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APPLICATION OF MOLECULAR TYPING TECHNIQUES IN THE 1998 DENGUE EPIDEMIC IN NICARAGUA

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Abstract. This report presents the results of applying the reverse transcriptase–polymerase chain reaction (RT-PCR) to the analysis of clinical specimens during the 1998 dengue epidemic in Nicaragua. The RT-PCR was validated through comparison with viral isolation, resulting in a sensitivity of 100% and a specificity of 90%. In-country application of the RT-PCR permitted the rapid identification of dengue-3 virus as the cause of the epidemic at the beginning of 1998 and the detection of the reintroduction of dengue-2 virus in the middle of the year. Nineteen isolates of dengue-3 and one of dengue-2 were characterized using the restriction site-specific (RSS)–PCR technique. This showed that the dengue-3 strain belonged to the ''Sri Lanka'' subtype and that the dengue-2 strain belonged to the ''Jamaica'' subtype, both of which have been associated with hemorrhagic dengue in the Americas. The application of these simple PCR-based strain typing methods in a country endemic for dengue virus infections can help to characterize the transmission dynamics of this important emerging infectious disease problem and provide this information to local health authorities in a timely manner so that appropriate control measures can be implemented.

Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are the most important mosquito-borne viral diseases affecting humans worldwide and constitute a major public health problem in tropical and subtropical regions. The 4 serotypes of dengue virus (DV), a member of the *Flaviviridae* family of single-stranded RNA viruses, cause a wide spectrum of disease in humans from the acute febrile illness DF to the life-threatening DHF/DSS. It is estimated that 2.5 billion people are at risk for dengue infection, of which nearly 100 million people are infected with DV and 250,000 progress to DHF/DSS annually.[1] In Latin America, there has been an alarming increase in dengue transmission over the past 20 years, particularly in urban environments.[2]

Dengue has become one of the principal health problems in Nicaragua. It affects hundreds of people annually during the rainy season, when the density of the mosquito vector increases, and epidemics affecting thousands of people occur periodically. In 1985, the first epidemic of dengue in Nicaragua occurred, caused by serotypes 1 and 2, and 17,000 cases including 7 deaths were reported.[3] After that, sporadic cases were observed until 1990, when more than 4,000 cases were reported as a result of the introduction of serotype 4.[4] At the end of 1994 and during the rainy season of 1995, more than 20,000 cases of dengue were notified, caused principally by the circulation of dengue-3.[4,5] During 1996 and 1997, the incidence of dengue was relatively low; however, at the beginning of 1998, the number of cases increased abruptly and remained high throughout the year.

Seroepidemiologic surveillance of dengue is performed mainly by the detection of DV-specific IgM antibodies, while the detection of circulating serotypes is done traditionally by viral isolation and identification.[6,7] Over the past few years, reverse transcriptase–polymerase chain reaction (RT-PCR) assays have been used for the identification of DV serotypes in supernatants of infected cells and in clinical specimens.[5,8–12] This technique is useful for rapidly obtaining information about circulating DV serotypes. However, it is important to isolate the virus to confirm its identity and to study it in more detail. Each DV serotype is further divided into subtypes based on genetic variation. These subtypes are studied to understand the origin and evolution of viral strains and to investigate the correlation between DV subtypes and disease severity of the resulting epidemic. To determine the subtype, regions of the viral genome are usually sequenced to generate phylogenetic trees using sophisticated and labor-intensive methods.[13–19] Recently, a simple PCR-based method was developed to identify DV subtypes, called restriction site-specific (RSS)-PCR.[20]

In this paper, we present the application of RT-PCR and RSS-PCR in Nicaragua during the dengue epidemic of 1998 to rapidly identify the circulating DV serotypes and subtypes and use the information obtained from such analyses to contribute to improved understanding and management of the epidemic.

