Virology 253, 86–95 (1999)
Article ID viro.1998.9481, available online at http://www.idealibrary.com on IDEAL®

Rapid Subtyping of Dengue Viruses by Restriction Site-Specific (RSS)–PCR

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Received July 29, 1998; returned to author for revision September 2, 1998; accepted October 20, 1998

Dengue is a major public health problem worldwide. It is caused by four dengue virus serotypes, each further divided into distinct genetic subtypes. Strain typing is important for understanding the epidemiology and viral factors associated with disease transmission. However, most of the existing subtyping methods are expensive and technically unwieldy for timely, practical applications in developing countries. Here we describe a simple, rapid, PCR-based subtyping method, restriction site-specific (RSS)–PCR, which we used to analyze dengue virus serotypes 2 and 3. For each serotype, four primers targeted to sequences spanning polymorphic endonuclease restriction sites in the envelope gene were used to reverse transcribe and amplify viral RNA. These RT–PCR products generated distinct electrophoretic band patterns for different strains. Analysis of 73 dengue-2 strains and 54 dengue-3 strains representing a broad geographic distribution over several decades revealed that the RSS–PCR fingerprints reproducibly fell into 7 and 3 groups, respectively. These groups correlated well with previous phylogenetic classifications. This one-step assay should be widely accessible and allow more detailed epidemiologic investigations in dengue-endemic countries. This novel PCR approach to subtyping organisms based on restriction site polymorphisms should be applicable to other pathogens. © 1999 Academic Press

INTRODUCTION

Dengue fever and its more severe form, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), are considered among the most important and widespread reemerging infectious diseases in developing countries. Approximately 100 million cases of dengue fever and 250,000 cases of DHF/DSS are estimated to occur annually in tropical regions worldwide, caused by the mosquito-borne dengue virus (Monath and Heinz, 1996). The mechanism of DHF pathogenesis is still poorly understood, but it is influenced by viral determinants of virulence and host factors, including immunopathological processes (Halstead, 1988; Monath, 1994). Dengue virus is a single-stranded, enveloped RNA flavivirus whose ~11-kb genome encodes three structural (C, prM/M, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The dengue viruses are divided into four antigenically distinct serotypes, which in turn can be grouped into several subtypes based on genetic differences detected by sequencing (genotypes) (Deubel *et al.,* 1993; Lanciotti *et al.,* 1994; Lewis *et al.,* 1993; Rico-Hesse, 1990) or RNase T1 oligonucleotide fingerprinting (topotypes) (Henchal *et al.,* 1986; Monath *et al.,* 1986; Trent *et al.,* 1989; Trent *et al.,* 1983; Walker *et al.,* 1988).

When applied to clinical isolates obtained from endemic settings, strain typing is a powerful tool for determining the geographic distribution of strains and understanding the epidemiology of infectious diseases. Strain typing can provide insights into relationships between disease manifestations and biological characteristics of an organism (Friedman *et al.,* 1997). In diseases such as dengue fever and DHF/DSS, where no appropriate animal model exists, this population-based epidemiologic approach to subtyping may identify viral factors that contribute to disease severity. In particular, a rapid molecular strain-typing method that can be applied in a field setting during an epidemic to analyze a large number of strains in a timely fashion may help in understanding dengue virus epidemiology and pathogenesis in more detail.

The existing strain-typing methods based on dengue viral genetic differences are constrained by technical requirements or cost considerations and cannot be easily applied to investigate a dengue epidemic where it is occurring. Thus, even though phylogenetic classifications can be determined by sequencing fragments of the envelope (E) gene (Chungue *et al.,* 1993, 1995; Deubel *et al.,* 1993) or the E/NS1 junction (Rico-Hesse, 1990, 1997) instead of the entire E or NS1 genes (Blok *et al.,* 1991; Lanciotti *et al.,* 1994, 1997; Lewis *et al.,* 1993), sequencing capability is still required. Nonsequencing methods to distinguish strains such as restriction analysis of labeled viral cDNA require radioactivity and large amounts of viral RNA (Vorndam *et al.,* 1994b). Typing by RT–PCR followed by restriction analysis (Vorndam *et al.,* 1994a)

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0042-6822/99 $30.00
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requires expensive and labile restriction enzymes, while single-strand conformation polymorphism (Farfan *et al.*, 1997) involves more elaborate electrophoresis and detection procedures. The method we report here offers simple and rapid typing of strains using widely available reagents and equipment, which should enable investigation of outbreaks of dengue in its endemic setting. This novel approach to strain typing can be applied to other organisms as well, particularly where restriction enzyme polymorphisms are associated with distinct subtypes.

