JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1998, p. 2634–2639 Vol. 36, No. 9
0095-1137/98/$04.00+0

Typing of Dengue Viruses in Clinical Specimens and Mosquitoes by Single-Tube Multiplex Reverse Transcriptase PCR

EVA HARRIS,[1]* T. GUY ROBERTS,[1] LEILA SMITH,[1] JOHN SELLE,[1] LAURA D. KRAMER,[2] SONIA VALLE,[3] ERICK SANDOVAL,[3] AND ANGEL BALMASEDA[3]

Program in Molecular Pathogenesis, University of California, San Francisco, San Francisco, California 94143-0422[1]; Center for Vector-Borne Disease Research, University of California, Davis, Davis, California 95616[2]; and Centro Nacional de Diagnóstico y Referencia, Ministerio de Salud, Managua, Nicaragua[3]

Received 17 February 1998/Returned for modification 15 May 1998/Accepted 8 June 1998

In recent years, dengue viruses (serotypes 1 to 4) have spread throughout tropical regions worldwide. In many places, multiple dengue virus serotypes are circulating concurrently, which may increase the risk for the more severe form of the disease, dengue hemorrhagic fever. For the control and prevention of dengue fever, it is important to rapidly detect and type the virus in clinical samples and mosquitoes. Assays based on reverse transcriptase (RT) PCR (RT-PCR) amplification of dengue viral RNA can offer a rapid, sensitive, and specific approach to the typing of dengue viruses. We have reduced a two-step nested RT-PCR protocol to a single-tube reaction with sensitivity equivalent to that of the two-step protocol (1 to 50 PFU) in order to maximize simplicity and minimize the risk of sample cross-contamination. This assay was also optimized for use with a thermostable RT-polymerase. We designed a plasmid-based internal control that produces a uniquely sized product and can be used to control for both reverse transcription or amplification steps without the risk of generating false-positive results. This single-tube RT-PCR procedure was used to type dengue viruses during the 1995 and 1997-1998 outbreaks in Nicaragua. In addition, an extraction procedure that permits the sensitive detection of viral RNA in pools of up to 50 mosquitoes without PCR inhibition or RNA degradation was developed. This assay should serve as a practical tool for use in countries where dengue fever is endemic, in conjunction with classical methods for surveillance and epidemiology of dengue viruses.

Over the last 20 years, classic dengue fever and the more severe form, dengue hemorrhagic fever-dengue shock syndrome (DHF-DSS), have emerged as the most important arthropod-borne viral diseases in humans (22). During this period, dengue fever has spread throughout tropical regions worldwide, principally in urban settings. Up to 100 million cases of classic dengue fever are estimated annually, and roughly 450,000 cases of DHF-DSS are reported annually, while approximately 2.5 billion people live in areas at risk for epidemic dengue virus transmission (9, 22). The dramatic spread of epidemic dengue fever and the emergence of DHF-DSS occurred after World War II in Southeast Asia, where DHF is now one of the leading causes of hospitalization and death. This pattern of epidemic dengue fever and emerging DHF is being repeated in Latin America (10), where it is spreading throughout the region at an alarming rate.

Dengue fever is caused by four distinct serotypes of dengue virus, which are transmitted to humans by the domestic mosquitoes *Aedes aegypti* and *Aedes albopictus* (22). The lack of a vaccine or a cure for dengue fever make the development of laboratory-based surveillance systems all the more important to provide an early warning of dengue fever epidemics and to furnish information for effective vector control measures (9). It is crucial to determine which serotypes of dengue virus are circulating where and when since previous infection with one of the four dengue serotypes can be an important risk factor for developing DHF-DSS upon infection with a heterotypic serotype (11, 23). The current "gold standard" for typing dengue virus involves isolation of the virus in cultured cells or mosquitoes followed by indirect immunofluorescence. However, this requires cell culture facilities or mosquito colonies, which are difficult to maintain in laboratories in developing countries. The most rapid serological techniques, such as immunoglobulin M enzyme-linked immunosorbent assay with a single serum sample, do not furnish information about the serotype of the virus. The plaque reduction neutralization technique allows typing but is costly and difficult to perform.

Single-step reverse transcriptase (RT) PCR (RT-PCR) detection and typing of dengue virus offers a sensitive, specific, and rapid alternative that requires only one acute-phase serum sample. This technique can be made cost-effective by following a low-cost methodology (12–14). Early detection of dengue virus in patient serum allows the possibility of mounting a rapid response aimed at vector control in the affected areas. This assay is also useful for typing the virus and providing important information for epidemiological studies. In addition, rapid assays for the detection of dengue virus in mosquitoes are useful for investigation of the virus and its vector in nature (6). Recently, a number of molecular approaches to the detection and characterization of dengue viral RNA have been described (8, 15, 18, 20, 24, 26, 28, 30, 34). Here we present a modified RT-PCR assay for the single-step detection and typing of dengue virus in clinical specimens and mosquitoes. This assay has been simplified for use in countries where dengue fever is endemic.

