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Rapid Subtyping of Dengue Virus Serotypes 1 and 4 by Restriction Site-Specific PCR

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We previously reported a simple subtyping method, restriction site-specific PCR (RSS-PCR), for dengue virus serotypes 2 and 3; here we describe its application for subtyping dengue virus serotypes 1 and 4. Three major RSS-PCR types were observed for dengue virus serotype 1 and two types were observed for dengue virus serotype 4, in agreement with previous strain classifications based on sequence analysis. Because of its simplicity, this method is amenable to rapid subtyping and application to epidemiological studies of dengue in countries where dengue is endemic.

Dengue viruses are single-stranded, enveloped RNA flaviviruses which are traditionally classified into four serotypes, designated dengue-1, -2, -3, and -4, based on antigenic characteristics (2, 19). Numerous methods, including RNase T_1 oligonucleotide fingerprinting, restriction enzyme analysis, and nucleotide sequencing of different genomic fragments, have demonstrated strain variation within each dengue serotype, dividing them into distinct genetic subtypes (4, 5, 10, 11, 12, 13, 16, 17, 18). Dengue virus classification into subtypes is useful for studying the global distribution and movement of dengue serotypes, which contributes to the identification of viral factors that influence disease severity and risk factors associated with the transmission of particular strains.

Accurate characterization of strain difference usually requires labor-intensive typing procedures, which are difficult to perform in a timely manner during epidemic periods. Recently, a new PCR-based approach to rapidly subtype dengue viruses was developed (8) and consists of a single reverse transcriptase PCR (RT-PCR) amplification using four primers that target regions spanning polymorphic endonuclease restriction sites within the envelope (E) gene. This method, called restriction site-specific PCR (RSS-PCR), is simple, rapid, requires minimal laboratory equipment, and uses widely available reagents. The successful results obtained with dengue-2 and dengue-3 led us to develop the RSS-PCR method for dengue-1 and dengue-4 strains.

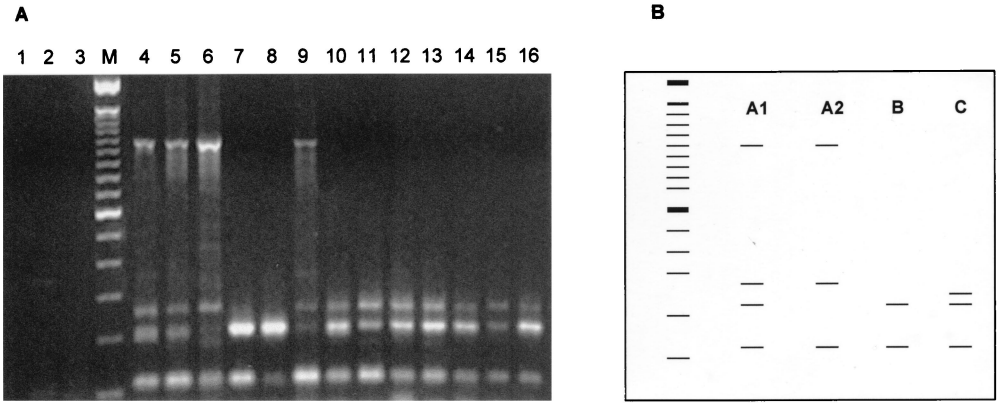

FIG. 1. RSS-PCR patterns of dengue-1 strains of different geographic origins. (A) Agarose gel electrophoresis of RSS-PCR products. Lane 1, dengue-2 Brazil 1998 (64022); lane 2, dengue-3 Indonesia 1989 (430); lane 3, dengue-4 Puerto Rico 1986 (081); lane M, 100-bp ladder (Gibco BRL); lanes 4 to 16, dengue-1 [lane 4, Indonesia 1977 (407-1); lane 5, Philippines 1984 (901); lane 6, Thailand 1979 (213); lane 7, Thailand 1973 (735); lane 8, Thailand 1974 (106); lane 9, Western Pacific 1980; lane 10, Nigeria 1985; lane 11, Sri Lanka 1985; lane 12, Mexico 1982 (086); lane 13, Brazil 1986 (543); lane 14, Brazil RJ 1995 (49217); lane 15, Brazil ES 1998 (60548); lane 16, Brazil BA 1996 (57291)]. Lanes 4, 5, and 9, pattern A1; lane 6, pattern A2; lanes 7 to 8, pattern B; lanes 10 to 16, pattern C. (B) Schematic diagram representing the different RSS-PCR patterns.