RESULTS

Primer design

The restriction site-specific (RSS)–PCR technique is based on a single RT–PCR amplification using four primers that target regions spanning polymorphic restriction sites. The resulting products are electrophoresed in agarose gels to generate specific patterns of bands. To design primers for dengue-2, we selected a prototype strain (16681) and analyzed the restriction sites in the E gene region, with particular emphasis on long (>6 bp) recognition sequences. The frequency of these sites was assessed in other dengue-2 E gene sequences in the GenBank database. Restriction enzymes that displayed distinct numbers of sites in the different dengue-2 strains were selected, and primers (19–20 nt) were designed around these restriction sites, based on the sequence of the prototype strain (Table 3). Since dengue-3 viruses have less sequence variation than dengue-2 viruses, a larger region of the dengue type 3 genome was examined, encompassing the prM, M, and E genes. All restriction enzymes that displayed polymorphism in site frequency among the dengue-3 sequences in the GenBank database were selected. The sequences around these restriction sites were examined, and those that contained the greatest sequence variation among the strains were chosen as templates for primer design (Table 3).

Analysis of dengue-2 viruses

The dengue viruses used in this study were compared with respect to country and date of isolation to dengue strains that had been previously classified into subtypes (genotypes or topotypes). An initial group of 20 dengue-2 strains in our collection whose geographic source and year of isolation matched those of previously classified strains were analyzed to optimize primers and PCR conditions. The pattern of bands generated for each strain was assigned an RSS–PCR designation (A–G). A representative example of each RSS–PCR pattern for dengue-2 is shown (Fig. 1A), along with a schematic diagram summarizing the results (Fig. 1B). When the resulting RSS–PCR patterns were compared with the subtype designation of the matching strain, distinct patterns were found to correlate with known subtypes. A second set of 53 uncharacterized strains were then tested in a blinded

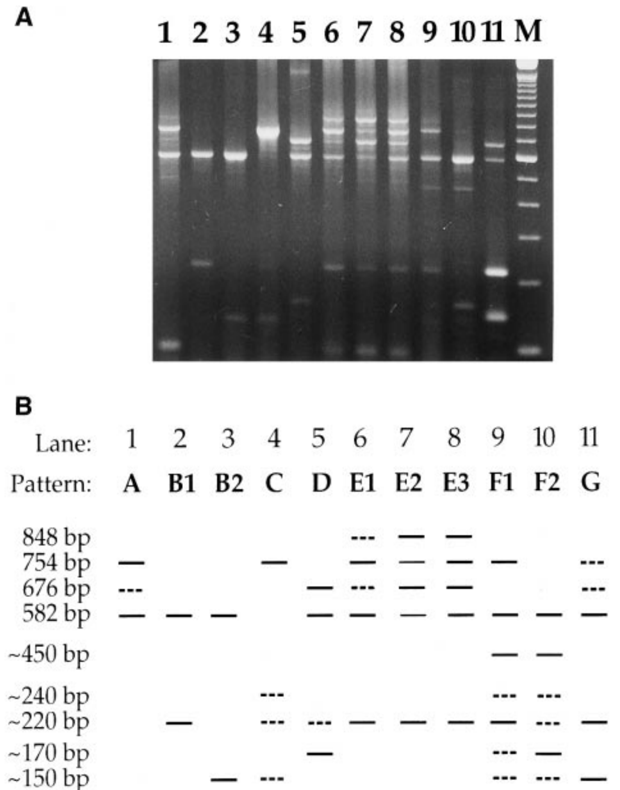

FIG. 1. RSS–PCR patterns of dengue-2 strains of different geographic origins. Viral RNA was extracted, reverse transcribed, and amplified with primers RSS1–RSS4. (A) Agarose gel electrophoresis of RSS–PCR products. Lane 1, Puerto Rico 1986 (1690); lane 2, Thailand 1980 (603); lane 3, Thailand 1980 (454); lane 4, Trinidad 1982 (8211085); lane 5, Philippines 1983 (305); lane 6, Indonesia 1976 (1122); lane 7, Indonesia 1977 (1209); lane 8, Indonesia 1977 (1208); lane 9, Fiji 1971 (491); lane 10, Puerto Rico 1969 (159); lane 11, Republic of Guinea, 1981 (PM33974); lane M, 100-bp ladder (Gibco BRL). (B) Schematic diagram representing the different RSS–PCR patterns. The dotted line designates bands that display sample-to-sample variation. The products predicted by pairs of primers are as follows: RSS3–RSS4, 848 bp; RSS2–RSS3, 754 bp; RSS1–RSS4, 676 bp; RSS1–RSS2, 582 bp.

fashion, and the resulting patterns were assigned an RSS–PCR designation (Table 1). No products were generated from dengue-1, dengue-3, or dengue-4 viruses.