MATERIALS AND METHODS

Virus strains and specimens. Virus stocks were kindly provided by the Centers for Disease Control and Prevention (CDC) (serotype 1 dengue virus [dengue-1; strain Hawaii], serotype 2 dengue virus [dengue-2; strain 16681], serotype 3 dengue virus [dengue-3; strain H-87], and serotype 4 dengue virus [dengue-4; strain 703-4]) or were isolated in Nicaragua during the 1995 and 1997-1998 outbreaks. Human serum samples were obtained from patients clinically suspected of having dengue fever within 0 to 4 days from the time of onset of

* Corresponding author. Present address: Division of Public Health Biology and Epidemiology, School of Public Health, University of California, Berkeley, 140 Warren Hall, Berkeley, CA 94720. Phone: (510) 643-9773. Fax: (510) 642-6350. E-mail: eharris@uclink4.berkeley.edu.

symptoms. The samples had been collected for routine diagnosis of dengue fever at the Centro Nacional de Diagnóstico y Referencia in Managua, Nicaragua, in June and July 1995 and December 1997 to May 1998.

Mosquito inoculation. Mosquitoes (*A. aegypti* Rock) were inoculated with approximately 10^3 PFU of dengue-2 (strain 16681) by the method of Rosen and Gubler (29). Adults were maintained at 27°C and were provided with 10% sucrose as nourishment. Live mosquitoes were frozen at −70°C on days 1, 2, 3, 4, 6, 7, and 21. A group of mosquitoes that had died 3 days postinoculation were frozen on day 5. Uninfected *A. aegypti* were reared in the laboratory in Nicaragua from eggs laid by field-collected females.

Viral growth. *A. albopictus* C6/36 cells (16) were grown in minimal essential medium (MEM) (Gibco BRL, Grand Island, N.Y.) containing Earle's salts, L-glutamine, and nonessential amino acids and supplemented with 0.11% sodium bicarbonate, 100 U of penicillin per ml, 75 U of streptomycin per ml, and 10% fetal bovine serum (FBS) (Gemini Bioproducts, Inc., Calabasas, Calif.). Baby hamster kidney cells (BHK21-15) (19) were grown in MEM as described above, except that 0.124% sodium bicarbonate and 5% FBS were used. Viruses were propagated in C6/36 cells, and after incubation at 28°C for 7 days, the cellular supernatant was clarified by centrifugation, supplemented with 20% FBS, and stored at −70°C until use. For plaque assays, monolayers of BHK21-15 cells were grown to 90 to 95% confluence in six-well plates and were inoculated in duplicate with 200 μl of cellular supernatant containing serial dilutions of the viral stock. After 2 h at 37°C, the cells were overlaid with MEM containing 1% SeaPlaque agarose (FMC BioProducts, Rockland, Maine) and 5% FBS and were incubated for 7 days at 37°C in 5% CO_2. The cells were then fixed in 10% formadehyde for 2 h and stained with a solution containing 0.27% crystal violet. Virus isolation was performed by inoculating C6/36 cells with a 5- to 20-fold dilution of a serum specimen. After 7 days, isolated virus was serotyped with monoclonal antibodies and by RT-PCR.

RNA extraction. RNA was extracted from serum or the supernatant of infected cells by combining 300 μl of the sample sequentially with 300 μl of lysis buffer (6 M guanidine isothiocyanate, 50 mM sodium citrate, 1% Sarkosyl, 20 μg of *Escherichia coli* tRNA per ml, 100 mM β-mercaptoethanol), 60 μl of 2 M sodium acetate (pH 4.0), 600 μl of water-saturated phenol, and 240 μl of chloroform and mixing after the addition of each of the reagents. After a 15-min centrifugation, the aqueous phase was transferred to a new tube and was mixed with an equal volume of isopropanol. Following a 20-min centrifugation at 4°C, the supernatant was removed and the pellet was washed in 75% ethanol, air dried, and resuspended in 25 μl of RNase-free sterile distilled water.

RNA was extracted from pools of infected or uninfected mosquitoes macerated in 100 μl of phosphate-buffered saline. Prior to maceration, pools of uninfected mosquitoes were spiked with exogenous viral particles. The macerates were clarified by centrifugation, mixed with 100 μl of lysis buffer (see above), and extracted with a 1:1 mixture of phenol and chloroform. Five microliters of acid-washed size-selected silica particles (13, 33) were added to each sample, and the mixture was incubated for 5 min, pelleted by centrifugation, and washed twice (50% ethanol, 10 mM Tris [pH 7.4], 1 mM EDTA, 50 mM NaCl). After resuspension in 10 μl of RNase-free distilled water, the samples were incubated for 5 min at 50 to 55°C and centrifuged. The eluate supernatant was transferred to a new tube, and the pellet was resuspended in 5 μl of water immediately prior to amplification. Alternatively, the washed pellet can be resuspended in 15 μl of water and used directly for amplification.

Reverse transcription and PCR amplification. (i) Two-enzyme RT-PCR. The reaction mixture contained 50 mM KCl, 10 mM Tris (pH 8.5), 0.1% Triton X-100, 0.01% gelatin, each of the four deoxynucleotide triphosphates at a concentration of 200 μM, 1.5 mM $MgCl_2$, 30 mM tetramethylammonium chloride (5), 0.5 M betaine (25), 5 mM dithiothreitol, 5′ primer D1 and 3′ primer TS1 at a concentration of 1 μM each, 3′ primers TS2, TS3, and DEN4 at a concentration of 0.5 μM each, 0.0017 to 0.025 U of RAV-2 RT (Amersham Corp., Arlington Heights, Ill.) per μl, and 0.025 U of *Taq* DNA polymerase (*Taq* DNA polymerase [Promega Corp., Madison, Wis.]; AmpliTaq [Perkin-Elmer Corp., Foster City, Calif.]) per μl. Reverse transcription was conducted at 42°C for 60 min, followed by 40 amplification cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 5 min. A total of 2.5 to 5 μl of extracted RNA was used as a template in a 25-μl reaction volume. Amplification was conducted in 0.6-ml tubes (Robbins Scientific Corp., Sunnyvale, Calif.) with a model 480 thermal cycler (Perkin-Elmer, Norwalk, Conn.) or a PTC-200-60 thermocycler (MJ Research, Inc., Watertown, Mass.).