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TABLE 1. Dengue-1 strains used in this study[a]

Strain	Location	Yr	RSS pattern	Source
(475)	Philippines	1983	A1	1
(500)	Philippines	1983	A1	1
(506)	Philippines	1983	A1	1
(290)	Philippines	1984	A1	1
(684)	Philippines	1984	A1	1
(901)	Philippines	1984	A1	1
(933)	Philippines	1984	A1	1
(094)	Thailand	1973	B	1
(735)	Thailand	1973	B	1
(106)	Thailand	1974	B	1
(595)	Thailand	1975	A1	1
(213)	Thailand	1979	A2	1
(101)	Thailand	1980	A2	1
(213)	Thailand	1980	A2	1
(266)	Thailand	1980	B	1
(878)	Thailand	1980	B	1
(190-1)	Indonesia	1976	A1	1
(448-1)	Indonesia	1976	A1	1
(406-1)	Indonesia	1977	A1	1
(407-1)	Indonesia	1977	A1	1
(077-1)	Indonesia	1978	A1	1
(159-1)	Indonesia	1978	A1	1
(086)	Mexico	1982	C	1
(894)	Mexico	1982	C	1
(679)	Mexico	1983	C	1
(598)	Mexico	1984	C	1
(022)	Mexico	1985	C	1
(350)	Mexico	1985	C	1
(210)	Mexico	1986	C	1
(454)	Mexico	1986	C	1
(666)	Mexico	1988	C	1
(461)	Brazil	1986	C	1
(543)	Brazil	1986	C	1
(578)	Brazil	1986	C	1
(426)	Aruba	1985	C	1
(495)	Aruba	1985	C	1
(810)	Aruba	1986	C	1
(648)	Puerto Rico	1985	C	1
(405)	Puerto Rico	1986	C	1
(009)	Puerto Rico	1986	C	1
(454)	Puerto Rico	1986	C	1
	Sri Lanka	1985	C	1
	Nigeria	1985	C	1
	Jamaica	1981	C	1
	Western Pacific	1980	A1	1
28605	RJ (Brazil)	1986	C	2
28641	RJ (Brazil)	1986	C	2
27923	RJ (Brazil)	1986	C	2
32426	RJ (Brazil)	1986	C	2
39372	RJ (Brazil)	1990	C	2
39474	RJ (Brazil)	1990	C	2
49657	RJ (Brazil)	1995	C	2
53378	RJ (Brazil)	1995	C	2
64313	RJ (Brazil)	1999	C	2
64286	RJ (Brazil)	1999	C	2
64305	RJ (Brazil)	1999	C	2
56722	RJ (Brazil)	1996	C	2
56717	RJ (Brazil)	1996	C	2
58610	RJ (Brazil)	1997	C	2
58542	RJ (Brazil)	1997	C	2
60984	RJ (Brazil)	1998	C	2
60741	RJ (Brazil)	1998	C	2
64289	RJ (Brazil)	1999	C	2
64316	RJ (Brazil)	1999	C	2
51325	RJ (Brazil)	1995	C	2
49217	RJ (Brazil)	1995	C	2
57399	ES (Brazil)	1996	C	2
57217	ES (Brazil)	1996	C	2
59835	ES (Brazil)	1997	C	2
59934	ES (Brazil)	1997	C	2
60548	ES (Brazil)	1998	C	2
60538	ES (Brazil)	1998	C	2
57291	BA (Brazil)	1996	C	2
58067	BA (Brazil)	1997	C	2
58724	RN (Brazil)	1997	C	2
58438	RN (Brazil)	1997	C	2
58485	MG (Brazil)	1997	C	2
58522	MG (Brazil)	1997	C	2
60616	MG (Brazil)	1998	C	2
60619	MG (Brazil)	1998	C	2
60440	CE (Brazil)	1998	C	2
60447	CE (Brazil)	1998	C	2