MATERIALS AND METHODS

Origin and processing of clinical specimens. Serum specimens from patients suspected of having dengue are regularly sent to the Virology Laboratory of the Centro Nacional de Diagnóstico y Referencia (CNDR) of the Nicaraguan Ministry of Health for routine diagnosis from the National Surveillance System and from sentinel hospitals and health centers. The leftover portion of a random selection of these samples was analyzed by RT-PCR (n = 520). All samples were coded and no personal identifiers were used. Only blood specimens drawn within the first 4 days since onset of symptoms were analyzed. Of these 520 specimens, 116 were also analyzed by viral isolation for the validation of the RT-PCR assay, and 20 viral isolates were analyzed by RSS-PCR to determine the genetic subtype.

Extraction of viral RNA. Viral RNA was extracted as previously described.[5] Briefly, 300 μl of serum were mixed sequentially with 300 μl of lysis buffer (6 M guanidinium isothiocyanate, 50 mM sodium citrate, 1% Sarkosyl, 20 μg/ml of *Escherichia coli* tRNA, 100 mM β-mercaptoethanol), 60 μl of 2 M sodium acetate (pH 4), 600 μl of water-saturated phenol, and 240 μl of chloroform. After 15 min of centrifugation at 10,000 rpm, the aqueous phase was trans-

ferred to a new tube and mixed with an equal volume of isopropanol. After a 20-min centrifugation at 10,000 rpm at 4°C, the pellet was washed with 75% ethanol, dried, and resuspended in 20 μl of RNase-free distilled water.

Reverse transcription and PCR amplification. A simplified single-tube procedure was used to reverse transcribe and subsequently amplify viral RNA.[5] Five microliters of extracted RNA were added to 20 μl of an RT-PCR mixture consisting of 50 mM potassium chloride, 10 mM Tris (pH 8.5), 0.01% gelatin, 200 μM of each of the 4 deoxynucleotide triphosphates, 1.5 mM magnesium chloride, 30 mM tetramethylammonium chloride, 0.5 M betaine, 5 mM dithiothreitol, 1 μM of the 5′ primer D1 and 3′ primer TS1, 0.5 μM of each of the 3′ primers TS2, TS3, and DEN4, 0.005 units/μl of reverse transcriptase RAV-2 (Amersham Corp., Arlington Heights, IL) and 0.025 units/μl of *Taq* DNA polymerase (AmpliTaq®; Perkin Elmer Corp., Foster City, CA). Reverse transcription was performed at 42°C for 60 min, followed directly by 40 cycles of amplification consisting of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, ending with a final extension of 72°C for 5 min. The reverse transcription/amplification was performed in 0.5-μl tubes in a thermocycler model UNO (Biometra, Göttingen, Germany). Ten microliters of the product were analyzed by electrophoresis in a 1.5% agarose gel in 1× TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) containing 4 ng/ml of ethidium bromide. The expected size of the amplified products was as follows: 482 basepairs for dengue-1, 119 basepairs for dengue-2, 290 basepairs for dengue-3, and 389 basepairs for dengue-4. Positive controls for the reverse transcriptase-PCR included RNA extracted from reference strains of each of the 4 serotypes and the plasmid pBD3L,[5] which produces an amplicon of 350 basepairs with primers D1 and TS3 and serves as an additional control for the amplification process.

Restriction site-specific–polymerase chain reaction. The protocol described by Harris and others[20] for PCR-based subtyping of dengue-2 and dengue-3 viruses was followed. Extraction of RNA was performed as described above, using 300 μl of supernatants of cells infected with previously isolated viral strains. Each reaction consisted of 5 μl of extracted RNA combined with 45 μl of a PCR mixture prepared exactly as above, except that 0.025 units/μl of reverse transcriptase RAV-2 and 0.5 μM of each of 4 RSS-PCR primers (RSS1–4 for dengue-2 and RSS5–8 for dengue-3) were used. Reverse transcription and amplification were performed in a single tube as above, except with 30 cycles of amplification. Ten microliters of the products were analyzed by electrophoresis as above. The RSS-PCR patterns were interpreted as previously described.[20]