Analysis of the RSS–PCR patterns with respect to the geographic source and date of isolation of the strains revealed geographic and temporal clustering (Table 4). RSS–PCR type A contained post-1981 Caribbean strains from Jamaica (1981–1983), Puerto Rico (1984–1986), and the Dominican Republic (1986). Type B consisted of Thai isolates from 1964–1985 and was divided into two subgroups, depending on the size of the low-molecular-weight fragment. RSS–PCR type C contained three strains from Trinidad isolated in 1978–1982, while type D contained 14 Philippine strains from 1981–1984. Type E was further divided into three patterns, depending upon the relative intensities of the four characteristic fragments, and was made up of dengue-2 viruses from Sri Lanka (1982–1985), Indonesia (1975–1985), the Philippines (1975), and Burkina Faso (1982). RSS–PCR type F

TABLE 1

Dengue-2 Viruses Used in This Study

Strain (CDC No.)	Location	Year	RSS pattern	Source[a]
1349	Burkina Faso	1982	E2	2
1715 (054)	Dominican Republic	1986	A	1
(491)	Fiji	1971	F1	1
(004)	Fiji	1971	F1	1
(545)	Fiji	1971	F1	1
(071)	Fiji	1971	F1	1
(525)	Fiji	1971	F1	1
1051 (070)	Indonesia	1976	E3	1
1261 (162)	Indonesia	1978	E1	1
1208 (880)	Indonesia	1977	E1	1
1013 (978)	Indonesia	1975	E1	1
1016 (453)	Indonesia	1975	E2	1
1017 (885)	Indonesia	1976	E2	1
1039 (621)	Indonesia	1976	E3	1
1174 (480)	Indonesia	1977	E3	1
1209 (608)	Indonesia	1977	E2	1
1121 (832)	Indonesia	1976	E1	1
1256 (285)	Indonesia	1978	E3	1
619 (655)	Indonesia	1985	E1	1
033 (042)	Indonesia	1984	E3	1
1122 (348)	Indonesia	1976	E1	1
1329	Jamaica	1982	A	2
1410 (124)	Jamaica	1983	A	1
594	Jamaica	1981	A	1
1421 (044)	Mexico	1983	F1	1
200787 (563)	Mexico	1983	F1	1
021.AP2/2124 (813)	Philippines	1983	D	1
012.AP2/1207 (483)	Philippines	1984	D	1
031.AP2/2172 (889)	Philippines	1983	D	1
120.AP2/2770 (463)	Philippines	1983	D	1
040.AP3/2201 (519)	Philippines	1983	D	1
072.AP2/2322 (305)	Philippines	1983	D	1
167.AP2/10649 (658)	Philippines	1983	D	1
169.AP2/10665 (731)	Philippines	1983	D	1
S-35179	Philippines	1975	E1	1
036.AP1/12269 (215)	Philippines	1984	D	1
018.AP2/12118 (596)	Philippines	1984	D	1
032.AP2/12214 (768)	Philippines	1984	D	1
060.AP2/3135 (729)	Philippines	1984	D	1
S-10099	Philippines	1981	D	1
020.AP1/2169 (654)	Philippines	1983	D	1
152	Puerto Rico	1969	F2	1
159 (622)	Puerto Rico	1969	F2	2
1328 (489)	Puerto Rico	1977	F1	1
1470 (133)	Puerto Rico	1984	A	1
1642 (202)	Puerto Rico	1985	A	1
1814 (742)	Puerto Rico	1986	A	1
1692 (728)	Puerto Rico	1986	A	1
1690 (160)	Puerto Rico	1986	A	1
1710 (555)	Puerto Rico	1985	A	1
1781 (411)	Puerto Rico	1986	A	1
PM33974	Rep. Guinea	1981	G	2
HD10674	Senegal	1970	G	2
1592 (975)	Sri Lanka	1985	E2	1
1355 (180)	Sri Lanka	1982	E2	1
1674 (782)	Sri Lanka	1985	E2	1
1672 (532)	Sri Lanka	1985	E2	1
1583 (629)	Sri Lanka	1985	E2	1
S-7848 (D-15)	Tahiti	1971	F1	1
1250 (016)	Tahiti	1972	F1	1
S-7850 (D-32)	Tahiti	1971	F1	1
218	Thailand	1980	B2	1

TABLE 1—*Continued*

Strain (CDC No.)	Location	Year	RSS pattern	Source[a]
16681	Thailand	1964	B2	1
244	Thailand	1980	B2	1
215	Thailand	1980	B2	1
454	Thailand	1980	B2	1
603	Thailand	1980	B1	1
158	Thailand	1980	B2	1
135	Thailand	1980	B1	1
1251 (718)	Tonga	1974	F1	1
789859 (144)	Trinidad	1978	C	1
818394 (419)	Trinidad	1981	C	1
8211085 (801)	Trinidad	1982	C	1

[a] (1) S. Kliks, University of California, San Francisco; this collection of viral strains was originally obtained from the Division of Vector-Borne Infectious Diseases, Center for Disease Control and Prevention, Fort Collins, and the Armed Forces Research Institute of the Medical Sciences (AFRIMS), Bangkok, Thailand.
(2) R. Rico-Hesse, Southwest Foundation for Biomedical Research (San Antonio, Texas).

contained viruses from the South Pacific [Tahiti (1971–1972), Tonga (1974), and Fiji (1971)] as well as pre-1981 isolates from Puerto Rico (1969–1977). Finally, type G contained two African isolates, from the Republic of Guinea (1981) and Senegal (1970). These clusters correlated well with previous classifications based on sequence analysis, oligonucleotide fingerprinting, and antigen signature analysis (Table 5).