Continued

TABLE 1—*Continued*

[a] Source 1, School of Public Health, University of California, Berkeley. Strains were originally obtained from the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colo., and the Armed Forces Research Institute of the Medical Sciences, Bangkok, Thailand, and were kindly donated by S. Kliks, University of California, San Francisco. Source 2, Flavivirus Laboratory, Department of Virology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. All strains were isolated during epidemics in the following states of the country: Bahia (BA), Ceará (CE), Espírito Santo (ES), Minas Gerais (MS), Rio de Janeiro (RJ), and Rio Grande do Norte (RN).

Dengue-1 (Table 1) and dengue-4 (Table 2) strains representing a broad geographical distribution were obtained from existing collections; Brazilian and Nicaraguan viruses were isolated from sera by inoculation into the *Aedes albopictus* cell line C6/36 (9) and were identified by immunofluorescence using type-specific monoclonal antibodies (6). Viral seeds were propagated once in C6/36 cells grown in Leibovitz-15 or minimal essential medium (Gibco BRL, Grand Island, N.Y.) containing 10% fetal bovine serum. Primers were designed based on the sequence around polymorphic restriction sites in the E gene region of dengue-1 and dengue-4 as described previously

TABLE 2. Dengue-4 strains used in this study

Strain	Location	Yr	RSS pattern	Source[a]
(H241)	Philippines	1956	A2	1
(377)	Thailand	1977	A2	1
(561)	Thailand	1977	A2	1
(664)	Thailand	1979	A2	1
(840)	Thailand	1980	A2	1
(961)	Thailand	1984	A2	1
(171)	Thailand	1984	A2	1
(013)	Thailand	1984	A2	1
(789)	Thailand	1985	A1	1
(511)	Thailand	1985	A2	1
(280)	Thailand	1988	A2	1
(554)	Mexico	1984	B	1
(410)	Mexico	1984	B	1
(292)	Mexico	1984	B	1
(081)	Puerto Rico	1986	B	1
(437)	Puerto Rico	1986	B	1
(281)	Puerto Rico	1986	B	1
(703)	Nicaragua	1999	B	2

[a] Source 1, School of Public Health, University of California, Berkeley. Source 2, Department of Virology, Centro Nacional de Diagnóstico y Referencia, Ministry of Health, Managua, Nicaragua.

TABLE 3. Sequences and positions of oligonucleotide primers used to amplify dengue-1 and dengue-4 strains

Primer	Sequence	Genome position[a]	Strand
RSS9	5′-CTG TTC TAG TGC AGG TTA	1897–1914	+
RSS10	5′-CAT TTT CCC TAT ACT GCT TCC	2124–2144	−
RSS11	5′-GTC ACA AAC CCT GCC GTC CT	1089–1108	+
RSS12	5′-CGC AGC TTC CAT GCT CCA AT	1013–1032	−
RSS21	5′-GGA C/TCA ACA GTA CAT TTG CCG GA	1196–1218	+
RSS22	5′-GTT TTC ATG CTC GGG GAA GAT	1292–1313	+
RSS23	5′-CTT CTG ATG TGT CTG CTC CT	1604–1623	−
RSS24	5′-GAG AAC TTT CCT GAA/G CAC ATC GT	1836–1858	−

[a] The sequences of primers RSS9 to -12 are from Caribbean strain CV1636/77 (3) (NCBI accession no. D00501), while their genome positions are given according to the nucleotide sequence numbering of strain 45AZ5 (accession no. DVU88537). The sequence of primers RSS21 to -24 are from Philippine strain H-241 (accession no. U18433), while their genome positions are given according to the nucleotide sequence numbering of Dominican strain 814669 (accession no. M14931).

(8). The sequences and genomic positions of primers RSS9 to RSS12 (dengue-1) and RSS21 to RSS24 (dengue-4) are listed in Table 3.