Viral isolation and identification. Tissue culture flasks (25 cm^3) containing *Aedes albopictus* C6/36 cells[21] were inoculated with serum from suspected patients (diluted 1/20 in sterile phosphate-buffered saline) and incubated at 34°C for 10 days in minimal essential medium (Gibco-BRL) containing Earle's salts, L-glutamate, non-essential amino acids, 0.11% sodium bicarbonate, 105 U/ml of penicillin, 75 U/ml of streptomycin, and 2% fetal bovine serum. The cells were fixed with acetone (stored at −20°C) on slides to perform indirect immunofluorescence using the technique described by Gubler and others.[22] First, a polyclonal antibody (a gamma-globulin fraction of human serum with high hemagglutination titers of anti-DV antibodies) was used to determine infection with DV. With positive samples, the process was repeated with monoclonal antibodies (D1: 15F3–1; D2: 3H5–1; D3: 5D4–11; D4: 1H10–6; American Type Culture Collection, Manassas, VA) to identify the serotype.

RESULTS

Validation of the RT-PCR assay. To confirm the utility of RT-PCR for detection and typing of dengue virus, a comparison with viral isolation was performed. Of 116 samples processed by both methods, 38 were positive by viral isolation. Of these, all 38 were positive by RT-PCR, yielding a sensitivity of 100%. Of 78 specimens negative by viral isolation, 70 were also negative by RT-PCR, for a specificity of 90% for the RT-PCR technique. No samples were negative by RT-PCR and positive by viral isolation, whereas 8 samples were positive by RT-PCR when no virus was isolated. Of these latter samples, 2 demonstrated high titers of IgM, and 2 contained high titers of IgG.

Application of the RT-PCR assay to identify circulating DV serotypes. The RT-PCR assay was used to analyze clinical samples during the 1998 epidemic in Nicaragua to identify the temporal and geographic distribution of DV serotypes. Figure 1A shows the results of RT-PCR analysis of the first cases that were reported during the month of January 1998. As seen in lanes 4, 6, 9, 10, and 11, serotype 3 was the cause of the epidemic at this time. These positive samples were later confirmed by viral isolation. During the month of July, the introduction of a new serotype (dengue-2) was detected, as shown in Figure 1B and Table 1. In Figure 1B, the results of 3 dengue-3 positive samples are presented (lanes 5, 6, and 11) and 2 dengue-2 positive samples (lanes 9 and 12), all of which are from the month of July. Of a total of 520 samples processed, 129 (25%) were positive by RT-PCR. Table 1 shows the temporal and geographic distribution of the strains analyzed. Dengue-3 was the dominant serotype throughout the epidemic, while in the middle of the year dengue-2 was detected in the city of Managua and later spread to other cities in the Pacific region of the country (Granada, León, and Chinandega).

Identification of dengue-2 and dengue-3 subtypes. Each dengue serotype consists of various subtypes, depending on genetic variation. A new technique, RSS-PCR,[20] was used to determine the subtypes of the dengue-2 and dengue-3 strains circulating in Nicaragua in 1998. Figure 2 (lanes 1–4) shows the application of RSS-PCR to analyze dengue-2 subtypes. Lane 1 contains a member of RSS-PCR type F, which coincides with the ''Puerto Rico'' subtype[18] and which represents the endemic dengue-2 subtype in Latin America.[23] Lane 2 contains a Thai strain (RSS-PCR type B2), and lane 3 contains a strain belonging to the ''Jamaica'' subtype (RSS-PCR type A), which was introduced to the Caribbean in 1981 and later spread throughout Central and South America. Lane 4 shows the result of the amplification of a Nicaraguan dengue-2 strain isolated in November 1998. The Nicaraguan isolate generated the RSS-PCR type A pattern, which was similar to the ''Jamaica'' subtype. Lanes 6–12 demonstrate the RSS-PCR analysis of dengue-3. Lanes 6–9 contain dengue-3 strains isolated in Nicaragua throughout the 1998 epidemic. Lanes 10–12 contain the patterns of den-