Epidemiologic and clinical correlations of dengue-2 RSS–PCR types

The global distribution of dengue-2 subtypes can be traced using RSS–PCR. For instance, the putative introduction and spread of a new genotype in Latin America and the Caribbean is documented in Fig. 2. The "native" American dengue-2 genotype (Rico-Hesse *et al.,* 1997), represented by Puerto Rico (1969 and 1977), is similar to South Pacific isolates [e.g., Tahiti (1972)], and all are typed as RSS–PCR type F (Fig. 2, lanes 1–3). In 1981, a new genotype of dengue-2 was introduced into the Caribbean [lane 4, Jamaica (1982)] and disseminated throughout the region [lane 5, Puerto Rico (1986), and lane 6, Dominican Republic (1986)] (Deubel *et al.,* 1993; Gubler and Trent, 1994; Rico-Hesse, 1990). The members of this genotype fall into RSS–PCR type A. However, it took some years for the new subtype to replace the original one, as evidenced by the presence of the "native" subtype (F) in Mexico in 1983 (lane 7). As another example, the previously proposed hypothesis that dengue fever in Burkina Faso (1982) was caused by strains that had originated in Sri Lanka or India (Deubel, 1992; Rico-Hesse, 1990) is consistent with the RSS–PCR classification of both Sri Lankan strains (1982–1985) and a 1982 isolate from Burkina Faso as RSS–PCR type E2 (Table 4).

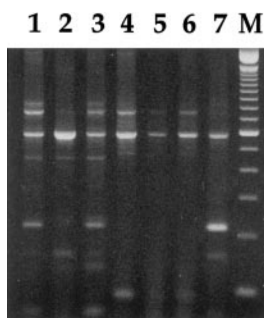

FIG. 2. Introduction of a new dengue-2 subtype into the Americas, as indicated by RSS–PCR patterns. Viral RNA was extracted, reverse transcribed, and amplified with primers RSS1–RSS4. Lane 1, Tahiti 1972 (1250); lane 2, Puerto Rico 1969 (159); lane 3, Puerto Rico 1977 (1328); lane 4, Jamaica 1982 (1329); lane 5, Puerto Rico 1986 (742); lane 6, Dominican Republic 1986 (1715); Mexico 1983 (200787); lane M, 100-bp ladder (Gibco BRL).

Analysis of clinical information available about the strains in our collection ($n = 49$) revealed that post-1981 Caribbean and Southeast Asian RSS–PCR types A and B, as well as types D (Philippines) and E (Indonesia and Sri Lanka), caused both dengue fever and DHF, as expected. However, the strains in RSS–PCR type F ($n = 9$) were associated only with dengue fever, consistent with reports that this "native" American genotype is correlated only with less severe disease (Rico-Hesse *et al.,* 1997).

Analysis of dengue-3 viruses

The dengue-3 strains in our collection were compared with respect to country and date of isolation to strains that had been classified into genotypes by sequence analysis of the E gene or fragments thereof (Chungue *et al.,* 1993; Lanciotti *et al.,* 1994). An initial set of 12 strains was examined using various combinations of primers designed to target polymorphic regions surrounding restriction sites. Three reproducible patterns were identified (A–C; Figs. 3A and 3B). No amplified fragments were obtained from dengue virus types 1, 2, and 4. An additional set of 42 uncharacterized dengue-3 strains was then analyzed, and the results are given in Table 2. RSS–PCR type A consisted of Indonesian and Philippine

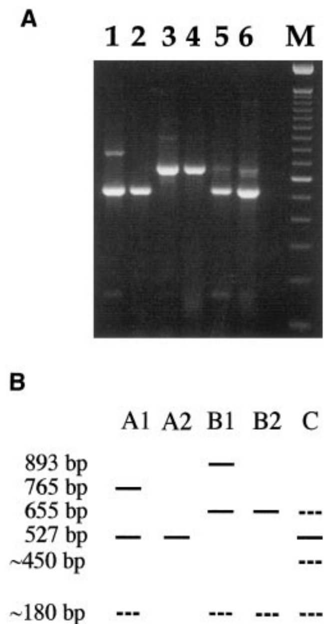

FIG. 3. RSS–PCR patterns of dengue-3 viruses of different geographic origins. Viral RNA was extracted, reverse transcribed, and amplified with primers RSS5–RSS8. (A) Agarose gel electrophoresis of RSS–PCR products. Lane 1, Indonesia 1977 (796); lane 2, Indonesia 1976 (847); lane 3, Thailand 1974 (498); lane 4, Thailand 1984 (500); lane 5, Sri Lanka 1981 (805); lane 6, Nicaragua 1998 (256); lane M, 100-bp ladder (Gibco BRL). (B) Schematic diagram representing the different RSS–PCR patterns. The dotted line designates bands that display sample-to-sample variation. The products predicted by pairs of primers are as follows: RSS5–RSS8, 893 bp; RSS6–RSS8, 765 bp; RSS5–RSS7, 655 bp; RSS6–RSS7, 527 bp.

