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Development and Evaluation of Serotype- and Group-Specific Fluorogenic Reverse Transcriptase PCR (TaqMan) Assays for Dengue Virus

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Five fluorogenic probe hydrolysis (TaqMan) reverse transcriptase PCR (RT-PCR) assays were developed for serotypes 1 to 4 and group-specific detection of dengue virus. Serotype- and group-specific oligonucleotide primers and fluorogenic probes were designed against conserved regions of the dengue virus genome. The RT-PCR assay is a rapid single-tube method consisting of a 30-min RT step linked to a 45-cycle PCR at 95 and 60°C that generates a fluorogenic signal in positive samples. Assays were initially evaluated against cell culture-derived dengue stock viruses and then with 67 dengue viremic human sera received from Peru, Indonesia, and Taiwan. The TaqMan assays were compared to virus isolation using C6/36 cells followed by an immunofluorescence assay using serotype-specific monoclonal antibodies. Viral titers in sera were determined by plaque assay in Vero cells. The serotype-specific TaqMan RT-PCR assay detected 62 of 67 confirmed dengue virus-positive samples, for a sensitivity of 92.5%, while the group-specific assay detected 66 of 67 confirmed dengue virus-positive samples, for a sensitivity of 98.5%. The TaqMan RT-PCR assays have a specificity of 100% based on the serotype concordance of all assays compared to cell culture isolation and negative results obtained when 21 normal human sera and plasma samples were tested. Our results demonstrate that the dengue virus TaqMan RT-PCR assays may be utilized as rapid, sensitive, and specific screening and serotyping tools for epidemiological studies of dengue virus infections.

Human cases of febrile illness resembling dengue fever (DF) have been recognized as a clinical entity for more than 200 years, and the mosquito *Aedes aegypti* has been recognized as the principal vector of dengue virus for at least 70 years (7, 9). Dengue illness is caused by any of four serologically related single-stranded (+)-sense, enveloped RNA viruses of the family *Flaviviridae*, and these viruses are designated Den-1, Den-2, Den-3, and Den-4 (12, 19). Dengue infection is transmitted by the bite of infected mosquitoes throughout tropical and subtropical regions of the world (7, 9).

Dengue virus has reemerged at an alarming rate during the past decade and has become the most important arbovirus disease in terms of morbidity, mortality, and economic cost, with an estimated 100 million dengue virus infections occurring annually (7, 9). Each of the four dengue virus serotypes can produce a spectrum of disease severity ranging from a mild to moderate febrile illness to a severe and fatal hemorrhagic disease (6, 19). The febrile syndrome, or DF, is the most common, and occurs more frequently among older children and adults as a self-limiting acute illness characterized by a sudden onset of nonspecific indicators, including headache, myalgia, arthralgia, and rashes (6). DF can range in severity from a minor infirmity to a temporarily incapacitating syndrome with residual fatigue and depression. At the severe end of the disease spectrum is dengue hemorrhagic fever, which primarily affects children under the age of 10 years (9). The clinical manifestations of dengue hemorrhagic fever include plasma leakage, a bleeding tendency, and liver involvement, which, if uncompensated, can lead to potentially life-threatening syndromes including disseminated intravascular coagulation and dengue shock syndrome (9). There are no vaccines for dengue, and treatment is limited to supportive therapies (6).

The current methods used by most laboratories for the diagnosis of acute dengue virus infections are the detection of virus or antibody in blood samples. A definitive serological diagnosis requires testing of acute- and convalescent-phase samples, usually collected at least 7 days apart, to demonstrate a fourfold or greater increase in antibody titer. However, the specific serotype of dengue virus responsible for the infection cannot be determined reliably by this method, particularly for

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TABLE 1. Probe and primer sequences for the dengue serotype specific assays

Primer or probe	Sequence (5′–3′)	Genomic region	GenBank reference no.
DEN-1-forward	GAC ACC ACA CCC TTT GGA CAA	NS5 (8586–8606)	M87512
DEN-1-reverse	CAC CTG GCT GTC ACC TCC AT	NS5 (8692–8673)	
DEN-1-probe	AGA GGG TGT TTA AAG AGA AAG TTG ACA CGC G	NS5 (8608–8638)	
DEN-2-forward	CAT GGC CCT KGT GGC G	C (237–251)	M29095
DEN-2-reverse	CCC CAT CTY TTC AGT ATC CCT G	C (305–284)	
DEN-2-probe	TCC TTC GTT TCC TAA CAA TCC	C (254–274)	
DEN-3-forward	GGG AAA ACC GTC TAT CAA TA	C (118–221)	M93130
DEN-3-reverse	CGC CAT AAC CAA TTT CAT TGG	C (241–221)	
DEN-3-probe	CAC AGT TGG CGA AGA GAT TCT CAA GAG GA	C (174–202)	
DEN-4-forward	TGA AGA GAT TCT CAA CCG GAC	C (187–207)	M14931
DEN-4-reverse	AAT CCC TGC TGT TGG TGG G	C (293–275)	
DEN-4-probe	TCA TCA CGT TTT TGC GAG TCC TTT CCA	C (247–273)	