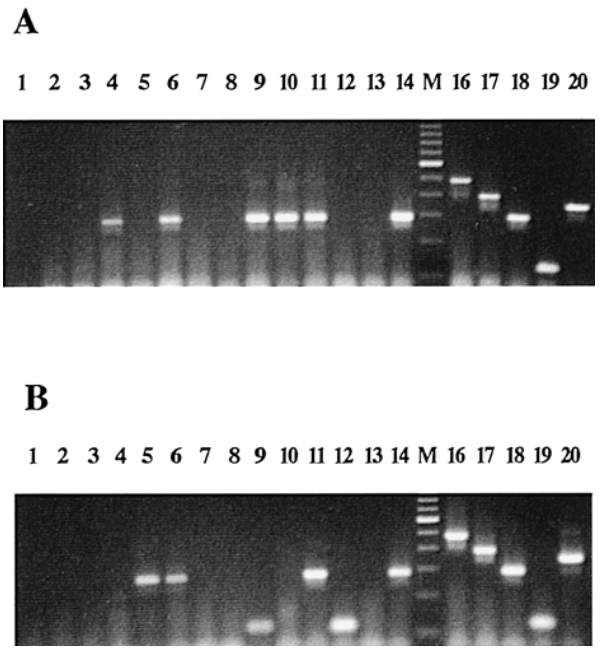

FIGURE 1. Reverse transcriptase–polymerase chain reaction analysis of serum from patients suspected of dengue infection in Nicaragua. **A,** samples from the beginning of the epidemic in January 1998, showing the presence of dengue-3. Lane 1, negative reagent control (water); lane 2, negative amplification control (water); lane 3, negative extraction control (water); lane 4, #0101; lane 5, #0161; lane 6, #0137; lane 7, #0171; lane 8, #0173; lane 9, #0120; lane 10, #0218; lane 11, #0221; lane 12, #0174; lane 13, #0176; lane 14, positive extraction control (dengue-3); lane M, 100-basepair [bp] ladder (Gibco–BRL, Gaithersburg, MD); lane 16, positive amplification control (dengue-1); lane 17, positive amplification control (dengue-4); lane 18, positive amplification control (dengue-3); lane 19, positive amplification control (dengue-2); lane 20, positive amplification control (plasmid pBD3L).[5] **B,** samples from July and August 1998, demonstrating the introduction of dengue-2. Lane 1, negative reagent control (water); lane 2, negative amplification control (water); lane 3, negative extraction control (water); lane 4, #5198; lane 5, #5270, lane 6, #5392; lane 7, #5000; lane 8, #5001; lane 9, #6978; lane 10, #5195; lane 11, #5428; lane 12, #5426; lane 13, #5196; lane 14, positive extraction control (dengue-3); lane M, 100-bp ladder; lane 16, positive amplification control (dengue-1); lane 17, positive amplification control (dengue-4); lane 18, positive amplification control (dengue-3); lane 19, positive amplification control (dengue-2); lane 20, positive amplification control (plasmid pBD3L).

gue-3 controls: a strain belonging to RSS-PCR type C, designated the ''Sri Lanka'' subtype by other methods[16] (lane 10), a strain of RSS-PCR type B, which contains isolates from Indonesia and the Philippines (lane 11), and a member of RSS-PCR type A, which contains viruses from Thailand (lane 12). As can be observed, the Nicaraguan dengue-3 strains belong to RSS-PCR type C, which corresponds to group III of Lanciotti and others;[16] all the Nicaraguan dengue-3 strains analyzed fell into this group. Note that the RSS-PCR patterns of Nicaraguan isolates of both dengue-2 and dengue-3 appear to contain an extra amplified fragment that is not seen in the pattern of the control strains (~180 basepairs for dengue-2 and ~130 basepairs for dengue-3).