isolates collected in 1976–1985 and 1984, respectively. RSS–PCR type B contained strains from Thailand (1973–1985) and the Philippines (1956), while type C contained strains from Sri Lanka (1981–1985) and recent isolates from Central America [Nicaragua (1995–1998), Guatemala (1997), and El Salvador (1998)]. These assignments correlated with previous classifications determined by sequence analysis of the E gene (Lanciotti *et al.,* 1994). Unfortunately, our strain collection did not contain isolates from subtype IV [Puerto Rico (1963 and 1977) and Tahiti (1965)]; thus, we were unable to determine the RSS–PCR pattern of this group. Strains from the current epidemic of dengue in Nicaragua were typed by RSS–PCR and continue to be processed on site as they are isolated. Identification of these strains, as well as a 1995 Nicaraguan isolate, as RSS–PCR type C (Fig. 3, lane 6; Table 2) is consistent with genetic classification of a 1994 strain from the Central American region as the Sri Lanka/India genotype (CDC, 1995).

DISCUSSION

Typing pathogens based on genetic differences can reveal important information related to disease transmission. The ability to detect a particular genotype in a community may identify risk factors associated with transmission of that strain. Characterization of the geographic distribution of different genotypes or clades can help in designing appropriate vaccine candidates and trial sites, as is being done with the human immunodeficiency virus (HIV). In addition, the detection of genetic clusters of an infectious agent may help identify pathogen factors associated with different disease manifestations. In the case of dengue, for which there is no appropriate animal model, population-based molecular epidemiologic studies become even more critical for identifying viral determinants associated with the clinical spectrum of the disease.

However, the existing typing methods for dengue virus are often applied well after the dengue epidemics are over. They are used mostly for phylogenetic analyses and not for identifying risk factors for transmission or clinical manifestations. This is because these techniques are technically demanding and usually can be performed only in research laboratory settings in developed countries or in advanced laboratories in developing countries. Hence, reliable epidemiologic information from any given region is not usually obtained expediently at the time of the outbreak. For the most part, these labor-intensive typing procedures preclude processing a large number of isolates; the few studies that have analyzed numerous strains from a given geographical location have done so retrospectively years after the strains were isolated (Rico-Hesse *et al.,* 1998; Trent *et al.,* 1989; Walker *et al.,* 1988). For dengue subtyping methods to be most useful for epidemiologic and pathogenesis investigations, a large number of strains need to be examined during the epidemic period. The RSS–PCR method should serve this purpose, since it was designed to be low cost and to require minimal laboratory equipment and small amounts of template. We have used RSS–PCR to type strains from the current epidemics in Nicaragua and El Salvador and found that the epidemics in both countries were caused by the same dengue-3 subtype. This RSS–PCR type was immediately shown to belong to the Sri Lanka subtype, which has been associated with DHF. This type of information can be used to alert authorities to the potential severity of epidemics caused by circulating strains and to mobilize resources to implement appropriate prevention and control measures.

The RSS–PCR technique has been validated against well-characterized sets of dengue type 2 and 3 viruses. Its reproducibility is evidenced by the observations that (1) the same samples repeatedly produced the same patterns, (2) the patterns remained stable over time (up to 9 years in the case of pattern E), and (3) laboratory storage and passage of the strains did not affect the results. The RSS–PCR method is highly specific, as primers designed for dengue-2 do not recognize the other serotypes, and similarly for the dengue-3 primers. The groups generated by RSS–PCR typing of both dengue-2 and dengue-3 viruses representing a worldwide distribution of isolates display geographic and temporal cluster-