Viral RNAs were extracted from the supernatants of infected cells using a QIAamp Viral RNA Mini Kit (Qiagen, Inc., Valencia, Calif.) according to the manufacturer's protocol or by lysis with guanidine isothiocyanate, extraction with organic solvents, and ethanol precipitation (7). The reaction mixture and electrophoresis conditions were as described previously (8), except that 25-μl reaction volumes were used. Briefly, 2.5 μl of viral RNA was added to 22.5 μl of an RT-PCR mixture consisting of 50 mM potassium chloride, 10 mM Tris (pH 8.5), 0.01% gelatin, 200 μM concentrations of each of the four deoxynucleoside triphosphates, 1.5 mM magnesium chloride, 30 mM tetramethylammonium chloride, 0.5 M betaine, 5 mM dithiothreitol, 0.5 μM concentrations of each of four RSS-PCR primers (RSS9 to -12 for dengue-1 and RSS21 to -24 for dengue-4), 0.025 U of RT RAV-2 (Amersham Corp., Arlington Heights, Ill.) per μl and 0.025 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer Corp., Foster City, Calif.) per μl. Reverse transcription was conducted at 42°C for 60 min, followed directly by 30 amplification cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 5 min. Amplification was conducted with 0.5-ml tubes (USA Scientific, Ocala, Fla.) in a model PTC-200 thermocycler (MJ Research, Inc., Watertown, Mass.).

We selected sets of strains in our collection that represented each of three dengue-1 genotypes previously described by E-NS1 sequence pairwise comparison (13), with one set including strains from Southeast Asia and the South Pacific, another containing viruses from Thailand and Taiwan, and a third containing isolates from the Americas, Africa, and Southeast Asia. The bands generated by the RSS-PCR assay using primers RSS9 to -12 showed distinct patterns for American and Asian strains. Figure 1 shows representative examples of each RSS-PCR pattern for dengue-1 viruses, along with a schematic diagram summarizing the results. The first group (type A), which includes viruses from the Philippines (1983 to 1984), Indonesia (1976 to 1978), Thailand (1975, 1979, and 1980), and the western Pacific (1980), was divided into two subgroups (A1 and A2), depending on the presence of an ~200-bp fragment. The second group (type B) contains isolates from Thailand (1973, 1974, and 1980), and the third group (type C) is composed of strains from the Americas, Africa, and Sri Lanka. These RSS primers are specific for dengue-1, as dengue-2, -3, and -4 did not generate amplified products in this assay (Fig. 1, lanes 1 to 3).

As with dengue-1, several primer sets were tested with dengue-4 strains from our collection that matched strains previously classified by sequence analysis of the E gene (10) with respect to location and date of isolation. The best results were obtained with primers RSS21 to -24 (Table 3), which were used to analyze the rest of our dengue-4 strains. Two patterns were generated, as shown in Fig. 2 and Table 2, one (type A) representing strains from Thailand (1977 to 1988) and the other (type B) representing viruses isolated in Mexico and the Caribbean. Type A was divided into two subgroups due to variation in one of the amplified fragments from a Thai isolate from 1984. Again, no product was obtained with the other three serotypes (Fig. 2).

The RSS-PCR method for dengue-1 and -4 fulfills the requisites of a molecular typing assay: the primers were specific to the serotype to which they were developed, the patterns were stable over time, repeated amplification of the same specimens reproducibly yielded the same results, and geographic and temporal clustering was observed. In addition, in areas with extensive dengue transmission, such as Thailand, the cocirculation of two distinct subtypes and of genetic variants within the same subtype was observed, which is consistent with previous reports (13, 15).

The dengue-1 RSS-PCR results, categorized according to country and year of isolation, are essentially the same as the sequence analysis results showing geographic and temporal clustering. A phylogenetic analysis of 40 dengue-1 strains from different geographic areas based on a 240-nt region from the E-NS1 junction defined three main genotypes and two additional ones, each represented by a single virus isolate (13). Similarly, another study comparing the sequences of a 179-nt region of the E genes of 35 dengue-1 isolates yielded three genotypes (4). The largest genotypic group in both analyses consisted of dengue-1 strains from the Americas, Africa, and Southeast Asia, and it corresponds to our RSS-PCR type C. The second genotype, containing viruses from the Philippines, Indonesia, Thailand, and the South Pacific, coincides with RSS-PCR type A, and the third group, consisting of Thai and Taiwanese viruses, corresponds to RSS-PCR type B. RSS-PCR revealed two distinct subtypes (A and B) circulating simultaneously in Thailand, in agreement with previous observations (13).

