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Direct sequencing of genomic cDNA fragments amplified by the polymerase chain reaction for molecular epidemiology of dengue-2 viruses

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Summary. A nucleotide fragment encoding amino acids 29 to 94 in the E-protein of 28 dengue-2 isolates of diverse geographic and host origins was examined by direct sequencing of a polymerase chain reaction (PCR)-amplified product, and compared to six previously published sequences. Nucleotide divergence ranged from 0 to 19.8% corresponding to a maximum of 9% divergence in the amino acid sequence. Taking a divergence of 6% between the nucleotide sequence as a cut off for genotype classification, six groups have been established. Southeast Asian and the Jamaican 1983 genotypes show a high rate of similarity ($> 95.2\%$). Our results suggest that virus of this group is now circulating as the dominant topotype in Brazil (1990) and in French Guyana (1986–1991). African strains fall into two groups, one endemic group (1970–1990) and one epidemic group (1986–1987). The three other groups correspond to viruses from Sri Lanka (1982) and the Seychelles (1977), from Puerto Rico (1973) and from Tahiti (1975). Our approach appears to be valuable characterizing dengue isolates, easily and rapidly.

Introduction

The flavivirus genus contains approximately 70 related members classified in complexes which include the most important mosquito-borne viral illnesses of man, dengue, yellow fever and Japanese encephalitis. Amongst these diseases, dengue is responsible for a growing health problem in the tropical world. The four dengue viruses are transmitted mainly by *Aedes aegypti* and *Aedes albopictus* in Asia, Africa, the Americas and South Pacific. Dengue infection is

responsible for mild febrile flu-like illness (dengue fever, DF) in millions of people each year, and for hemorrhagic manifestations and shock (dengue hemorrhagic fever, DHF; dengue shock syndrome, DSS) due to vascular permeability and disorders of hemostasis. These abnormalities can progress to death, primarily among children. Over 2,500,000 DHF cases with 42,751 deaths were reported to WHO between 1956 and 1989 [11]. In the absence of prophylactic measures, the uncontrolled spread of DHF/DSS from Southeast Asia to the Americas represents a threat for the New World. DHF/DSS was first recognized sporadically in Thailand and Manila in the early 1950 [9], then increased throughout tropical Asia, with large outbreaks, and is now endemic in the southeast part of Asia. The first cases of DHF/DSS were recorded in the dengue endemic Carribean bassin since the early 1970 [6] but emerged in epidemic form during the dengue-2 outbreak in Cuba 1981 [16], and recently in Venezuela [1].

Dengue viruses are characterized by a single-stranded positive-sense RNA associated with a core protein in a nucleocapsid surrounded by an envelope consisting of a lipid bilayer including anchored viral envelope (E) and membrane (M) proteins. The four dengue serotypes are classified by cross-neutralization using polyclonal antibodies [28]. Monoclonal antibodies and RNA oligonucleotide fingerprinting revealed that each serotype contained a number of geographic variants or topotypes [22, 34]. The distribution of dengue-1 and dengue-2 variants throughout the tropical belt has recently been investigated by direct sequencing of the viral genomic RNA [25]. Dengue-2 virus has received the most attention because of its frequent association with severe dengue symptoms. Two exhaustive analyses of Thai dengue-2 viruses demonstrated antigenic and genetic variation within a defined geographic area but no virulence-related marker was identified [35, 36]. Comparisons of structural genes of dengue-2 viruses isolated from patients with different levels of disease severity demonstrated a high degree of heterogeneity in the nucleotide and amino acid sequences but no correlation with the degree of virulence could be established [3, 30–32]. Recent advances in the characterization of the E protein structure and of the domains related to its biological functions may help to determine phenotypic markers involved in dengue antigenicity and virulence [20, 21, 26].

It has been suggested that DHF/DSS may be the consequence of the enhancement of the mononuclear cell infectivity by pre-existing cross-reactive non-neutralizing antibodies [10, 11]. Accordingly, the absence of numerous DHF/DSS cases in areas where the transmission of multiple endemic or serially epidemic serotypes exists could be due (1) to the incapacity of these dengue strains to be enhanced or (2) to genetic control of the immune responsiveness. Indeed, the appearence in the Carribean of an Asian-like dengue-2 genotype following the Cuban epidemic in 1981 and the increase in the number of severe cases of dengue infection are probably linked [25]. The presence of genetically distinct genotypes in Africa may also be related to the mild disease recorded in that continent [25, 27]. On the other hand, the lower susceptibility of blacks

to the severe manifestations of dengue may also be related to the absence of DHF/DSS in Africa, and, to a lesser extent, in the Americas [8, 16, 17].