(ii) Single-enzyme (r*Tth*) RT-PCR. The reaction mixture for r*Tth* RT-PCR contained 115 mM potassium acetate, 8% glycerol, 50 mM bicine (pH 8.2), the four deoxynucleotide triphosphates at a concentration of 200 μM each, 2 mM manganese acetate, primers D1 and TS1 at a concentration of 0.5 μM each, primers TS2, TS3, and DEN4 at a concentration of 0.25 μM each, and 0.05 U of r*Tth* DNA polymerase (Perkin-Elmer Corp.) per μl. One cycle of 60°C for 30 min for the reverse transcription was followed by a 2-min incubation at 94°C and 40 cycles of 94°C for 45 s, 50°C for 1 min, and 60°C for 1 min, with a final extension at 60°C for 7 min.

Primer sequences are as follows: D1, 5′-TCA ATA TGC TGA AAC GCG CGA GAA ACC G; TS1, 5′-CGT CTC AGT GAT CCG GGG G; TS2, 5′-CGC CAC AAG GGC CAT GAA CAG; TS3, 5′-TAA CAT CAT CAT GAG ACA GAG C (18); and DEN4, 5′-TGT TGT CTT AAA CAA GAG AGG TC. The expected sizes of the amplification products are 482 bp (dengue-1), 119 bp (dengue-2), 290 bp (dengue-3), and 389 bp (dengue-4). Ten microliters of the 25-μl reaction mixtures was electrophoresed on 1.5% agarose gels in 1× TBE (89 mM Tris borate, 2 mM EDTA [pH 8.3]) with a 100-bp ladder as a size standard (100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, and 2,072 bp; Gibco BRL) or Amplisize DNA size standards (50, 100, 200, 300, 400, 500, 700, 1,000, 1,500, and 2,000 bp; Bio-Rad Laboratories, Richmond, Calif.).

Plasmid construction. To clone the dengue virus amplicons, dengue viral RNA was amplified with 5′ and 3′ primers containing the restriction sites *Eco*RI and *Bam*HI, respectively, at their 5′ ends. The products were treated with proteinase K, digested with the appropriate restriction enzymes, gel purified, and ligated with pBluescript (KS II; Stratagene Cloning Systems, La Jolla, Calif.) that had been digested with *Eco*RI and *Bam*HI and gel purified. To construct a control plasmid with a dengue-3 fragment ~50 bp larger than the original amplicon, a 54-bp fragment was amplified from *Leishmania braziliensis* with primers 13A and MP3H (1), treated with proteinase K (Promega Corp.), and then incubated with T4 DNA polymerase (New England Biolabs, Inc., Beverly, Mass.) and the four nucleotides. The plasmid containing the dengue-3 insert (pDB3) was linearized within the insert by digestion with *Sty*I, and the overhanging ends were filled in by incubation with T4 DNA polymerase and the four nucleotides. After treatment of the vector with calf intestinal alkaline phosphatase (New England Biolabs, Inc.), the 54-bp *L. braziliensis* fragment was ligated into pBD3 to create pBD3L.

For in vitro transcription of the 350-bp dengue-3 fragment containing the insert, pBD3L was linearized with *Xba*I immediately downstream of the dengue-3 insert and was incubated with T3 RNA polymerase (Promega Corp.) and the four nucleotides for 60 min at 37°C. To remove the plasmid DNA from the in vitro transcription reaction, the reaction mixture was treated with RQ1 DNase (Promega Corp.) in the presence of 10 mM calcium chloride for 60 min at 37°C. To confirm that only RNA remained, an aliquot was treated with RNase for 30 min at 37°C.

RESULTS

Single-tube RT-PCR assay. A one-tube RT-PCR protocol was developed to reverse transcribe dengue viral RNA and amplify four differently sized type-specific products. This protocol is an adaptation of a two-step nested RT-PCR assay described by Lanciotti et al. (18). Five oligonucleotide primers are included in the one-step assay: one 5′ primer that targets a region of the capsid gene conserved in all four dengue virus serotypes and four 3′ primers, each of which is complementary to sequences unique to each serotype. These primers are positioned such that a differently sized product is generated from each type, as shown in Fig. 1A, lanes 1 to 4 (dengue-2, 119 bp; dengue-3, 290 bp; dengue-4, 389 bp; dengue-1, 482 bp). Several modifications were made to the original protocol. The dengue-4-specific primer was redesigned to avoid hairpin formation so as to increase the yield of the dengue-4 product. Cosolvents, such as tetramethylammonium chloride (5) and betaine (25), were included in the reaction mixture to improve the sensitivity of the assay. The concentrations of the different primers were adjusted to optimize the amplification of all four products.