secondary dengue virus infections. Also, virus isolation by cell culture inoculation or less frequently direct intrathoracic mosquito inoculation of acute-phase sera followed by serotype identification with immunofluorescence antibody testing using monoclonal antibodies usually takes at least a week. An advantage of virus isolation over serological diagnosis is that the serotype of the infecting virus can be reliably identified, and the isolate is available for other studies such as genotyping. Advances in molecular biology and especially nucleotide sequencing have enabled comparisons to be made of sequences representing numerous flaviviruses, including all four dengue virus serotypes, as well as sequences from dengue virus isolates from diverse geographic regions (4, 12, 19). Based on this type of information, a number of traditional reverse transcriptase PCR (RT-PCR) assays to identify dengue virus RNA have been reported over the past decade (8). RT-PCR assays can provide a same-day serotype-specific laboratory diagnosis of dengue infection and have a sensitivity similar to that of viral isolation in cell culture (17). Recently, fully automated RT-PCR assays that utilize fluorogenic probe hydrolysis (TaqMan) technology to detect dengue virus RNA also have been developed. Advantages of TaqMan technology over standard RT-PCR methods are that the assay is a single-tube method that greatly reduces the risk of contamination and a fluorogenic signal is produced in positive samples that are monitored in real time, enabling quantitative measurements to be made.

The goal of our study was to develop a set of fluorogenic probe hydrolysis-based RT-PCR (TaqMan) assays to screen and type dengue viruses. As a screening tool, a group-specific assay was developed that would detect dengue virus RNA from all four serotypes, and a set of four serotype-specific assays was established for the definitive identification of dengue viruses.

MATERIALS AND METHODS

Sequence selection and alignment. Dengue nucleotide sequences were retrieved from GenBank and aligned using CLUSTAL X (version 1.8 [1]) and BioEdit (www.mbio.ncsu.edu/RnaseP/info/programs/BIOEDIT/bioedit.html) sequence alignment software. Separate alignments were prepared for each dengue virus serotype assay using Den-1 West Pac (U88535) and S275/90 (M87512); Den-2 New Guinea C (M29095), ThNH-p14/93 (AF022439), DEN2JAM (M20558) and China isolate 04 (AF119661); Den-3 H87 (M93130), CH53489 (AF008555), Z026 MALAY94-3 (AB010990), and JM086 MALAY93-3 (AB010982); and Den-4 814669 (M14931) and H241-P (S66064). Assay target regions were first identified by visual inspection of sequence alignments, and then exact primer and probe sequences were selected by using a primer design software (NetPrimer; Premier Biosoft International, Palo Alto, Calif.) that enabled prediction of oligonucleotide melting temperatures; thermodynamic energies;

G+C content; and potentials for dimerization, cross-linking, and secondary structure. A Den-1 target sequence was identified within the nonstructural protein 5 (NS5) genomic region, while Den-2, -3, and -4 target sequences were identified in the capsid (C) region (Table 1). Primer and probe design characteristics and restrictions recommended by PE Biosystems (Foster City, Calif.) were utilized.

While considering design options for the dengue virus group assay, a relatively homologous region was noticed in a sequence alignment of the 3′ untranslated region (UTR) (Fig. 1). This region was considered to be a potential target except for a few mismatched bases among the aligned serotypes and strains. To overcome these mismatches, a multiplex format was utilized that was based on a universal primer set and two probes with slightly different sequences (Table 2). The universal primer set was designed with a single degeneracy in the forward primer to account for a mismatch encountered in Den-3 (strain H87) and two degeneracies in the reverse primer sequence to account for mismatches encountered among Den-4 sequences. Within the probe binding area, there is a single base mismatch that would not permit the use of a single universal probe. To overcome the mismatch, two probes of equal length (27 bp) differing by a single base substitution were used. The probe with specificity for Den-1 and Den-3 was labeled with a 5′ 6-carboxyfluorescein (6-FAM) fluorophore. The second probe, specific for Den-2 and Den-4, was labeled with a 5′ 2,7-dimethoxy-4,5-dichloro-6-carboxyfluoroscein (JOE) fluorophore. Both probes were labeled with a 3′ 6-carboxytetramethyl-rhodamine (TAMRA) quencher molecule. By mixing the two probes in equimolar ratios the assay became a multiplex assay with two fluorophores producing emission spectra at separate wavelengths (6-FAM at 525 nm, JOE at 548 nm). The multiplex assay specifically targets the 3′ noncoding region of the dengue virus genome and is designed to detect all four members of the dengue virus group.

