools consisting of five mosquitoes were removed daily, beginning 2 days after inoculation, and frozen for RT-PCR analysis. At 10 days after inoculation, dengue type 2 virus infection was verified by testing of a random sample of these mosquitoes by a direct immunofluorescence assay (DFA) of head-squash material with a conjugate prepared from high-titer human serum.

Detection of dengue viruses in mosquitoes by RNA capture prior to amplification. Amplification of RNA isolated from dengue virus-infected mosquitoes initially yielded negative results. Because the mosquitoes were known to be infected, as verified by the DFA, we postulated that an inhibitory component was present in the isolated RNA. To resolve this problem, we used a dengue virus RNA capture step prior to the RT-PCR. The D2 consensus primer was 3′ end labeled with biotin-14-dATP by use of terminal deoxynucleotidyl transferase as described above for DIG-UTP. The labeled oligonucleotide was immobilized on strepavidin-coated magnetic beads (Dynabeads; Dynal, Great Neck, N.Y.) by combining 100 μl of the bead suspension (binding capacity, 200 pmol of labeled oligonucleotide) with 200 pmol of the biotinylated oligonucleotide. After 10 min of incubation at room temperature, the beads were washed four times in bead wash buffer (0.2 M Tris [pH 7.5], 0.2 M NaCl) by use of a magnetic particle concentrator (Dynal). The RNA samples were mixed with 2 pmol of the oligonucleotide-bead complex in bead wash buffer, and the mixture was heated to 70°C for 5 min and slowly cooled to 42°C for 5 min to allow the RNA to anneal. The beads were washed twice with bead wash buffer and mixed with 10 μl of water, and the mixture was heated to 70°C to elute the RNA.

Detection and typing of dengue viruses from viremic human serum. Human serum samples were obtained from patients with clinically characterized and virologically confirmed dengue infection and were tested by the RT-PCR assay. These samples had previously been shown to contain dengue viruses by isolation in C6/36 *A. albopictus* cell cultures or by intrathoracic inoculation of mosquitoes and the DFA as described above. Dengue virus serotypes were determined by an indirect immunofluorescence assay (IFA) with dengue virus type-specific monoclonal antibodies (6). We tested samples obtained from persons with either classical dengue fever or DHF-DSS during several epidemics in Southeast Asia and Puerto Rico. The samples from Southeast Asia had been stored frozen at −70°C with occasional to multiple thawings over a 10- to 15-year period. The samples from Puerto Rico were from more recent cases (less than 1 year before our test) of dengue fever in Puerto Rico.

RESULTS

Specificity of the RT-PCR. RNA isolated from each of the four dengue virus reference strains was subjected to the RT-PCR assay. The correctly sized DNA product (511 bp) was obtained for each of the dengue viruses after amplification with consensus primers D1 and D2 (Fig. 1A). Each DNA product was correctly typed when assayed by either dot blot hybridization with the type-specific probes (Fig. 2) or a second round of amplification with the type-specific primers (Fig. 1B). The specificity was also verified by performing the nested PCR assay on 33 unique dengue virus isolates representative of most of the defined geographic topotypes (Table 2) (14). In addition, the RT-PCR assay was tested for specificity by attempting amplification reactions with purified RNA from five dengue virus-related flaviviruses (West Nile, Japanese encephalitis, St. Louis encephalitis, yellow fever, and Edge Hill). Viruses of the Japanese encephalitis complex (Japanese encephalitis, West Nile, and St. Louis encephalitis) were amplified in the first-round amplification reaction with consensus primers D1 and D2 to generate DNA products of 511 bp for West Nile and St. Louis encephalitis virus and 550 bp for Japanese encephalitis virus, in agreement with the published sequences (Fig. 1A). Yellow fever and Edge Hill viruses were not amplified with the consensus primers (Fig. 1A). A faint DNA band of approximately 150 bp was observed for Edge Hill but was likely due to nonspecific amplification, since products of this size were occasionally observed in other PCRs. The DNA product obtained after first-round amplification of Japanese encephalitis, West Nile, and St. Louis encephalitis viruses did not react with the dengue virus type-specific oligonucleotide probes in dot blot hybridization experiments (Fig. 2). In addition, no DNA products were obtained when these amplified DNAs were used as targets in the nested PCR amplification with the dengue virus type-specific oligonucleotide primers (Fig. 1B). First-round amplification of related flaviviruses did not alter the specificity of the assay, since the amplified DNA products generated did not react with the dengue virus type-specific probes or primers.