Because of the low level of dengue virus replication in cell cultures, genetic investigations usually require the extraction of purified viral RNA from large batches of cultures, which involves a considerable amount of work and is not without risk. We have recently developed a rapid, sensitive and specific method for identifying dengue viruses, using the polymerase chain reaction (PCR) coupled to a dot-hybridization with type-specific probes [5]. The choice of the gene sequence to be amplified was dictated by its high antigenicity and variability [20, 21, 26]. We used the strategy of a direct sequencing of the amplified genes in an attempt to identify the geographic origin of new dengue-2 viruses and to follow their molecular evolution. The results of this study are discussed here.

Materials and methods

Cells and viruses

Aedes pseudoscutellaris AP 61 cells were grown in Leibovitz L 15 medium supplemented with 10% tryptose phosphate and 10% fetal bovine serum. The isolates of dengue-2 analyzed in this study are summarized in Table 1. Clinical and epidemiologic informations are also presented when available. Viruses were passed first by inoculating subconfluent AP 61 or C 6/36 cells with either a 1 : 10 dilution of patient serum or with supernatant fluid from triturates of infected mosquitoes in Hanks medium. Viruses were subsequently passaged once in AP 61 cells and the type-identity confirmed by indirect fluorescent antibody tests using monoclonal antibodies [12].

Virus propagation and RNA extraction

Subconfluent AP 16 cells in 25 cm^2 plastic flasks were infected with each virus isolate at a multiplicity of 0.01 to 0.1 PFU per cell for 1 h and 5 ml of Leibovitz medium containing 3% fetal calf serum were added. Five to seven days later, the supernatant was discarded and the cells were washed three times with cold TNE 1 × (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA). Cell were lysed in 500 µl of 0.1 × TNE containing 0.5% Nonidet P 40 and 0.5% sodium deoxycholate at 4 °C. Cell nuclei were pelleted by centrifugation at 2,500 rpm for 5 min at 4 °C in a microfuge. Proteins in the supernatant were solubilized in 1% SDS. RNA was recovered by two extractions with phenol equilibrated in TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA), two extractions with diethyl-ether and then precipitated with 2.5 volumes of ethanol/0.3 M ammonium acetate at −20 °C. The RNA was then pelleted by centrifugation at 10,000 rpm for 10 min at 4 °C, rinsed with ethanol 75°, dried, and resuspended in 100 µl of sterile glass-distilled water. The total RNA concentration was approximately 1 µg/µl corresponding to 10 ng/µl of viral RNA (Deubel, unpubl. obs.).

Synthesis of double-stranded DNA

Details of the methods used to obtain dengue cDNA have been described [5]. Briefly, first strand cDNA was obtained by reverse transcription (RT) of about 100 ng (0.03 pmole) of viral RNA and 5 pmole of dengue-2-specific primer
5′(305)-CCCCATCCTCTGTCTACCATG-(285)3′
in RT buffer (70 mM KCl; 50 mM Tris-HCl pH 8.3; 10 mM $MgCl_2$; 10 mM dithiothreitol) containing 40 units of RNase inhibitor (RNasin; Promega), 0.25 mM each of the four deoxynucleotide triphosphate (dNTP; Pharmacia), and one unit of avian myeloblastosis

Table 1. Description of the dengue-2 virus isolates compared by genomic sequencing in this study

Stain	Code	Source[a]	Country of origin	Year of isolation	Grade of illness	Ref. for sequence[b]
1409	JAM	human	Jamaica	1983	DF	[4]
New Guinea C	NG	human	New Guinea	1944	DF	[7]
16681	THA 1	human	Thailand	1964	DSS	[3]
PUO-218	THA 2	human	Thailand	1980	DF	[7]
M1	MAL	human	Malaysia	1987	DHF	[30]
SL 767	SL	human	Sri Lanka	1982	DF	[3]
P 25600[c]	PR	human	Puerto Rico	1973	DF	
P 51526[c]	BUR	human	Burma	1976	fatal	
P 51527[c]	SEY	human	Seychelles	1977	DF	
P 51528[c]	TAH	human	Tahiti	1975	DF	
37	VN 1	human	Vietnam	1987	DHF	
57	VN 2	human	Vietnam	1987	DHF	
135	VN 3	human	Vietnam	1987	DHF	
326	VN 4	human	Vietnam	1987	DHF	
ArA 2022[d]	BF 1	*A. afr.*	Burkina Faso	1980		
ArA 6894[d]	BF 2	*A. aeg.*	Burkina Faso	1986		
ArA 19643[d]	IC	*A. aeg.*	Ivory Coast	1987		
HD 10674[d]	SEN 1	human	Senegal	1970	DF	
ArD 20761[d]	SEN 2	*A. lut.*	Senegal	1974		
AnD 34156[d]	SEN 3	*E. patas*	Senegal	1981		
ArD 34285[d]	SEN 4	*A. lut.*	Senegal	1981		
ArD 35884[d]	SEN 5	*A. lut.*	Senegal	1982		
ArD 75505[d]	SEN 6	*A. lut.*	Senegal	1990		
HD 76395[d]	SEN 7	human	Senegal	1990	DF	
HD 77284[d]	SEN 8	human	Senegal	1990	DF	
38910	BRA 1	human	Brazil	1990	DF	
39012	BRA 2	human	Brazil	1990	DF	
40247	BRA 3	human	Brazil	1990	fatal	
537907	FG 1	human	French Guyana	1986	DF	
541941	FG 2	human	French Guyana	1986	DF	
CaH 881[e]	FG 3	human	French Guyana	1988	DF	
118627	FG 4	human	French Guyana	1990	DF	
124602	FG 5	human	French Guyana	1991	DF	
126196	FG 6	human	French Guyana	1991	DF	