Perkin-Elmer EZ-RT-PCR reagent kits (PE Biosystems) were used to prepare master-mix recipes according to the manufacturer's guidelines for individual component concentrations. Final PCR conditions for a 50-μl reaction volume using 5 μl of template were as follows: manganese acetate, 3 mM; KCl, 115 mM; primers, 0.3 μM; probe, 0.15 μM; dATP, dCTP, and dGTP, 0.1 mM (each); dUTP, 0.2 mM; recombinant Tth DNA polymerase, 0.1 U/μl; and bovine serum albumin, 0.1 μg/μl in a 5× buffer (250 mM Bicine, 575 mM potassium acetate, 0.05 mM EDTA). The RT-PCR assay consisted of a 30-min RT step at 60°C linked to a 45-cycle PCR (95°C for 15 s and 60°C for 60 s).

Development optimization. The assay was optimized against RNA extracted from a panel of stock viruses maintained at the Naval Medical Research Center: Den-1, Hawaii; Den-2, New Guinea C; Den-3, H-87 (Philippines); and Den-4, Philippines. RNA was extracted from 140 μl of stock virus using the QIAamp viral RNA mini kit (Qiagen, Valencia, Calif.) following the manufacturer's instructions and stored at −70°C.

Human sera. A total of 67 dengue virus-positive human serum samples were received from existing collections at the U.S. Naval Medical Research Unit 2, Jakarta, Indonesia, the U.S. Naval Medical Research Center Detachment, Lima, Peru, and the National Taiwan University, Taipei, and were tested anonymously for evaluation of the TaqMan assays. All samples were collected from DF patients, including 31 from Indonesia, 28 from Peru, and 8 from Taiwan. Among these 67 samples, 30 were positive for Den-1, 10 were positive for Den-2, 23 were positive for Den-3, and 4 were positive for Den-4. A total of 21 normal human serum or plasma samples were also collected from healthy donors living in the United States and used as negative controls. Serum samples were thawed and tested simultaneously in C6/36 cells and by the TaqMan assays in a randomized,
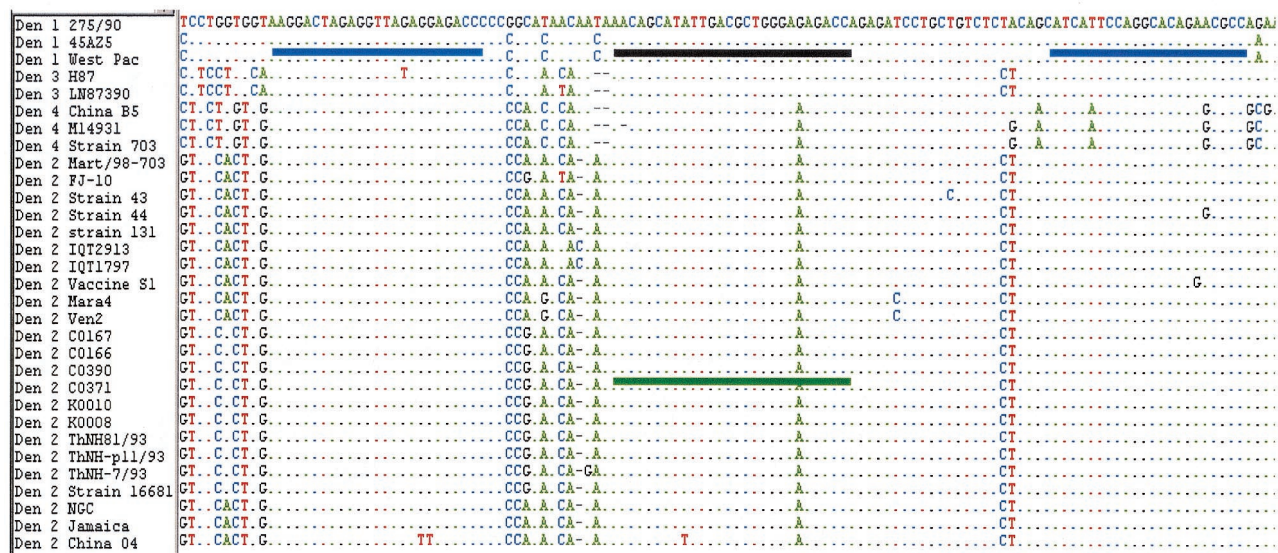
FIG. 1. Alignment of dengue virus 3′ UTR, target area of the dengue virus group assay. Primer positions are indicated in blue, and 6-FAM- and JOE-labeled probe positions are indicated in black and green, respectively.