Sensitivity of the RT-PCR. For postamplification detection and typing, the sensitivities of two methods were initially compared. In the first protocol, the amplified product was immobilized in quadruplicate on four nylon membranes and

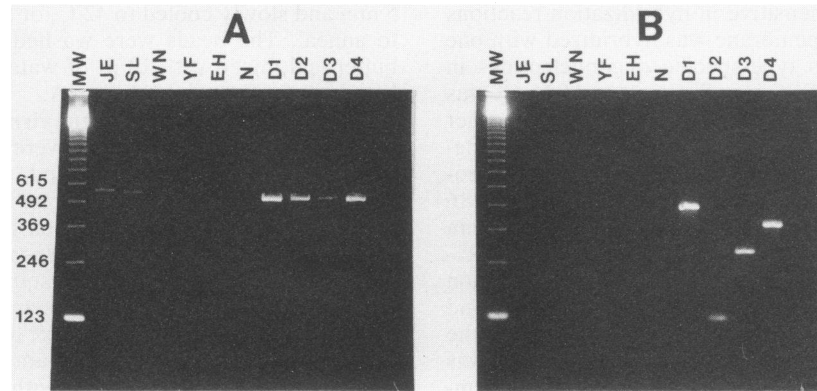

FIG. 1. Agarose gel analysis of the DNA product from RT-PCR of RNA samples isolated from dengue viruses and related flaviviruses. (A) After amplification with consensus primers D1 and D2. (B) After second-round amplification with type-specific primers TS1, TS2, TS3, and TS4. Molecular weight (MW) markers are shown on the left; DNA sizes are given in base pairs. Lanes show amplification of RNA from the following viruses: JE, Japanese encephalitis; SL, St. Louis encephalitis; WN, West Nile; YF, yellow fever; EH, Edge Hill; N, western equine encephalitis (negative control); D1, dengue type 1; D2, dengue type 2; D3, dengue type 3; and D4, dengue type 4.

hybridized with each of the four dengue virus type-specific probes labeled with DIG-UTP; initially ^{32}P-labeled probes were used, but these were later replaced with DIG-UTP probes of equal sensitivity. Using purified RNA as a standard, we consistently attained a sensitivity level of between 1,000 and 100,000 viral genome equivalents (Fig. 3). In the second protocol (nested), a small portion of the amplified product was subjected to an additional 20 cycles of amplification with the D1 consensus primer in combination with the four type-specific primers. Figure 4 displays the results of applying this nested PCR method with the same samples as those used in the hybridization analysis. Sensitivity attainable by this nested amplification method was greater; 100 viral genome equivalents were detected. The two protocols were also compared by testing 20 human viremic serum samples. The nested approach proved more sensitive by

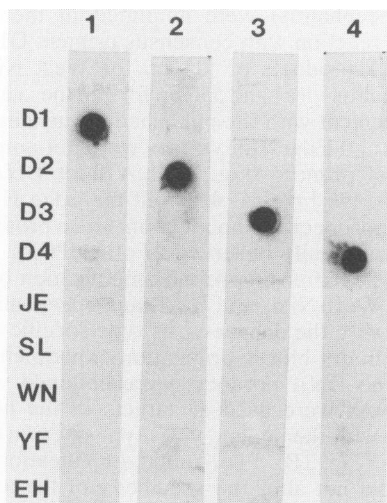

FIG. 2. Dot blot hybridization of the DNA product from RT-PCR of RNA samples isolated from dengue viruses and related flaviviruses. Abbreviations on the left are as defined in the legend to Fig. 1. Membranes were hybridized with probes specific for dengue viruses of types 1 (lane 1), 2 (lane 2), 3 (lane 3), and 4 (lane 4); all probes were labeled with DIG-UTP and detected with Lumi-Phos.