DISCUSSION

Virologic surveillance of dengue is traditionally performed via viral isolation and serotype identification, an expensive, time-consuming, and labor-intensive technique, which often requires more than 7 days to obtain results. This technique also requires that specimens be collected, transported, and stored properly so as to ensure that the virus remains viable and the sample remains sterile. Over the last several years, RT-PCR has been applied to detection and typing of dengue virus,[5,8–12] but until now, this tool has been used mostly on an experimental basis and not as a routine method in endemic countries. We have adapted the RT-PCR technique for routine use in dengue surveillance in the Virology Laboratory of the CNDR of the Nicaraguan Ministry of Health, simplifying it and reducing the cost of the process.[5,24]

Comparison of the RT-PCR assay, as performed under the existing conditions at the CNDR, with viral isolation (considered the reference standard) confirmed the utility of RT-PCR, since it resulted in high sensitivity (100%) and specificity (90%) compared with viral isolation. It is important to point out that of the 8 discordant cases (positive by RT-PCR and negative by viral isolation), 2 contained IgM antibodies and another 2 demonstrated high levels of IgG. Therefore, it is likely that in these cases the virus was neutralized, which prevented its isolation but not its detection by RT-PCR. The other 4 discordant cases probably resulted from improper specimen transportation and storage, which compromised the ability of the virus to be isolated, rather than from false-positive PCR results, since all the necessary precautions were taken to avoid contamination of the samples during the PCR procedure[24,25] and the results were repeated several times on different days with identical results. Thus, we believe that the RT-PCR is actually more sensitive than viral isolation under these conditions, and equally specific.

Routine application of RT-PCR was useful for understanding the 1998 dengue epidemic in Nicaragua. After 2 years of relatively low incidence, the number of dengue cases (confirmed by the detection of specific IgM antibodies) increased abruptly in the beginning of 1998. This was unexpected, since the dengue season usually begins later in the year (May) and since a seroepidemiologic survey carried out the previous year revealed a high level of dengue virus antibodies in the population (de los Reyes J, Balmaseda A, Huelva G, Gutiérrez CM, Cerda S, Amador JJ, unpublished results). The reintroduction of a serotype that had not circulated for a long time in Nicaragua was considered probable because dengue-3 had circulated continuously since 1994 and was therefore not expected to be causing such outbreaks.[4,5] However, when RT-PCR was used to analyze the first samples at the beginning of the epidemic, it was demonstrated within 2 days that the serotype responsible was in fact dengue-3 (Figure 1A). These results were later confirmed by viral isolation. The RT-PCR assay was applied routinely during the entire year as part of the national system of dengue viral surveillance; this allowed rapid typing of samples from regions most affected by the epidemic (Table 1). It also increased the quality of surveillance significantly because the conditions in health centers often result in specimens that are handled and stored inappropriately for viral isolation; however, these specimens are nonetheless adequate for RT-PCR.

Surveillance of dengue virus by RT-PCR allowed us to detect the introduction of a new serotype, dengue-2, in the

TABLE 1
Temporal and geographic distribution of dengue virus serotypes in Nicaragua, 1998*

SILAIS†		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Leon	D3	2	–	–	–	–	1	4	10	11	2	2	–	32
	D2	–	–	–	–	–	–	–	–	1	1	1	1	4
Managua	D3	3	–	1	–	–	1	4	10	30	16	2	1	68
	D2	–	–	–	–	–	–	2	2	4	–	1	1	10
Granada	D3	–	–	–	–	–	–	–	–	1	1	–	–	2
	D2	–	–	–	–	–	–	–	–	1	–	–	–	1
Rivas	D3	–	–	–	–	–	–	–	–	–	–	5	–	5
	D2	–	–	–	–	–	–	–	–	–	–	–	–	–
Jinotega	D3	–	–	–	–	–	5	1	–	–	–	–	–	6
	D2	–	–	–	–	–	–	–	–	–	–	–	–	–
Chinandega	D3	–	–	–	–	–	–	–	–	–	–	–	–	–
	D2	–	–	–	–	–	–	–	–	–	–	1	–	1
Total	D3	5	–	1	–	–	7	9	20	42	19	9	1	113
	D2	–	–	–	–	–	–	2	2	6	1	3	2	16

* – = 0.
† Department.