To test the sensitivity of the single-tube RT-PCR assay, plaque titration and RNA extractions were performed simultaneously with each dilution of a serial titration of viral stocks. One-tenth of the extracted RNA was amplified by RT-PCR. Plaque formation has commonly been used as a measure of the sensitivity of dengue viral RT-PCR assays (4, 24, 26, 28, 30), although it is not a direct indication of the number of viral particles. An example of the sensitivity of this assay is shown in Fig. 1A, lanes 6 to 9, where serial 10-fold dilutions of a dengue-3 stock were extracted, reverse transcribed, and amplified. The limit of detection was approximately 1 PFU for dengue-1, 50 PFU for dengue-2, 1 PFU for dengue-3, and 30 PFU for dengue-4. Side-by-side comparisons of the one-tube method and the original two-step protocol revealed that the two procedures had similar sensitivities (data not shown).

The single-tube RT-PCR assay was adapted for use with the thermostable RT-polymerase r*Tth* (Fig. 1B, lanes 1 to 4), obviating the need for two separate enzymes. Optimal results were obtained with one-half of the amount of r*Tth* recom-

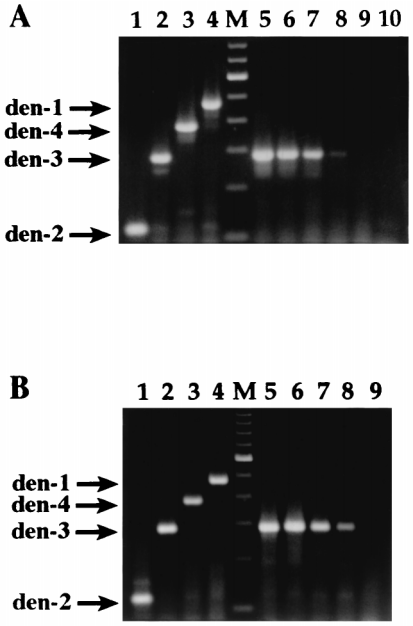

FIG. 1. Detection and typing of dengue virus by using two versions of the RT-PCR assay. (A) Reverse transcription with RAV-2 RT and amplification with *Taq* DNA polymerase; (B) reverse transcription and amplification with the bifunctional enzyme r*Tth*. (A and B) Lanes 1, dengue-2 (den-2); lanes 2, dengue-3 (den-3); lanes 3, dengue-4 (den-4); lanes 4, dengue-1 (den-1); lanes M, 100-bp ladder (lowest band shown, 100 bp); lanes 5 to 8, dengue-3 at 1,000, 100, 10, and 1 PFU, respectively. (A) Lane 9, 0 pfu; lane 10, water (negative control). (B) Lane 9, water. Expected product sizes are as follows: dengue-2, 119 bp; dengue-3, 290 bp; dengue-4, 389 bp; dengue-1, 482 bp.

mended by the manufacturer. The primer concentrations in this assay were reduced by 50% compared to the concentrations used in the two-enzyme assay described above, and no cosolvents were necessary. The sensitivity of the r*Tth* assay was similar to that of the two-enzyme protocol (Fig. 1B and data not shown).

Internal control plasmid. As a positive control for the RT-PCR assay, a plasmid containing a uniquely sized dengue-3 fragment was constructed. When amplified, this fragment can be differentiated from the authentic viral amplification product. The 290-bp dengue-3 product was cloned into pBluescript (pBD3), and a 54-bp fragment was inserted to create a 350-bp PCR substrate (pBD3L). Using this plasmid, an in vitro RNA transcript can be generated for use as a positive control for both the reverse transcription and amplification steps. Alternatively, the plasmid itself can be used as a positive control for the amplification step only. Figure 2 (lanes 1 and 2) shows the size difference between the original dengue-3 fragment and the fragment containing the insert, excised from pBD3 and pBD3L, respectively. The amplification products derived directly from plasmids pBD3 and pBD3L (lanes 3 and 4, respectively) and from the DNase-treated in vitro transcript of pBD3L (lane 5) are of the expected sizes. The last three lanes demonstrate that the in vitro transcript of pBD3L (lane 6) is sensitive to RNase (lane 8) but not DNase (lane 7).

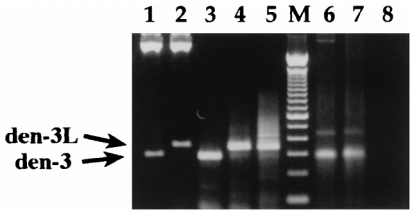

FIG. 2. Uniquely sized internal control. Lanes 1 and 2, authentic dengue-3 amplicon (den-3) and dengue-3 amplicon containing the 54-bp insert (den-3L), respectively, excised from pBD3 and pBD3L with *Eco*RI and *Bam*HI; lanes 3 to 5, RT-PCR products derived from pBD3, pBD3L, and the DNase-treated in vitro transcript of pBD3L, respectively; lane M, 100-bp ladder (lowest band shown, 100 bp); lane 6, in vitro transcript of pBD3L; lane 7, DNase-treated in vitro transcript of pBD3L; lane 8, RNase-treated in vitro transcript of pBD3L. Expected product sizes are as follows: dengue-3, 290 bp; dengue-3L, 350 bp.