TABLE 2. Comparison of RT-PCR and serological typing of geographically and temporally distinct dengue viruses

Strain	Location	Yr isolated	Serotype determined by both methods
16007	Thailand	1964	1
1041	Indonesia	1976	1
30893	Malaysia	1981	1
162.AP2	Philippines	1984	1
11651	Puerto Rico	1986	1
GML100063	Guatemala	1989	1
INS353117	Columbia[a]	1990	1
INS353178	Colombia[a]	1990	1
88970	Venezuela	1990	1
TC16681/64	Thailand	1964	2
489	Puerto Rico	1977	2
285	Indonesia	1978	2
042.AP4/2207	Philippines	1983	2
D85-044	Thailand	1985	2
1715	Dominican Republic	1986	2
88967	Venezuela	1990	2
CH53489D731	Thailand	1973	3
1300	Malaysia	1974	3
1340	Puerto Rico	1977	3
1178	Indonesia	1977	3
1280	Indonesia	1978	3
D80273	Thailand	1980	3
26237	Malaysia	1980	3
168.AP2	Philippines	1983	3
D84315	Thailand	1984	3
1594	Sri Lanka	1985	3
D86013	Thailand	1986	3
1053	Indonesia	1976	4
1132	Indonesia	1977	4
1152	Mexico	1985	4
072-090-85	Tahiti	1985	4
JQ1190	Venezuela	1990	4
JQ1090	Venezuela	1990	4

[a] South America.

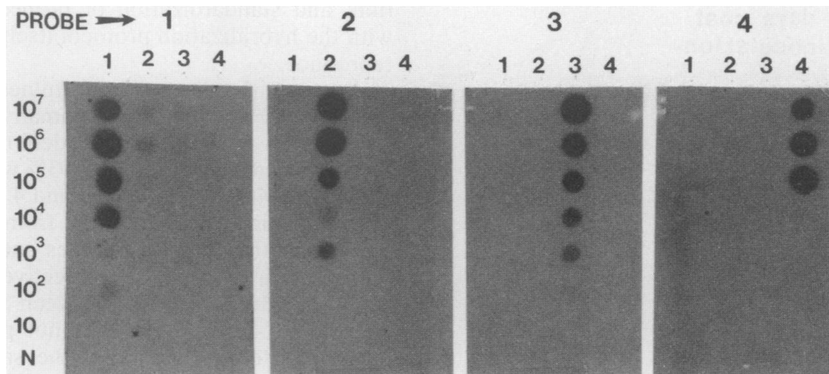

FIG. 3. Dot blot hybridization of the DNA product obtained after one round of RT-PCR amplification of quantitated dengue virus RNAs with consensus primers D1 and D2. The membranes contained identical samples in the same configurations. Lanes 1 to 4 show dengue viruses of types 1 to 4, respectively. Membranes were hybridized with probes specific for dengue viruses of types 1 (panel 1), 2 (panel 2), 3 (panel 3), and 4 (panel 4). All probes were labeled with DIG-UTP and detected with Lumi-Phos. The number of initial RNA molecules assayed is shown on the left.

correctly identifying five viremic serum samples that were found negative by the dot blot hybridization method (Table 3). The nested PCR method was used exclusively throughout the remainder of this study.