blinded fashion. Nucleic acid was isolated from human serum samples using previously described methods (2). Typically, this procedure utilized 100 μl of plasma or serum as the starting input material. Final nucleic acid extracts were obtained in a total volume of 50 μl.

Viral isolation and immunofluorescence assay. The positive and negative samples were diluted 1:10 in culture medium and inoculated onto the *Aedes albopictus* mosquito cell line C6/36 for confirmation of viral isolation as described previously (18). The cell cultures were incubated for 7 days at 28°C after a 1-h absorption period at 28°C. Cells were harvested after 7 days for staining in an indirect immunofluorescence assay as described previously (22). Cells were reacted with either dengue virus group-specific or dengue virus serotype-specific monoclonal antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse antibody was used as the detector.

Plaque assay in Vero cells. The titers of dengue virus in human serum samples were determined by inoculating samples at 1:5, 1:10, and 1:100 dilutions in culture medium onto Vero cell monolayers and assaying 7 days later (5). Cell monolayers were overlaid with agar and neutral red to determine the number of PFU per milliliter.

Dengue viruses and control flaviviruses. All four dengue virus serotypes were prepared in Vero cells as virus seed stocks, and virus titers were determined by the plaque assay. These viruses were used to spike normal human serum to determine the detection threshold of the TaqMan assay. Two other flaviviruses, yellow fever virus (YF-17D, vaccine strain) and Japanese encephalitis virus (JE-SA14-14-2, live attenuated vaccine strain), were also prepared in Vero cells and used as control viruses for flavivirus cross-reactivity studies with the dengue virus serotype-specific and dengue virus group-specific TaqMan assays.

Log dilutions of cell culture-derived stock viruses were prepared for evaluation by diluting extracted viral RNA in 1× Tris-EDTA. Standard curves were generated with each assay by running each log standard in duplicate.

Assay evaluation. Using the Perkin-Elmer 7700 instrument (PE Biosystems), specific PCR products were directly detected by monitoring the increase in fluorescence of a dye-labeled oligonucleotide probe. Assay specificity was evaluated by testing serotype-specific probe and primer sets against specificity panels that included Den-1, -2, -3, -4, JE, and YF viruses.

Each assay was initially optimized using RNA extracted from stock viruses

followed by sensitivity measurements using clinical samples. The results of the TaqMan assays were compared to results of samples confirmed positive by C6/36 viral isolation. The detection threshold for each TaqMan assay was also determined using log dilutions of RNA extracted from dengue stock viruses of known plaque titer (PFU per milliliter) which were run in duplicate as a standard curve for each assay.

Each standard curve yielded information on the assay performance such as the range of linearity and the correlation coefficient. The determination of the detection threshold was based on the lowest level at which viral RNA was consistently detected and remained within the range of linearity of a standard curve having a minimum correlation coefficient of 0.98. The value in PFU per milliliter for each RNA standard was based on the original titer of stock virus as determined by plaque assay. The lowest level of viral RNA detected by each assay was adjusted from PFU per milliliter to PFU per volume tested by taking into account the original viral titer, the volume used for the RNA extraction procedure (140 μl), and the volume of extracted RNA used as an analyte for testing (5 μl sample).

The extracted human serum samples were tested using the five dengue TaqMan assays in parallel with the routine methods. Results of standard virological and TaqMan methods were then compared.

RESULTS

Limit of detection testing with dengue RNA extracted from cell culture-derived stock viruses spiked in normal human serum indicated that each TaqMan assay had a range of linearity of at least 5 log dilutions in 1× Tris-EDTA (Table 3). Figures 2A and 2B represent typical amplification plots obtained with the dengue virus group-specific assay; serotype-specific assay plots are not shown. Figure 2A shows a logarithmic curve of the dengue virus group assay when testing Den-1 to -4 viruses, JE virus, YF virus, and no-template (blank) controls tested in

TABLE 2. Probe and primer sequences for dengue group-specific assay (3′ UTR)