month of July in Managua. Dengue-2 viruses, particularly the Southeast Asian and ''Jamaica'' genotypes, have been specifically associated with DHF[23,26] and therefore often cause increased concern. In addition, a serotype that has not circulated recently in a given population will encounter a larger number of susceptible hosts, many of whom may have experienced a primary infection with a different dengue serotype and may therefore be at increased risk for severe disease, according to the sequential infection theory.[27] Thus, the information about the introduction of dengue-2 was used to mobilize resources within the Nicaraguan Ministry of Health for more aggressive mosquito control so that the dengue crisis could be managed more effectively. The fact that dengue-2 was detected in various regions of Nicaragua after mid-1998 suggested the possibility of dengue-2 circulation the following year, when virus transmission begins again. In light of this information, the Program for Control of Vector-Borne Diseases of the Ministry of Health planned more aggressive antivectorial control measures for 1999.

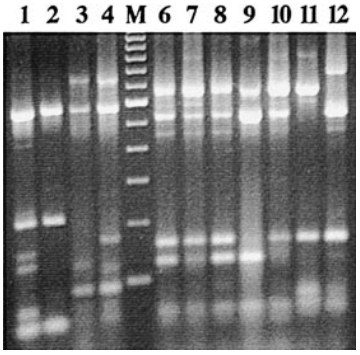

FIGURE 2. Analysis of dengue-2 and dengue-3 subtypes by restriction site-specific–polymerase chain reaction (RSS-PCR). Lanes 1–4, RSS-PCR of dengue-2. Lane 1, RSS-PCR type F (TO74 718); lane 2, RSS-PCR type B2 (TH80 218); lane 3, RSS-PCR type A (PR85 202); lane 4, Nicaraguan dengue-2 isolate #9622 (November 1998); lane M, 100-basepair ladder. Lanes 6–12, RSS-PCR of dengue-3. Lane 6, Nicaraguan dengue-3 #0256 (January, 1998); lane 7, Nicaraguan dengue-3 isolate #4431, (July, 1998); lane 8, Nicaraguan dengue-3 isolate #6627, (August, 1998); lane 9, Nicaraguan dengue-3 isolate #5680 (July, 1998); lane 10, RSS-PCR type C (SL85 393); lane 11, RSS-PCR type B (TH80 267); lane 12, RSS-PCR type A (IN85 316).

The experience of the Virology Laboratory of the CNDR with the application of RT-PCR for detection and typing of dengue virus may be useful to other laboratories in the region that present similar conditions or that cannot afford viral isolation or overseas processing of specimens to determine the circulating dengue serotype(s). However, it is critical to emphasize the importance of validating the RT-PCR assay, as performed under the conditions of each laboratory, against the reference standard (e.g., viral isolation) before implementing its routine use.

Determination of the genotype of circulating dengue viruses is also important, since strains originating in Southeast Asia have been more often associated with severe disease.[16,23] The RSS-PCR strain-typing technique[20] is relatively simple, like any PCR method, and as such is more accessible than sequence analysis and subsequent generation of phylogenetic trees for subtype identification. The application of the RSS-PCR method in Nicaragua allowed us to rapidly determine that the subtype causing the 1998 epidemic was the same dengue-3 virus that had been circulating in Nicaragua since 1994[4,28] and that it was a member of the ''Sri Lanka'' subtype, which is of Asian origin and has been associated with hemorrhagic fever. It also allowed us to quickly detect that the Nicaraguan dengue-2 isolate belonged to the ''Jamaica'' subtype that was introduced to the Americas from Southeast Asia in 1981 and that has been related to outbreaks of DHF in Latin America and the Caribbean. National health authorities were alerted immediately about the danger of the circulation of this viral strain. Interestingly, an additional band was observed in the RSS-PCR patterns generated by the Nicaraguan isolates that was not present in that of the control strains, indicating a certain degree of genetic variation in the Nicaraguan strains; this observation is being further investigated.

The use of simple PCR-based strain typing techniques in place of more expensive and sophisticated alternatives thus enabled a resource-poor country to obtain important epidemiologic information about dengue virus rapidly and on-site. The application of these simplified methods in-country

helped to characterize the transmission dynamics of dengue in real time during an epidemic and to provide this information in a timely fashion to the appropriate health authorities and the national vector control program.

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