Detection and typing of dengue type 2 virus in infected mosquitoes. Figure 5 displays the results of testing dengue type 2 virus-infected *A. aegypti* mosquitoes. As previously stated, the RNA isolated from these infected mosquitoes was originally found negative for dengue type 2 virus by the RT-PCR assay at all time points. However, when the RNA samples were captured on magnetic beads prior to RT-PCR amplification, they were amplified with consensus primers D1 and D2 and correctly typed by the nested PCR method. Samples were found positive for dengue type 2 virus starting at the earliest time point (day 2) and were positive throughout the remainder of the time points assayed (Fig. 5).

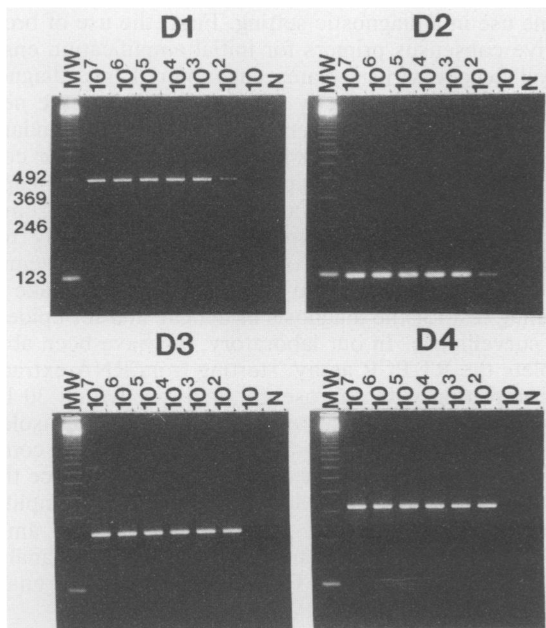

FIG. 4. Agarose gel analysis of the product from RT-PCR followed by second-round nested PCR of quantitated RNA samples. The number of initial RNA molecules assayed is indicated above each lane. RNAs were from dengue virus types 1 (D1), 2 (D2), 3 (D3), and 4 (D4). Molecular weight (MW) markers are shown on the left; DNA sizes are given in base pairs. N, tRNA negative control.

Detection and typing of dengue viruses in clinical samples. Ninety-three human viremic serum samples were tested by the RT-PCR assay. Table 4 summarizes the results comparing identification by the RT-PCR assay with identification by virus isolation in mosquitoes or cell cultures and subsequent typing by the IFA with type-specific monoclonal antibodies. In all but four instances, dengue viruses were correctly detected and typed by the RT-PCR assay, compared with virus isolation. One dengue type 1 virus sample and three samples containing dengue type 2 virus were not found positive by the RT-PCR method. Ten additional samples from Southeast Asia (data not included in Table 4) were originally dengue virus positive when isolated but negative when tested by the RT-PCR assay. Since the storage history of these samples may have reduced or eliminated virus titers, these samples were reinoculated into mosquitoes and assayed for viruses. Seven samples were negative, and three samples yielded questionable results (one or two fluorescent cells were observed in the DFA). These three samples were subsequently passed in C6/36 cells. After a suitable period of incubation, two tested positive for dengue type 2 virus and one tested positive for dengue type 1 virus.

DISCUSSION

In this report, we describe the development of a rapid and specific assay for detecting and typing dengue viruses. The

TABLE 3. Comparison of dot blot hybridization and nested PCR for detection and typing of dengue viruses in human serum samples

Method	No. of viremic serum samples of the following dengue virus type:			
	1	2	3	4
Virus isolation	2	6	7	5
RT-PCR and dot blot hybridization	1	6	6	2
RT-PCR and nested PCR	2	6	7	5