Primer or probe	Sequence (5′–3′)	Genomic target	GenBank reference no.
Group forward	AAG GAC TAG AGG TTA KAG GAG ACC C	10589–10613	M87512
Group reverse	GGC GYT CTG TGC CTG GAW TGA TG	10699–10677	M87512
Probe 1 Den-1-3 (5′-6-FAM)	AAC AGC ATA TTG ACG CTG GGA GAG ACC	10610–10636	M87512
Probe 2 Den-2-4 (5′-JOE)	AAC AGC ATA TTG ACG CTG GGA AAG ACC	10616–10642	M29095

TABLE 3. Dengue assay characteristics

Assay	Detection threshold (PFU/vol tested)	Correlation coefficient	Cross-reactivity	Serotype concordance (%)
Dengue 1	0.3	0.999	Not observed	100
Dengue 2	0.1	0.983	Not observed	100
Dengue 3	1.1	0.990	Not observed	100
Dengue 4	0.1	0.993	Not observed	100
Den group	0.1–1.0	≥ 0.980	Not observed	100

duplicate. For logarithmic plots (Fig. 2A and B), the x axis represents the relative signal intensity (ΔRn) plotted against the y axis, which is the PCR cycle number. Figure 2C is the standard curve generated when testing log dilutions of extracted Den-1 RNA in duplicate with the dengue virus group-specific assay. Figure 2C represents a typical standard curve graph where the threshold cycle is plotted against the starting quantity (PFU per milliliter). In Fig. 2C, the plaque titer of the stock virus used is indicated along with the concentration of the last point to remain linear on the standard curve. The last linear dilution on the standard curve has units of PFU per milliliter, which was converted to PFU per volume tested. The detection threshold for all assays was between 0.1 and 1.1 PFU/volume tested.

To evaluate TaqMan assays for the detection of dengue virus RNA from human sera, we tested 67 dengue virus isolation-positive sera collected from dengue patients from Peru, Indonesia, and Taiwan and 21 normal human sera from the United States. All samples were thawed for viral isolation testing by C6/36 cell inoculation and plaque titration in Vero cells at the same time that aliquots were lysed for RNA extraction for the TaqMan assays. Based on the 67 reisolation-positive samples, the serotype-specific TaqMan assays detected 62 of 67 positive samples, and group-specific assay detected 66 of 67 positive samples, for sensitivities of 92.5 and 98.5%, respectively. Taq-Man assays failed to amplify extracted viral RNA from JE or YF vaccine strains and had a specificity of 100% (21 of 21) based on results of testing the 21 normal human serum or plasma samples. The serotype concordance for TaqMan serotype-specific assays with the viral isolation method was 100% (62 of 62 or 67 of 67, respectively). Direct plaque titer assays in Vero cells were performed on 59 of the 67 isolation-positive samples; 8 of 67 were not tested by plaque titer assay due to insufficient sample volume. A comparison of C6/36 isolation, plaque titer assay, and TaqMan results from clinical samples is listed in Table 4.

DISCUSSION

In this study, we developed dengue virus group- and serotype-specific TaqMan assays that were used to screen and type for the presence of dengue virus RNA in a set of clinical samples. These assays were shown to be a sensitive and specific method for detecting and typing dengue virus RNA when compared to the standard culture methods. The TaqMan assays were also target specific for either dengue virus group or dengue virus serotype but did not cross-react with two related flaviviruses, JE and YF, and did not amplify normal human sera or plasma samples. Although three other reports docu-
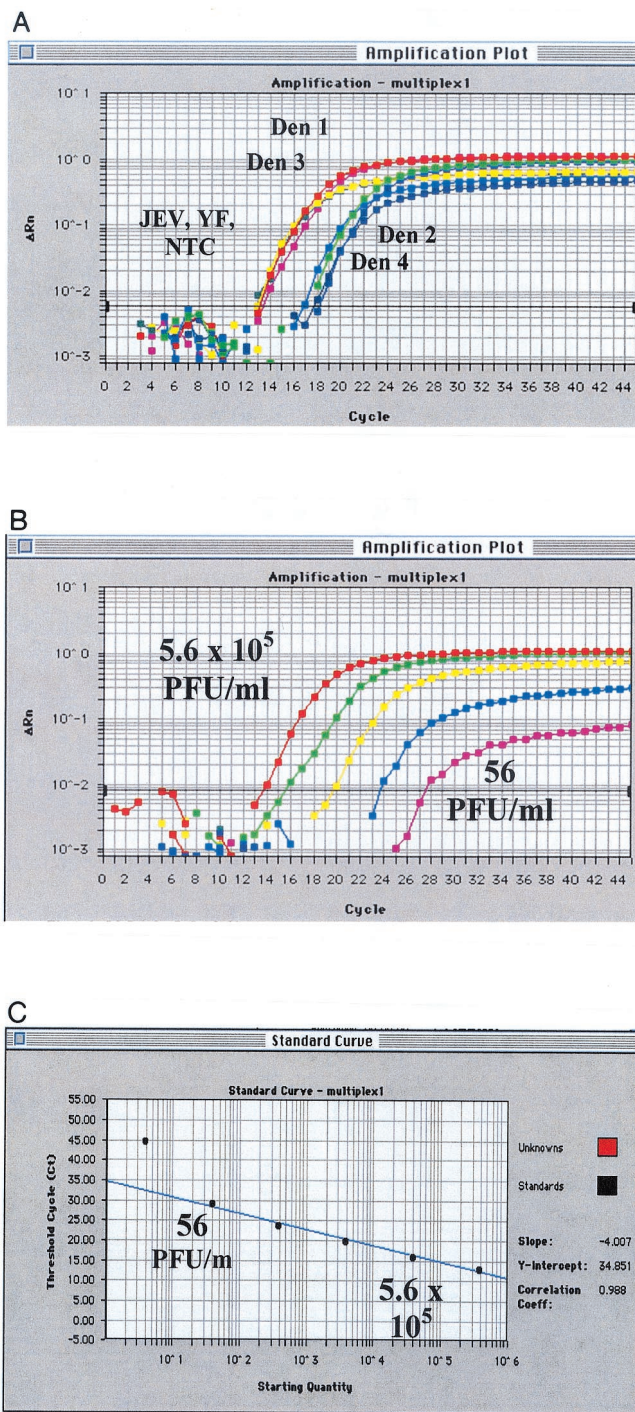