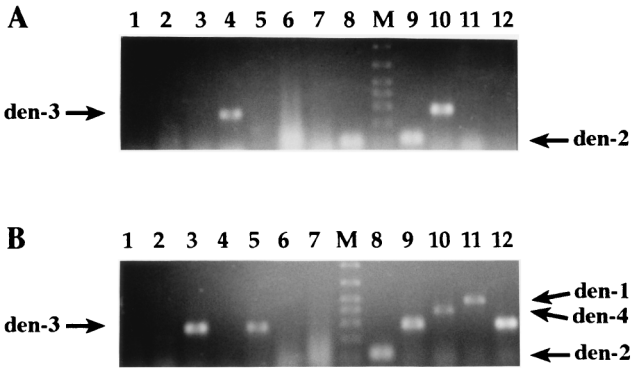

FIG. 3. RT-PCR detection and typing of dengue virus in serum from patients infected during the 1995 epidemic in Nicaragua. RNA was extracted from serum samples and was amplified by the two-enzyme single-tube RT-PCR assay as described in Materials and Methods. (A) Lane 1, negative control (water); lanes 2 to 8, 11, and 12, samples from patients from the Atlantic coast of Nicaragua (Bluefields); lane M, Amplisize DNA size standards (lowest band shown, 100 bp); lane 9, dengue-2 (den-2) RNA (positive control); lane 10, dengue-3 (den-3) RNA (positive control). (B) Lanes 1 to 7, samples from patients from the Atlantic coast of Nicaragua (Bluefields); lane M, Amplisize DNA size standards (lowest band shown, 100 bp); lane 8, dengue-2 (den-2) RNA (positive control); lane 9, dengue-3 (den-3) RNA (positive control); lane 10, dengue-4 (den-4) RNA (positive control); lane 11, dengue-1 (den-1) RNA (positive control); lane 12, sample from a patient from central Nicaragua (Chontales). Expected products sizes are as follows: dengue-2, 119 bp; dengue-3, 290 bp; dengue-4, 389 bp; dengue-1, 482 bp.

RT-PCR detection and typing of dengue virus in clinical specimens. During outbreaks of dengue fever in Nicaragua in 1995 and 1997-1998, serum specimens referred to the National Virology Laboratory at the Centro Nacional de Diagnóstico y Referencia, Ministry of Health, were analyzed by the two-enzyme RT-PCR assay. Viral RNA was extracted in duplicate directly from patient serum and was amplified in duplicate on different days to minimize the risk of artifactual results. Figure 3 shows representative results obtained for specimens collected in June and July 1995. Specimens were obtained from patients in Bluefields, on the Atlantic coast of Nicaragua (Fig. 3A, lanes 2 to 8, 11, and 12, and Fig. 3B, lanes 1 to 7), and Chontales, in central Nicaragua (Fig. 3B, lane 12). The results for positive (Fig. 3A, lanes 9 and 10; Fig. 3B, lanes 8 to 11) and negative (Fig. 3A, lane 1) controls were as expected. Duplicate aliquots of each specimen yielded consistent and reproducible results. Figure 3A demonstrates that two dengue virus serotypes (e.g., lane 4, dengue-3; lane 8, dengue-2) were circulating simultaneously in the same geographical area. Figure 3B shows that dengue-3 was circulating in two different regions of the country (lanes 3 and 5, Bluefields; lane 12, Chontales). This assay has been used for routine epidemiological surveillance in Nicaragua since 1995 and has been implemented by collaborators at the Centro Nacional de Enfermedades Tropicales in Santa Cruz, Bolivia (27). The RT-PCR assay with r*Tth* has also been successfully used to analyze RNA extracted from clinical specimens (data not shown).

Detection of dengue virus in mosquitoes. To prepare mosquito samples for RT-PCR amplification, a procedure for the

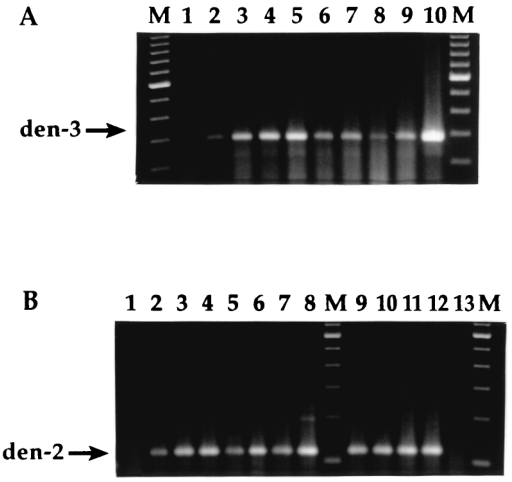

FIG. 4. (A) Detection of dengue virus in pools of mosquitoes by RT-PCR. A total of 350 PFU of dengue-3 (den-3) was added to pools of mosquitoes, which were then macerated. RNA was extracted as described in Materials and Methods, and one-fourth of the extract was amplified by RT-PCR (RAV-2 RT–*Taq* polymerase). Lanes 2, 4, 6, and 8, silica particle eluate; lanes 3, 5, 7, and 9, pellet. Lane 1, water (negative control); lanes 2 and 3, 0 mosquitoes; lanes 4 and 5, 5 mosquitoes; lanes 6 and 7, 25 mosquitoes; lanes 8 and 9, 50 mosquitoes; lane 10, dengue-2 RNA (positive control); lane M, 100-bp ladder (lowest band shown, 200 bp). (B) Detection of dengue virus in laboratory-infected mosquitoes. Mosquitoes were inoculated with dengue-2 (den-2; strain 16681) and were frozen at −70°C on the indicated days postinoculation. RNA was extracted and amplified by RT-PCR (RAV-2 RT–*Taq* polymerase). Lane 1, 5 uninfected mosquitoes; lane 2, one mosquito, one day postinoculation; lane 3, one mosquito, 2 days postinoculation; lane 4, one mosquito, 3 days postinoculation; lane 5, one mosquito, 4 days postinoculation; lane 6, one mosquito, 6 days postinoculation; lane 7, one mosquito, 7 days postinoculation; lane 8, one mosquito, 21 days postinoculation; lane M, 100-bp ladder (lowest band shown, 100 bp); lane 9, five mosquitoes, 2 days postinoculation; lane 10, five mosquitoes, 7 days postinoculation; lane 11, five mosquitoes frozen 2 days after natural death; lane 12, dengue-2 RNA (positive control); lane 13, water (negative control).