TABLE 2

Dengue-3 Viruses Used in This Study

Strain (CDC No.)	Location	Year	RSS pattern	Source[a]
ES14	El Salvador	1998	C	4
MES98	El Salvador	1998	C	5
20/8	Guatemala	1997	C	3
24/3	Guatemala	1997	C	3
25/1	Guatemala	1997	C	3
26/6	Guatemala	1997	C	3
27/9	Guatemala	1997	C	3
29/3	Guatemala	1997	C	3
29/12	Guatemala	1997	C	3
29/13	Guatemala	1997	C	3
32/80	Guatemala	1997	C	3
1385	Guatemala	1997	C	3
1834	Guatemala	1997	C	3
1033 (395)	Indonesia	1976	A1	1
1047 (847)	Indonesia	1976	A2	1
1153 (796)	Indonesia	1977	A1	1
1275 (233)	Indonesia	1978	A1	1
1280 (439)	Indonesia	1978	A1	1
1244 (739)	Indonesia	1978	A1	1
1241 (929)	Indonesia	1978	A1	1
(316)	Indonesia	1985	A1	1
(430)	Indonesia	1985	A1	1
(672)	Malaysia	1983	A1	1
EH95	Nicaragua	1995	C	2
4227	Nicaragua	1997	C	2
4431	Nicaragua	1997	C	2
218	Nicaragua	1998	C	2
256	Nicaragua	1998	C	2
1709	Nicaragua	1998	C	2
H-87	Philippines	1956	B2	1
(270)	Philippines	1984	A2	1
(380)	Philippines	1984	A2	1
(787)	Philippines	1984	A2	1
(959)	Philippines	1984	A1	1
(805)	Sri Lanka	1981	C	1
(969)	Sri Lanka	1982	C	1
(383)	Sri Lanka	1985	C	1
(904)	Sri Lanka	1985	C	1
CH53489D73-1 (492)	Thailand	1973	B1	1
CH53875D73-81 (726)	Thailand	1973	B1	1
2773D74-137 (498)	Thailand	1974	B1	1
1308 (689)	Thailand	1977	B1	1
1309 (793)	Thailand	1978	B1	1
D80-260 (267)	Thailand	1980	B1	1
D80-273 (35)	Thailand	1980	B1	1
(500)	Thailand	1980	B2	1
D84-137 (21)	Thailand	1984	B1	1
(085)	Thailand	1984	B1	1
(315)	Thailand	1984	B1	1
(500)	Thailand	1984	B1	1
(734)	Thailand	1984	B1	1
(931)	Thailand	1984	B1	1
(413)	Thailand	1985	B1	1
(641)	Thailand	1985	B1	1

[a] (1) See Table 1.
(2) A. Balmaseda, Departamento de Virología, Centro Nacional de Diagnóstico y Referencia, Ministerio de Salud, Managua, Nicaragua.
(3) L. Castillo, Laboratorio de Virología, Ministerio de Salud Pública y Asistencia Social, Guatemala City, Guatemala.
(4) C. de Lozano, Departamento de Virología, Ministerio de Salud Pública, San Salvador, El Salvador.
(5) M. Diamond, Department of Medicine, University of California (San Francisco, CA).

TABLE 3

Sequence and Position of Oligonucleotide Primers Used to Amplify Dengue-2 and Dengue-3 Viruses

Primer	Sequence	Genome position[a]	Strand	Specificity
RSS1	5′-GGA TCC CAA GAA GGG GCC AT	1696–1715	+	Dengue-2
RSS2	5′-GGC AGC TCC ATA GAT TGC T	2277–2259	−	Dengue-2
RSS3	5′-GGT GTT GCT GCA GAT GGA A	1524–1542	+	Dengue-2
RSS4	5′-GTG TCA CAG ACA GTG AGG T	2371–2353	−	Dengue-2
RSS5	5′-CCA ACA TAA CAA CTG ACT C	1131–1149	+	Dengue-3
RSS6	5′-GGC AAG GGA AGC (C/T)TG GTA	1259–1276	+	Dengue-3
RSS7	5′-CTA CAT TTT AAG TGC CCC G	1785–1767	−	Dengue-3
RSS8	5′-GAC AGG CTC CTC CTT CTT G	2023–2005	−	Dengue-3

[a] The genome positions of primers RSS1–4 are given according to the published sequence of dengue virus type 2, strain 16681 (Blok *et al.,* 1989), while the positions of primers RSS5–8 correspond to the published sequence of dengue virus type 3, strain H87 (Osatomi and Sumiyoshi, 1990).

ing (Table 4). The RSS–PCR types correlate well with subtype assignments made using other methods, such as genomic sequencing (Blok *et al.,* 1991; Chungue *et al.,* 1993; Deubel *et al.,* 1993; Lanciotti *et al.,* 1994; Lewis *et al.,* 1993; Rico-Hesse, 1990, 1997), oligonucleotide fingerprinting (Monath *et al.,* 1986; Trent *et al.,* 1983), and antigen signature analysis (Monath *et al.,* 1986) (Table 5).

Several observations bear discussion. For instance, a specific RSS–PCR pattern was generated from African strains (G), consistent with a previously assigned genotype (Rico-Hesse, 1990); however, other phylogenetic analyses excluded this group due to the failure of primers to amplify African strains (Lewis *et al.,* 1993). Interestingly, while dendrograms derived from sequence data placed Thai and post-1981 Caribbean ("Jamaica") strains in the same subtype (III) (Lewis *et al.,* 1993; Rico-Hesse, 1990), both RSS–PCR and oligonucleotide fingerprinting (Trent *et al.,* 1983) generated two groups—A ("Jamaica") and B (Thailand). Last, while isolates from Trinidad have been previously classified as the "Puerto Rico" subtype, they appear to differ somewhat genetically from the other members of the group at both sequence and antigenic levels (Lewis *et al.,* 1993; Monath *et al.,* 1986; Rico-Hesse, 1990); by RSS–PCR analysis, these strains actually form a distinct group (C).