FIG. 2. (A) Dengue virus group-specific assay. Den-1 to -4, JEV, and YF are shown. Results represent typical amplification plots obtained with the dengue virus group-specific assay. The PE 7700 instrument reading, set to FAM filter, shows specific detection of all four serotypes but not JEV and YF. The probe with Den-2 and Den-4 specificity has a JOE fluorochrome label but is still detected with the FAM filter. (B) A log plot of Den-1 stock virus tested by the group assay. Results represent typical amplification plots obtained with the dengue virus group-specific assay. Correlation coefficient = 0.988 (FAM filter). (C) A log plot and standard curve of Den-1 stock virus tested by the group assay. Correlation coefficient = 0.988 (FAM filter). Results represent a typical standard curve graph where the threshold cycle is plotted against the starting quantity (PFU/ml).

TABLE 4. Comparison of results obtained from clinical samples

Plaque titer range (PFU/ml)	No. of positive specimens detected by:		
	C6/36 isolation	TaqMan group-specific assay	TaqMan serotype-specific assay
<25	9	8	8
25–99	4	4	3
10^2–$<10^3$	11	11	11
10^3–$<10^4$	6	6	6
10^4–$<10^5$	21	21	19
$\geq 10^5$	8	8	8
Total	59	58	55

ment the development of TaqMan assays for dengue virus, none report the development of a group assay (3, 11, 13), and in the first of these reports the authors state that they were unable to locate a region of the genome where suitable universal primers could be applied for a group assay. This is the first scientific report in which a group-specific TaqMan assay is reported with specificity to all viruses within the dengue virus group.

The detection threshold for the clinical serum samples based on the plaque assay (25 PFU/ml) was estimated to be equivalent to the detection threshold for the dengue virus-spiked normal serum, although a direct conversion of units from PFU per milliliter to RNA copies per milliliter cannot be determined without further investigation. A group-specific assay utilizing the TaqMan format could serve as an excellent screening tool for laboratory diagnosis of dengue illness. This rapid tool would greatly benefit reference laboratories in areas where multiple tropical diseases are endemic. In such areas where multiple diseases cocirculate, differential diagnosis is important so that limited resources can be directed in focused disease control efforts. An outbreak of a dengue-like illness could be clinically indistinguishable from an outbreak of hepatitis, yellow fever, or malaria. Furthermore, differential diagnosis would be time-consuming and costly. The ability to make a rapid and definitive diagnosis could shift limited public health resources more quickly to more directed intervention strategies.