The dengue-4 isolates we analyzed by RSS-PCR fell into two subtypes, consistent with the two genotypes that resulted from sequence analysis of the entire E gene (10). By both methods, American isolates were contained in a different group than that of Thai viruses. Another phylogenetic analysis derived from sequence comparison of a small 179-nt region of the E gene revealed two subgroups that differed in sequence by 4.9%, again grouping the isolates from the Americas, South Pacific, and Indonesia separately from those from the Philippines, Sri Lanka, and Thailand (4). That dengue-4 yielded fewer subtypes than dengue-1, -2, and -3 is not surprising, since

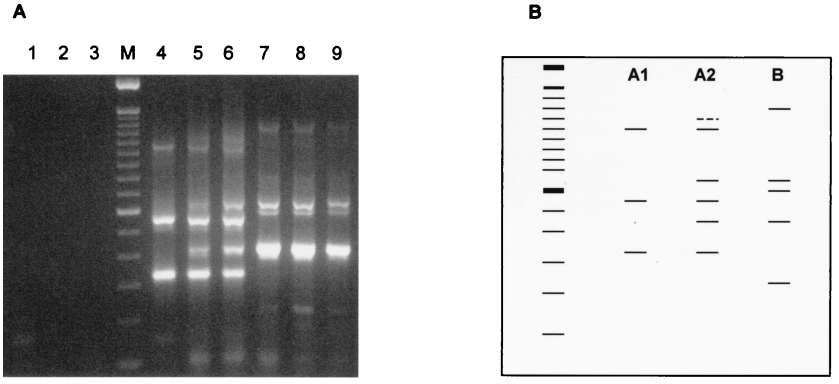

FIG. 2. RSS-PCR patterns of dengue-4 strains of different temporal and geographic origins. (A) Agarose gel electrophoresis of RSS-PCR products. Lane 1, dengue-1 Brazil 1986 (543); lane 2, dengue-2 Brazil 1998 (63731); lane 3, dengue-3 Indonesia 1989 (430); lane M, 100-bp ladder (Gibco BRL); lanes 4 to 9, dengue-4 [lane 4, Thailand 1985 (789); lane 5, Thailand 1977 (377); lane 6, Thailand 1988 (280); lane 7, Puerto Rico 1986 (437); lane 8, Mexico 1984 (440); lane 9, Nicaragua 1999 (703)]. Lane 4, pattern A1; lanes 5 to 6, pattern A2; lanes 7 to 9, pattern C. (B) Schematic diagram representing the different RSS-PCR patterns. The dotted line designates bands that display sample-to-sample variation.

dengue-4 is reported to have less sequence variation in the E gene than the other dengue serotypes (4). A minor difference was observed in pattern A (the absence of a 420 bp-fragment in type A1 compared to A2), indicating a certain degree of genetic variation in Thai isolates (1984).

The occurrence of new dengue epidemics every year emphasizes the need for a simple assay that can facilitate analysis of a large number of samples in order to obtain more detailed epidemiologic information during epidemic periods. The relation between dengue subtype and disease severity has not been extensively studied for dengue-1 or dengue-4 strains, but it warrants further investigation since an association between viruses of Southeast Asian origin and dengue hemorrhagic fever has been reported for dengue-2 and -3 (4, 11, 14). RSS-PCR generates a classification of dengue virus subtypes similar to that obtained using the more labor-intensive and costly sequence analysis approach. In this report, RSS-PCR proved useful in quickly identifying recent isolates of dengue-1 from Brazil (type C) and dengue-4 from Nicaragua (type B). This technique should be valuable as a simple alternative for the rapid characterization of viral isolates and for epidemiologic analysis.

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