extraction of viral RNA from pools of mosquitoes without degradation of the RNA or inhibition of the PCR amplification was required. With a combination of a guanidine-based lysis buffer, organic solvents, and silica particles, a protocol that allows the reproducible isolation of very small quantities of viral RNA in the presence of up to 50 *A. aegypti* mosquitoes, without RT-PCR inhibitors, was developed. Pools of uninfected mosquitoes were spiked with exogenous viral particles, and extracts containing RNA from <100 PFU of dengue-3 were amplified (Fig. 4A). Eluates from silica particles and the silica pellets themselves functioned equally well. RT-PCR amplification of RNA extracted from laboratory-infected mosquitoes demonstrated that dengue viral RNA could be detected in a single mosquito at as early as 1 day postinoculation (Fig. 4B, lane 1), and at as late as 21 days postinoculation, our last time point for detection (lane 8). Pools of five mosquitoes yielded the expected positive results (lanes 9 and 10) as well. Dengue viral RNA was also amplified from infected mosquitoes frozen 2 days after natural death (Fig. 4B, lane 11). The results for negative (Fig. 4A, lane 1; Fig. 4B lanes 1 and 13) and positive (Fig. 4A, lane 10; Fig. 4B, lane 12) controls were as expected. Extracts could be stored frozen for at least 6 months without RNase inhibitors with no detectable loss of RNA integrity.

DISCUSSION

We have adapted a rapid RT-PCR assay for the typing of dengue virus in patient serum and mosquitoes for use under the difficult conditions often prevailing in countries where dengue fever is endemic. In general, PCR-based techniques can be more rapid, sensitive, and specific than alternative techniques when they are made accessible by a low-cost methodology that involves the in-house preparation of reagents and materials, recycling, simplification of procedures, and strict enforcement of simple but effective procedures for minimizing DNA contamination (12–14). Molecular techniques are particularly useful for the detection and typing of dengue virus, and several RT-PCR protocols have been described. Identification of the four serotypes can be achieved by (i) nested amplification of a primary product generated with universal dengue virus primers (18, 20, 34), (ii) hybridization of a universal RT-PCR product with type-specific probes (8, 15, 28), (iii) simultaneous amplification with four sets of type-specific primers (24), or (iv) use of a single 5′ universal primer and four type-specific 3′ primers (18, 30). However, the performance of multistep nested amplification increases the risk of cross-contamination, especially during routine analyses, while hybridization procedures entail additional cost and can be compromised by low water quality. Therefore, we have simplified the reverse transcription and amplification procedures and minimized the number of primers required for the detection and typing of dengue viruses in a single tube.

The single-tube multiplex assay described herein was adapted from a previously reported nested RT-PCR protocol (18). The conditions of sample extraction and amplification were modified such that the sensitivity of the single-step assay was comparable to that of the original nested protocol and to those of other RT-PCR assays for dengue virus detection (18, 24, 30). For instance, one of the primers was redesigned to improve the efficiency of amplification, and cosolvents were included in the reaction mixture to optimize the sensitivity. In addition, this RT-PCR assay was adapted for use with the bifunctional RT-polymerase r*Tth* (Perkin-Elmer Corp.). This thermostable enzyme is easier to transport and store in-country than other RTs, such as RAV-2 (Amersham Corp.), which are extremely labile. We constructed an internal amplification control that generates a uniquely sized product and eliminates the risk of false-positive results due to cross-contamination. This control has been useful for on-site troubleshooting of both the reverse transcription and the amplification processes.

The utility of this single-step assay has been demonstrated in laboratories in developing countries, including Nicaragua (Fig. 3), Bolivia (27), Ecuador (21), and Guatemala (31). Prior to the introduction of the simplified dengue virus RT-PCR assay, the National Virology Laboratory at the Nicaraguan Ministry of Health had been unable to type circulating dengue viruses due to the lack of the cell culture facilities or mosquito colonies necessary for classical viral isolation procedures. Only a small percentage of serum samples had been sent to laboratories outside the country for typing, a costly and lengthy process. Serotype information is particularly important, since all four dengue virus serotypes have been reported recently in Nicaragua (2, 17). Since 1995, RT-PCR has been used for epidemiological surveillance of specimens from selected patients suspected of dengue virus infection and for rapid diagnosis for particular patients, while routine diagnosis is still performed by the immunoglobulin M enzyme-linked immunosorbent assay. Samples positive by RT-PCR are subsequently processed for viral isolation in order to obtain viral stocks for future analysis, now that the necessary facilities are available. We have found that the virus in specimens that are received by the National Virology Laboratory from regional health centers in suboptimal conditions for culture nonetheless can be successfully amplified, suggesting that although virus infectivity is compromised, viral RNA can still be detected.