The results we obtained highlight the potential for the dengue TaqMan assay as a rapid, specific, and sensitive tool for the epidemiological and diagnostic investigation of dengue virus. These assays have several advantages over traditional identification methods. First, the TaqMan RT-PCR assays are more rapid than traditional methods. The TaqMan RT-PCR assays take approximately 5 h (including RNA extraction) versus 7 to 10 days for virus isolation and immunofluorescence antibody testing. In addition, up to 96 samples can be tested simultaneously, giving the TaqMan RT-PCR the advantage in regard to high-throughput testing. Second, the TaqMan RT-PCR assay results are at least equal in sensitivity and specificity when compared to traditional culture methods (data not shown). Third, the TaqMan RT-PCR assay is cost-effective when considered in balance with the savings in labor cost realized in comparison to routine cell culture methods. Fourth, the TaqMan RT-PCR assays are more easily standardized in comparison to traditional methodologies, which are often difficult to standardize and prone to significant test-to-test variation.

Fifth, the TaqMan RT-PCR assay can be made highly portable using several commercially available instruments.

There are limitations in determining the analytical sensitivity of the TaqMan assays in terms of PFU per milliliter (biologically active), or more properly, RNA copies per milliliter (nucleotide targets). The quantitative relationship between a PFU and an RNA copy is unknown for dengue virus. In addition, development of a reliable quantitative standard that is stable and reproducible has its own challenges. Viral RNA is labile and subject to rapid degradation by RNase activity. Therefore, the use of extracted RNA from culture material is unreliable even if stored and aliquoted properly (16, 21). There are several possible solutions to creating a stable internal positive control standard, each offering advantages and limitations. One is to create an RNA-positive control by incorporating a dengue virus genomic target fragment into a vector (such as pBluescript KS [Stratagene, La Jolla, Calif.]). Following the use of the T7 or SP6 promoters present within the vector and using T7 or SP6 RNA polymerase, positive-sense viral RNA transcripts could be generated in vitro. These synthetic RNA molecules could be quantitated and employed as a standard to determine the detection threshold of the TaqMan assays. Another method to create a stable internal positive control for quantitation is to use the plasmid itself containing the targeted viral nucleotide (DNA) sequence. The number of plasmid copies can also be quantitated, diluted serially, and used as a source material for quantitation during TaqMan amplification and detection. The first method discussed, which creates synthetic dengue virus RNA molecules, falls short in that the resultant RNA is quite susceptible to degradation and would not accurately assess the number of RNA molecules entering the assay. Plasmids are inherently more stable than free RNA in solution but are an inadequate control for the RT portion of the RT-PCR assay. Incorporating a highly stable quantitative standard RNA of known concentration that is resistant to RNA degradation, such as an "Armored RNA" product (Ambion, Inc., Austin, Tex.), would be a more reliable way to approach true quantitation for these assays (16, 21). Further empirical study is necessary to assess the efficiency of the RNA extraction step, the RT step, and the PCR amplification and detection step before we are able to develop a truly quantitative standard for the dengue TaqMan assay. Nevertheless, the TaqMan assays we have developed are ready for use immediately in diagnostic settings.

TaqMan assays are a powerful tool for the identification of a variety of clinically relevant viruses (10, 14, 15, 20). However, it is possible to create assays that result in high numbers of false negatives if multiple genotypes and strains of geographic isolates are not considered in the initial assay design. For example, if only the Den-2 New Guinea C strain sequence and stock virus were used for the design and development of a Den-2 assay, it is quite likely that mismatches would be found among the diversity of Den-2 strains or genotypes. These mismatches in the region of sequence targeted by the primer and probe set could yield false-negative results, or at the very least, a decreased sensitivity to the heterologous strains. This is especially true if the assay is targeted to a less conserved gene or sequence, such as the *E* gene in dengue virus, which displays a high degree of sequence variation among strains. In the development of our dengue TaqMan assays (serotype specific and

group), the above-mentioned factors were considered in the design of primer and probe sets and resulted in an assay that should detect a broad range of geographic isolates and strains of dengue virus (Fig. 1). In this study we sought to develop rapid, sensitive, and specific fluorogenic probe-based RT-PCR assays to screen and serotype a representative range of dengue viruses that are found in nature. In summary, the results demonstrate that the goal of producing sensitive and specific dengue virus group- and serotype-specific TaqMan assays has been achieved. Qualitative and quantitative dengue virus TaqMan assays could have tremendous utility for the epidemiological investigation of dengue illness and especially for the study of the viremic response with candidate live-attenuated dengue virus vaccines. This study also suggests that our dengue TaqMan assays would be valuable in testing acute-phase serum samples from patients clinically suspected to have dengue infection and providing the diagnostic results on the same day.