The information obtained from the RSS–PCR typing of our collection of dengue-2 viral strains provides some examples of how this technique may be applied in understanding the epidemiology and possibly the pathogenesis of dengue. The similarity of South Pacific dengue-2 isolates with pre-1981 Caribbean strains (both RSS–PCR type F) and the replacement of this native American or "Puerto Rico" genotype by the "Jamaica" subtype (RSS–PCR type A) that was introduced into the Caribbean in 1981 are consistent with previous observations based on other methods (Deubel *et al.,* 1993; Lewis *et al.,* 1993; Rico-Hesse, 1990, 1997; Trent *et al.,* 1983). The spread of dengue-2 strains identified as RSS–PCR

TABLE 4

Geographic and Temporal Distribution of Dengue-2 RSS–PCR Patterns

Country	Year	RSS pattern										
		A	B1	B2	C	D	E1	E2	E3	F1	F2	G
Thailand	1964–1985	0	**6**	**2**	0	0	0	0	0	0	0	0
Jamaica	1981–1983	**3**	0	0	0	0	0	0	0	0	0	0
Puerto Rico	1969–1977	0	0	0	0	0	0	0	0	**1**	**2**	0
	1984–1986	**7**	0	0	0	0	0	0	0	0	0	0
Dominican Republic	1986	**1**	0	0	0	0	0	0	0	0	0	0
Trinidad	1978–1982	0	0	0	**3**	0	0	0	0	0	0	0
Sri Lanka	1982–1985	0	0	0	0	0	0	**5**	0	0	0	0
Indonesia	1975–1985	0	0	0	0	0	6	**4**	**4**	0	0	0
Philippines	1975	0	0	0	0	0	**1**	0	0	0	0	0
	1981–1984	0	0	0	0	**14**	0	0	0	0	0	0
Burkina Faso	1982	0	0	0	0	0	0	**1**	0	0	0	0
Tahiti	1971–1972	0	0	0	0	0	0	0	0	**4**	0	0
Tonga	1974	0	0	0	0	0	0	0	0	**1**	0	0
Fiji	1971	0	0	0	0	0	0	0	0	5	0	0
Republic of Guinea	1981	0	0	0	0	0	0	0	0	0	0	**1**
Senegal	1970	0	0	0	0	0	0	0	0	0	0	**1**

TABLE 5

Comparison of RSS–PCR Types with Previous Classifications of Dengue-2 Viruses

RSS–PCR	E gene sequence[a]	E/NS1 sequence[b]	Oligonucleotide fingerprinting[c]	Antigen sig.[d]
A Post-1981 Jamaica, Caribbean	III Thailand/Jamaica	Thailand/Jamaica	Jamaica/W. Africa	Jamaica
B Thailand	III Thailand/Jamaica	Thailand/Jamaica	Burma/Thailand	Burma/Thailand
C Trinidad	V[e] Trinidad/Puerto Rico/ S. Pacific	Trinidad/Puerto Rico/ S. America/ South Pacific[e]	—[f]	Puerto Rico/ South Pacific[e]
D Philippines	I–II Philippines/Taiwan	Philippines/Taiwan	Philippines	Philippines
E Indonesia/Sri Lanka/ Burkina Faso	IV Indonesia/Sri Lanka/ Burkina Faso/ Seychelles	Indonesia/Sri Lanka/ Burkina Faso/ Seychelles	Seychelles	—[f]
F Puerto Rico/Mexico/ S. Pacific	V Puerto Rico/ South Pacific	Puerto Rico/ South America/ South Pacific	Puerto Rico/ South Pacific	Puerto Rico/ South Pacific
G Africa	—[f]	Africa	Jamaica/W. Africa	—[f]

[a] Lewis *et al.* (1993).
[b] Rico-Hesse (1990); Rico-Hesse *et al.* (1997).
[c] Monath *et al.* (1986); Trent *et al.* (1983).
[d] Monath *et al.* (1986).
[e] The Trinidad strains showed greater genetic distance from the other strains in the cluster.
[f] Not done or not resolved.

type E into West Africa from Sri Lanka also reflects transmission pathways detected by sequencing (Deubel, 1992; Lewis *et al.,* 1993; Rico-Hesse, 1990). The ability to obtain this information rapidly in dengue-endemic countries should enhance our understanding of the global spread of dengue fever.

The association of subtypes with disease severity is also upheld by RSS–PCR classifications. The Caribbean "Puerto Rico" strains and the related South Pacific isolates (e.g., Tonga, 1974) that form part of RSS–PCR type F (CDC subtype V) have been associated only with classic dengue fever (Gubler *et al.,* 1978; Rico-Hesse *et al.,* 1997). Analysis of the clinical information from isolates in our collection also indicated that type F strains were associated only with dengue fever. In contrast, the RSS–PCR types corresponding to "Jamaica" and Southeast Asian subtypes known to cause DHF were correlated with more severe disease in our strain collection as well.