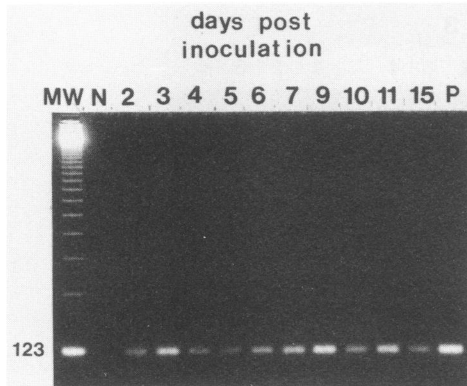

FIG. 5. Agarose gel analysis of the product from RT-PCR amplification of RNAs isolated from infected mosquitoes and captured on magnetic beads. Lanes: MW, molecular size markers (in base pairs); N, uninfected mosquitoes; 2 to 15, RNAs isolated from mosquitoes on the day postinfection shown above each lane; P, dengue type 2 virus positive control.

method relies on a combination of two steps: generation of a cDNA copy of the RNA genome by RT and subsequent *Taq* polymerase-mediated amplification of this cDNA. The two reactions are combined in a single reaction vessel, significantly reducing the assay time, lowering the risk of contamination problems, and facilitating the handling of large numbers of specimens. The use of primers homologous to conserved dengue virus RNA sequences ensures that all strains of dengue virus will be amplified in the first-round amplification reaction. The fact that viruses of the Japanese encephalitis serogroup were also amplified by the consensus primers confirms the broad reactivity of these primers. The use of type-specific primers for viruses of the Japanese encephalitis complex in similar two-round amplification assays would generate similar detection and typing tests for these viruses.

The specificity of our assay relies on the ability of the type-specific primers to recognize RNA sequences unique to each dengue virus type. This specificity was confirmed by testing 33 geographically unique virus isolates (characterized by RNA fingerprinting techniques [14]) as well as 93 previously identified viremic serum samples (Table 4). No cross-reactivity was detected between the type-specific primers and heterologous dengue virus types; only a single amplified product was obtained in each typing reaction. Typing of dengue viruses by the nested PCR method with a mixture of type-specific primers is superior to hybridization both in sensitivity and in ease of manipulation. Correct typing requires only electrophoresis of the amplified product on an agarose gel, whereas the hybridization method introduces a filter hybridization protocol requiring the labeling, purification, and standardization of probes. These probes, along with the hybridization protocol itself, are usually difficult to reproduce.

TABLE 4. Comparison of the RT-PCR assay and virus isolation for the identification and typing of dengue viruses from human serum

Method	No. of serum samples of the following dengue virus type:			
	1	2	3	4
RT-PCR	16	39	17	17
Virus isolation	17	42	17	17

The potential diagnostic usefulness of our assay is demonstrated by the analysis of human serum samples containing dengue virus. The assay demonstrates sensitivities of 94% with dengue type 1 virus, 93% with dengue type 2 virus, and 100% with dengue type 3 and 4 viruses, compared with virus isolation. The samples from Southeast Asia were originally titrated in mosquitoes and possessed virus titers ranging from 10^3 to 10^8 50% infective doses per ml of serum. A meaningful correlation between the original virus titers and the RT-PCR results was not possible because of the uncertain storage history of the samples. However, it is noteworthy that several of the samples which tested positive in the RT-PCR originally possessed virus titers as low as 10^3 50% infective doses per ml of serum. The four RT-PCR-negative samples that were found positive by virus isolation (false-negatives) may have been the result of the presence of fewer than 100 complete virus particles, the approximate sensitivity limit of the test. Another possibility is that these serum samples contained an inhibitor of the enzymatic amplification that copurified with the template RNA. A bead capture step could be used to eliminate this problem, as was done with RNA isolated from dengue virus-infected mosquitoes; however, insufficient sample volumes prevented execution of the bead capture step on these samples.

Although false-positive PCR results have been reported (13) in PCR-based assays, this problem was circumvented by routinely exercising numerous precautionary measures (physical separation of pre- and post-PCR manipulations, UV irradiation of reaction mixtures, and the use of positive-displacement pipettes) and including several samples without DNA to carefully monitor each assay.