REFERENCES

1. **Aiyar, A.** 2000. The use of CLUSTAL W and CLUSTAL X for multiple sequence alignment. Methods Mol. Biol. **132:**221–241.
2. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
3. **Chen, R. F., W. T. Yeh, M. Y. Yang, and K. D. Yang.** 2001. A model of the real-time correlation of viral titers with immune reactions in antibody-dependent enhancement of dengue-2 infections. FEMS Immunol. Med. Microbiol. **30:**1–7.
4. **Duebel, V.** 1997. The contributions of molecular techniques to the diagnosis of dengue infection, p. 335–366. *In* D. J. Gubler and G. Kuno (ed.), Dengue and dengue hemorrhagic fever. CAB International, Wallingford, United Kingdom.
5. **Eckels, K. H., W. E. Brandt, V. R. Harrison, J. M. McCown, and P. K. Russell.** 1976. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. Infect. Immun. **14:**1221–1227.
6. **George, R., and L. Lum.** 1997. Clinical spectrum of dengue infection, p. 89–113. *In* D. J. Gubler and G. Kuno (ed.), Dengue and dengue hemorrhagic fever. CAB International, Wallingford, United Kingdom.
7. **Gubler, D. J.** 1998. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev. **11:**480–496.
8. **Guzman, M. G., and G. Kouri.** 1996. Advances in dengue diagnosis. Clin. Diagn. Lab. Immunol. **3:**621–627.
9. **Halstead, S. B.** 1997. Epidemiology of dengue and dengue hemorrhagic fever, p. 23–44. *In* D. J. Gubler and G. Kuno (ed.), Dengue and dengue hemorrhagic fever. CAB International, Wallingford, United Kingdom.
10. **Hawrami, K., and J. Breuer.** 1999. Development of a fluorogenic polymerase chain reaction assay (TaqMan) for the detection and quantitation of varicella zoster virus. J. Virol. Methods **79:**33–40.
11. **Houng, H. H., D. Hritz, and N. Kanesa-thasan.** 2000. Quantitative detection of dengue 2 virus using fluorogenic RT-PCR based on 3′-noncoding sequence. J. Virol. Methods **86:**1–11.
12. **Kuno, G., G. J. Chang, K. R. Tsuchiya, N. Karabatsos, and C. B. Cropp.** 1998. Phylogeny of the genus *Flavivirus*. J. Virol. **72:**73–83.
13. **Laue, T., P. Emmerich, and H. Schmitz.** 1999. Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. J. Clin. Microbiol. **37:**2543–2547.
14. **Morris, T., B. Robertson, and M. Gallagher.** 1996. Rapid reverse transcription-PCR detection of hepatitis C virus RNA in serum by using the TaqMan fluorogenic detection system. J. Clin. Microbiol. **34:**2933–2936.
15. **Pas, S. D., E. Fries, R. A. De Man, A. D. Osterhaus, and H. G. Niesters.** 2000. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J. Clin. Microbiol. **38:** 2897–2901.
16. **Pasloske, B. L., C. R. Walkerpeach, R. D. Obermoeller, M. Winkler, and D. B. DuBois.** 1998. Armored RNA technology for production of ribonuclease-resistant viral RNA controls and standards. J. Clin. Microbiol. **36:**3590–3594.
17. **Sudiro, T. M., H. Ishiko, S. Green, D. W. Vaughn, A. Nisalak, S. Kalayana-rooj, A. L. Rothman, B. Raengsakulrach, J. Janus, I. Kurane, and F. A. Ennis.** 1997. Rapid diagnosis of dengue viremia by reverse transcriptase-polymerase chain reaction using 3′-noncoding region universal primers. Am. J. Trop. Med. Hyg. **56:**424–429.
18. **Tesh, R. B.** 1979. A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am. J. Trop. Med. Hyg. **28:**1053–1059.
19. **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.
20. **van Elden, L. J., M. Nijhuis, P. Schipper, R. Schuurman, and A. M. van Loon.** 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J. Clin. Microbiol. **39:**196–200.
21. **Walkerpeach, C. R., M. Winkler, D. B. DuBois, and B. L. Pasloske.** 1999. Ribonuclease-resistant RNA controls (armored RNA) for reverse transcription-PCR, branched DNA, and genotyping assays for hepatitis C virus. Clin. Chem. **45:**2079–2085.
22. **Wu, S. J., G. Grouard-Vogel, W. Sun, J. R. Mascola, E. Brachtel, R. Putvatana, M. K. Louder, L. Filgueira, M. A. Marovich, H. K. Wong, A. Blauvelt, G. S. Murphy, M. L. Robb, B. L. Innes, D. L. Birx, C. G. Hayes, and S. S. Frankel.** 2000. Human skin Langerhans cells are targets of dengue virus infection. Nat. Med. **6:**816–820.