This RT-PCR technique was also used by scientists at the Nicaraguan Ministry of Health in October 1995 to investigate an outbreak of hemorrhagic fever in northern Nicaragua which was initially thought to be caused by dengue virus. When these scientists demonstrated, using RT-PCR and other serological, virological, and entomological methods, that dengue virus was in fact not the cause, international interest was generated. Teams of scientists from CDC and the Instituto de Medicina Tropical "Pedro Kouri," Havana, Cuba, collaborated with Nicaraguan investigators, leading to the discovery that the etiological agent was in fact *Leptospira* (3). In August 1997, Bolivian scientists at the Centro Nacional de Enfermedades Tropicales used this RT-PCR assay to type dengue virus in Bolivia for the first time and to identify dengue-2 as the serotype responsible for the 1997 epidemic in Santa Cruz, corroborating the results reported by CDC (27). Thus, in-country access to simplified PCR-based techniques is useful for immediate public health purposes as well as for long-term epidemiological studies.

An RNA isolation method was required for the detection of dengue virus in infected mosquitoes without RNA degradation or PCR inhibition. The reported methods for the isolation of RNA from mosquitoes require the use of costly reagents to avoid inhibition (18), do not remove nucleases (7, 32), or consume the entire extraction in a single amplification reaction (7). Therefore, an extraction procedure that uses chaotropic agents and organic extraction to remove nucleases and that includes additional steps to remove substances potentially inhibitory to the amplification was developed. The resulting extracts are stable over time without the addition of expensive nuclease inhibitors. RT-PCR conducted with RNA extracted by this procedure exhibited excellent sensitivity and showed no evidence of inhibition of amplification even in the presence of large numbers of mosquitoes (Fig. 4A). Dengue viral RNA from a single inoculated mosquito could be detected after as little as 24 h and as many as 21 days. Furthermore, the extraction method was used to recover viral RNA from infected mosquitoes frozen after natural death, suggesting that even mosquitoes that have died during field collection can still furnish valuable information about viral infection.

Due to the lack of a vaccine or a cure for dengue fever, the development of laboratory-based surveillance systems is critical in order to provide early warning of dengue fever epidemics (9). Such information can enable preventive measures (e.g., mosquito control) and enhance preparedness on the part of physicians, hospitals, and the public. However, effective surveillance requires that countries where dengue fever is endemic have access to appropriately adapted modern technologies. Therefore, we have modified a dengue virus RT-PCR assay to make it suitable for use under existing conditions in laboratories in countries where dengue fever is endemic.

ACKNOWLEDGMENTS

We thank Nina Agabian and Alcides Gonzalez for support; Dennis Trent at the Center for Vector-Borne Diseases of CDC for prototype viral strains and Robert Lanciotti for advice; Srisakul Kliks and Bob Chiles for C6/36 and BHK21 cell lines and advice on cell culture; and Deborah Lans and Gloria Guevara for technical assistance. We also thank our collaborators Alberto Gianella and Carlos Peredo at the Centro Nacional de Enfermedades Tropicales in Santa Cruz, Bolivia, and Jose Pellegrino from the Instituto de Medicina Tropical "Pedro Kouri."

Financial support for this work was provided in part by the American Society for Biochemistry and Molecular Biology, the Fogarty International Center of the National Institutes of Health (grant D43 TW00905), and donations from Perkin-Elmer Corp., Promega Corp., and Amersham Corp. for Applied Molecular Biology workshops.