Other reports in which PCR was used to identify dengue viruses have appeared (3, 4). Our assay possesses several differences which we believe make it more amenable to routine use in a diagnostic setting. First, the use of broadly reactive consensus primers for initial amplification ensures that all dengue virus isolates encountered in a diagnostic laboratory will be correctly identified. Second, the nested PCR method is both more sensitive and easier to standardize than either hybridization or restriction digestion for confirmation of the amplification product. Finally, RNA capture on magnetic beads prior to amplification allows circumvention of potential PCR inhibitors, which are likely to be encountered in the analysis of a large number of specimens.

The accuracy and speed of the RT-PCR assay make it an appealing test for the diagnosis of dengue and for epidemiologic surveillance. In our laboratory, we have been able to complete the RT-PCR assay, starting from RNA extraction and completing with agarose gel analysis, within 30 h. In diagnostic laboratories currently using traditional isolation or serological methods, this assay could be used to complement existing techniques or in some cases to replace them. In addition, the basic methodology of directly amplifying RNA into double-stranded DNA can be used to amplify larger regions of the genome for rapid sequence analysis, which is potentially useful for both epidemiologic analysis and evolutionary studies.

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REFERENCES

1. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
2. **Duebel, V., R. M. Kinney, and D. W. Trent.** 1986. Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue type 2 virus, Jamaica genotype. Virology **155:**365–377.
3. **Duebel, V., M. Laille, J. Hugnot, E. Chungue, J. Guesdon, M. T. Drouet, S. Bassot, and D. Chevrier.** 1990. Identification of dengue sequences by genomic amplification: rapid diagnosis of dengue virus serotypes in peripheral blood. J. Virol. Methods **30:**41–54.
4. **Eldadah, Z. A., D. M. Asher, M. S. Godec, K. L. Pomeroy, L. G. Goldfarb, S. M. Feinstone, H. Levitan, C. J. Gibbs, and D. C. Gajdusek.** 1991. Detection of flaviviruses by reverse-transcriptase polymerase chain reaction. J. Med. Virol. **33:**260–267.
5. **Gubler, D. J.** 1988. Dengue, p. 223–260. *In* T. P. Monath (ed.), The arboviruses: epidemiology and ecology, vol. 2. CRC Press, Boca Raton, Fla.
6. **Gubler, D. J., G. Kuno, G. E. Sather, M. Valez, and A. Oliver.** 1984. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. Am. J. Trop. Med. Hyg. **33:**158–165.
7. **Halstead, S. B.** 1988. Pathogenesis of dengue: challenges to molecular biology. Science **239:**476–481.
8. **Henchal, E. A., and J. R. Putnak.** 1990. The dengue viruses. Clin. Microbiol. Rev. **3:**376–396.
9. **Mason, P. W., P. C. McAda, T. W. Mason, and M. J. Fournier.** 1987. Sequence of the dengue-1 virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. Virology **161:**262–267.
10. **Monath, T. P.** 1990. Flaviviruses, p. 763–814. *In* B. N. Fields, D. N. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology, 2nd ed. Raven Press, Ltd., New York.
11. **Mullis, K. B., and F. A. Faloona.** 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol. **155:**335–350.
12. **Osatomi, K., and H. Sumiyoshi.** 1990. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology **176:**643–647.
13. **Sarkar, G., and S. S. Sommer.** 1990. Shedding light on PCR contamination. Nature (London) **343:**27.
14. **Trent, D. W., C. L. Manske, G. E. Fox, M. C. Chu, S. C. Kliks, and T. P. Monath.** 1990. The molecular epidemiology of dengue viruses; genetic variation and microevolution. Appl. Virol. Res. **2:**293–315.
15. **Zhao, B., E. Mackow, A. Buckler-White, L. Markoff, R. M. Chanock, C. Lai, and Y. Makino.** 1986. Cloning full length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. Virology **155:**77–88.