Since this assay is a PCR-based procedure, a number of factors can influence the patterns obtained, including the concentration of template and primers, the type of polymerase, the amplification profile, and the duration of electrophoresis. The assay should be standardized in each laboratory, and consistency maintained in the methods and reagents. We recommend including a control strain known to generate a pattern consisting of two or more bands in every PCR amplification and gel electrophoresis. The concentration of the template is probably the single most important variable. An excess of template can lead to nonspecific extra bands; whereas insufficient template can result in amplification of only the most prominent fragments. Either situation can lead to misclassification of the RSS–PCR pattern. Quantitation of the DNA template is complicated by the fact that reverse transcription and amplification are combined into a single procedure conducted in the same tube. Because small amounts of viral RNA are usually extracted for this assay, quantification of the RNA starting material is also difficult. Thus, it may be necessary to amplify the undiluted RNA as well as a fivefold dilution to ensure an interpretable result. For instance, when higher molecular weight bands are visible and the pattern contains additional bands, the template RNA should be diluted and the amplification repeated.

RSS–PCR presents a simple and rapid approach to typing microorganisms in general. Since the primers are

based on the existence of polymorphic restriction sites, a universal feature, this method should be applicable to molecular typing of other pathogenic organisms as well. This technique should facilitate large-scale molecular epidemiologic studies of dengue virus that can be conducted on-site in dengue-endemic countries.

MATERIALS AND METHODS

Viral strains

The strains of dengue virus used in this study and their sources are listed in Tables 1 and 2. Viruses were propagated in C6/36 *Aedes albopictus* mosquito cells (Igarashi, 1985) grown in MEM medium (Gibco BRL, Grand Island, NY) containing Earle's salts, L-glutamine, and nonessential amino acids, supplemented with 0.11% sodium bicarbonate, 100 units/ml penicillin, 75 units/ml streptomycin, and 10% fetal bovine serum (FBS; Gemini Bioproducts, Inc., Calabasas, CA). 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.

RNA extraction

RNA was extracted from the supernatant of infected cells essentially as in Harris *et al.* (1998). Briefly, a 300-μl aliquot of the sample was combined sequentially with 300 μl of lysis buffer (6 M guanidine isothiocyanate, 50 mM sodium citrate, 1% Sarkosyl, 20 μg/ml *Escherichia coli* tRNA, and 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 mixed after the addition of each of the reagents. After a 15-min centrifugation, the aqueous phase was transferred to a new tube and 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.

Reverse transcription and PCR amplification

A reaction mixture was prepared containing 50 mM KCl, 10 mM Tris (pH 8.5), 0.01% gelatin, 200 μM each of the four deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 30 mM tetramethylammonium chloride (TMAC) (Chevet *et al.,* 1995), 0.5 M betaine (Mytelka and Chamberlin, 1996), 5 mM dithiothreitol (DTT), 0.5 μM each of four RSS primers, 0.005–0.025 U/μl of RAV-2 reverse transcriptase (Amersham Corp., Arlington Heights, IL), and 0.025 U/μl *Taq* DNA polymerase (AmpliTaq, Perkin–Elmer Corp., Foster City, CA). Primers RSS1–RSS4 were used to amplify dengue-2 virus, while primers RSS5–RSS8 were used to analyze dengue-3 strains (Table 3). Reverse transcription was conducted at 42°C for 60 min, followed by 30 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. Five microliters of extracted RNA was used as template in a 50-μl reaction volume. Amplification was conducted in 0.6-ml tubes (Robbins Scientific Corp., Sunnyvale, CA) using a Model 480 thermal cycler (Perkin–Elmer, Norwalk, CT) or a PTC-200-60 thermocycler (MJ Research, Inc., Watertown, MA). The primer sequences and their genomic positions are listed in Table 3. Ten microliters of the PCR product was electrophoresed on 1.5% agarose gels in 1× TBE (89 mM Tris borate, 2 mM EDTA, pH 8.3) until the bromophenol blue dye had migrated two-thirds the length of the gel. A 100-bp ladder was used as a size standard (Gibco BRL).

Interpretation of RSS–PCR patterns

To test the reproducibility of the RSS–PCR technique, each strain was amplified more than once to ensure that the same pattern was obtained. A control strain with a known pattern was amplified along with the test strains, and only when the control strain generated the expected patterns were the patterns of the test strains included in the analysis. In some situations, there was sample-to-sample variation with respect to the presence of a single band. In this situation, we designated the pattern with and without the band as belonging to the same RSS group and indicated the band in question as a dotted line in the schematic diagram (Figs. 1B and 3B). When two patterns were obtained from strains isolated in the same geographical area and temporal period that differed by only one fragment, these two similar patterns were considered to be part of a related RSS group and designated, for example, B1 and B2.

ACKNOWLEDGMENTS

We are grateful to Srisakul Kliks and Rebeca Rico-Hesse for generously providing strains and advice. We thank Deborah Lans for technical assistance and Leticia Castillo, Michael Diamond, Angel Balmaseda, and Celina de Lozano for viral strains. This research was supported by Fogarty International Center Grant TW-00905, the John D. and Catherine T. MacArthur Foundation, and the New England Biolabs Foundation.

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