REFERENCES

1. **Belli, A., B. Rodriguez, H. Avilés, and E. Harris.** 1998. Simplified PCR detection of New World *Leishmania* from clinical specimens. Am. J. Trop. Med. Hyg. **58:**102–109.
2. **Centers for Disease Control and Prevention.** 1995. Dengue type 3 infection—Nicaragua and Panama, October-November, 1994. Morbid. Mortal. Weekly Rep. **44:**21–24.
3. **Centers for Disease Control and Prevention.** 1995. Outbreak of acute febrile illness and pulmonary hemorrhage—Nicaragua, 1995. JAMA **274:**1668.
4. **Chan, S.-Y., I. M. Kautner, and S.-K. Lam.** 1994. The influence of antibody levels in dengue diagnosis by polymerase chain reaction. J. Virol. Methods **49:**315–322.
5. **Chevet, E., G. Lemaitre, and M. D. Katinka.** 1995. Low concentrations of tetramethylammonium chloride increase yield and specificity of PCR. Nucleic Acids Res. **23:**3343–3344.
6. **Chow, V. T. K., Y. C. Chan, R. Yong, K. M. Lee, L. K. Lim, Y. K. Chung, S. G. Lam-Phua, and B. T. Tan.** 1998. Monitoring of dengue viruses in field-caught *Aedes aegypti* and *Aedes albopictus* mosquitoes by a type-specific polymerase chain reaction and cycle sequencing. Am. J. Trop. Med. Hyg. **58:**578–586.
7. **Chungue, E., C. Roche, M.-F. Lefevre, P. Barbazan, and S. Chanteau.** 1993. Ultra-rapid, simple, sensitive, and economical silica method for extraction of dengue viral RNA from clinical specimens and mosquitoes by reverse transcriptase-polymerase chain reaction. J. Med. Virol. **40:**142–145.
8. **Deubel, V., M. Laille, J. P. Hugnot, E. Chungue, J. L. 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.
9. **Gubler, D. J., and G. G. Clark.** 1995. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg. Infect. Dis. **1:**55–57.
10. **Gubler, D. J., and D. W. Trent.** 1994. Emergence of epidemic dengue/dengue hemorrhagic fever as a public health problem in the Americas. Infect. Agents Dis. **2:**383–393.
11. **Halstead, S. B.** 1988. Pathogenesis of dengue: challenges to molecular biology. Science **239:**476–481.
12. **Harris, E.** 1996. Developing essential scientific capability in countries with limited resources. Nat. Med. **2:**737–739.
13. **Harris, E.** A low-cost approach to PCR: appropriate transfer of biomolecular techniques, in press. Oxford University Press, New York, N.Y.
14. **Harris, E., M. López, J. Arévalo, J. Bellatin, A. Belli, J. Moran, and O. Orrego.** 1993. Short courses on DNA detection and amplification in Central and South America: the democratization of molecular biology. Biochem. Educ. **21:**16–22.
15. **Henchal, E., S. Polo, V. Vorndam, C. Yaemsiri, B. Innis, and C. Hoke.** 1991. Sensitivity and specificity of a universal primer set for the rapid diagnosis of dengue virus infections by polymerase chain reaction and nucleic acid hybridization. Am. J. Trop. Med. Hyg. **45:**418–428.
16. **Igarashi, A.** 1985. Mosquito cell cultures and the study of arthropod-borne togaviruses. Adv. Virus Res. **30:**21–39.
17. **Kouri, G., M. Valdez, L. Arguello, M. G. Guzman, L. Valdes, M. Soler, and J. Bravo.** 1991. Dengue epidemic in Nicaragua, 1985. Rev. Inst. Med. Trop. São Paulo **33:**365–371.
18. **Lanciotti, R., C. Calisher, D. Gubler, G. Chang, and V. Vorndam.** 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J. Clin. Microbiol. **30:**545–551.
19. **MacPherson, I. A., and M. G. P. Stoker.** 1962. Polyoma transformation of hamster cells—an investigation of genetic factors affecting cell competence. Virology **16:**147–151.
20. **Meiyu, F., C. Huosheng, C. Cuihua, T. Xiaodong, J. Lianhua, P. Yifei, C. Weijun, and G. Huiyu.** 1997. Detection of flaviviruses by reverse transcriptase-polymerase chain reaction with the universal primer set. Microbiol. Immunol. **41:**209–213.
21. **Moleon-Borodowsky, I., and L. E. Plaza.** Personal communication.
22. **Monath, T. P., and F. X. Heinz.** 1996. Flaviviruses, p. 961–1034. *In* B. N. Fields, D. M. Knipe, P. M. Howley, et al. (ed.), Fields virology. Lippincott-Raven Publishers, Philadelphia, Pa.
23. **Morens, D. M.** 1994. Antibody-dependent enhancement of infection and the pathogenesis of viral disease. Clin. Infect. Dis. **19:**500–512.
24. **Morita, K., M. Tanaka, and A. Igarashi.** 1991. Rapid identification of dengue virus serotypes by using polymerase chain reaction. J. Clin. Microbiol. **29:**2107–2110.
25. **Mytelka, D. S., and M. J. Chamberlin.** 1996. Analysis and suppression of DNA polymerase pauses associated with a trinucleotide consensus. Nucleic Acids Res. **24:**2774–2781.
26. **Pao, C., D.-S. Yao, C.-Y. Lin, and C.-C. King.** 1992. Amplification of viral RNA for the detection of dengue types 1 and 2 virus. J. Infect. **24:**23–29.
27. **Peredo, C., T. Garron, J. L. Pellegrino, E. Harris, and A. Gianella.** Detection and identification of dengue 2 virus from Santa Cruz, Bolivia, by a one-step RT-PCR method. Submitted for publication.
28. **Pierre, V., M.-T. Drouet, and V. Deubel.** 1994. Identification of mosquito-borne flavivirus sequences using universal primers and reverse transcriptase/

polymerase chain reaction. Res. Virol. **145:**93–104.
29. **Rosen, L., and D. Gubler.** 1974. The use of mosquitoes to detect and propagate dengue viruses. Am. J. Trop. Med. Hyg. **23:**1153–1160.
30. **Seah, C. L. K., V. T. K. Chow, H. C. Tan, and Y. C. Chan.** 1995. Rapid, single-step RT-PCR typing of dengue viruses using five NS3 gene primers. J. Virol. Methods **51:**193–200.
31. **Torres, O.** Personal communication.
32. **Vodkin, M. H., T. Streit, C. J. Mitchell, G. L. McLaughlin, and R. J. Novak.** 1994. PCR-based detection of arboviral RNA from mosquitoes homogenized in detergent. BioTechniques **17:**114–116.
33. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76:**615–619.
34. **Yenchitsomanus, P. T., P. Sricharoen, I. Jaruthasana, S. N. Pattanakitsakul, S. Nitayaphan, J. Mongkolsapaya, and P. Malasit.** 1996. Rapid detection and identification of dengue viruses by polymerase chain reaction (PCR). Southeast Asian J. Trop. Med. Public Health **27:**228–236.