[a] Isolates were from human, *Erythrocebus (E.) patas* monkey; and *Aedes (A.) luteocephalus (lut.), africanus (afr.), aegypti (aeg.)* mosquitoes

[b] If not given otherwise, this paper

[c] Strains provided by Dr. Léon Rosen, Hawaii

[d] Strains provided by Dr. Jean-Pierre Digoutte, Senegal

[e] Strain provided by Dr. Jean-Paul Moreau, French Guyana

virus reverse transcriptase (Boehringer). Numbers flanking the primer sequence indicate the map site at which the ending 5'-nucleotides hybridize on the dengue-2 E gene [4]. DNA molecules were then subjected to a 32-cycle amplification (denaturation at 94 °C for 15 s, annealing at 55 °C for 60 s, and elongation at 72 °C for 45 s) by polymerase chain reaction (PCR) in PCR buffer (50 mM KCl; 10 mM Tris-HCl pH 8.3; 2.5 mM $MgCl_2$; 0.01% w/v gelatin) using one fifth of the cDNA-RNA hybrid mixture, 50 pmole of the first primer, 50 pmole of a second primer of genomic-sense
5'(39)-GGGGTTTCAGGAGGAAGCTGGGTTGAC-(65)3',
0.5 mM dNTP and one unit of *Thermus aquaticus* (Taq) polymerase (Perking Elmer Cetus) [5, 29]). Subsequently, the DNA was purified after gel electrophoresis on low melting point agarose by phenol extraction and ethanol precipitation [5].

Sequencing of the amplified product

Sequencing reactions were carried out on the double strand DNA by the dideoxy method using either the genomic-sense dengue-2-specific 39–65 primer or the anti-genomic sense 305–285 primer and T 7 polymerase (Sequenase) as described previously [5]. About 300 ng (3 pmole) of the amplified and purified DNA was heat-denatured in boiling water in the presence of 20 ng (3 pmole) of primer for 2 min then instantly cooled on ice. The DNA sequencing was performed with a Sequenase kit (US Biochemical). Samples were denatured for 2 min at 80 °C and loaded on a 6% polyacrylamide/bisacrylamide (38/2)-8 M urea sequencing gel in TBE 1 × (100 mM Tris-borate pH 8.0, 2.5 mM EDTA). A first loading of each set of sample was electrophoresed at 1000 V until the xylene cyanol dye reached the bottom of the gel (long run). Then, a second loading of the samples in free wells of the gel was carried out and electrophoresed further until the bromophenol blue reached the bottom of the gel (short run). The gel was fixed in ethanol/acetic acid/water (20/10/70) for 10 min, vacuum-dried, and autoradiographed on Fuji X-film.

Dendrogram and sequence similarity

Phylogenic trees generated after multiple nucleotide and amino acid sequence alignments were calculated using CLUSTAV V program [14].

Results

Specificity of the DNA priming and amplification, amplicon sequencing

Primer oligonucleotides have been selected for RT/PCR reactions according to their highly conserved sequences in dengue-2 topotypes [3] and high melting temperature to avoid negative results due to mutations. They flank a "hot spot" region of less nucleotide homology in the E gene amongst dengue-2 variants [3] and encode the hydrophilic peptide 29–94 which has important immunological reactivity [21]. Success of the RT/PCR reaction was observed with all dengue-2 variants while giving a clearcut single DNA band of the expected size of 266 base pairs in 1% agarose gel (Fig. 1 A). The amount of amplified material (amplicon) corresponded to about 3 µg (30 pmole) as quantitated by direct comparison of its ethidium bromide staining intensity to the intensity of standard DNA. The amplified DNA band was excised from the gel and 3 pmoles were used for direct sequencing by primer extension. Both primers of nt 39–65 and nt 305–285 that were used for RT/PCR amplification were tested for their ability to obtain accessible sequence data. In order to reduce the strong C-G

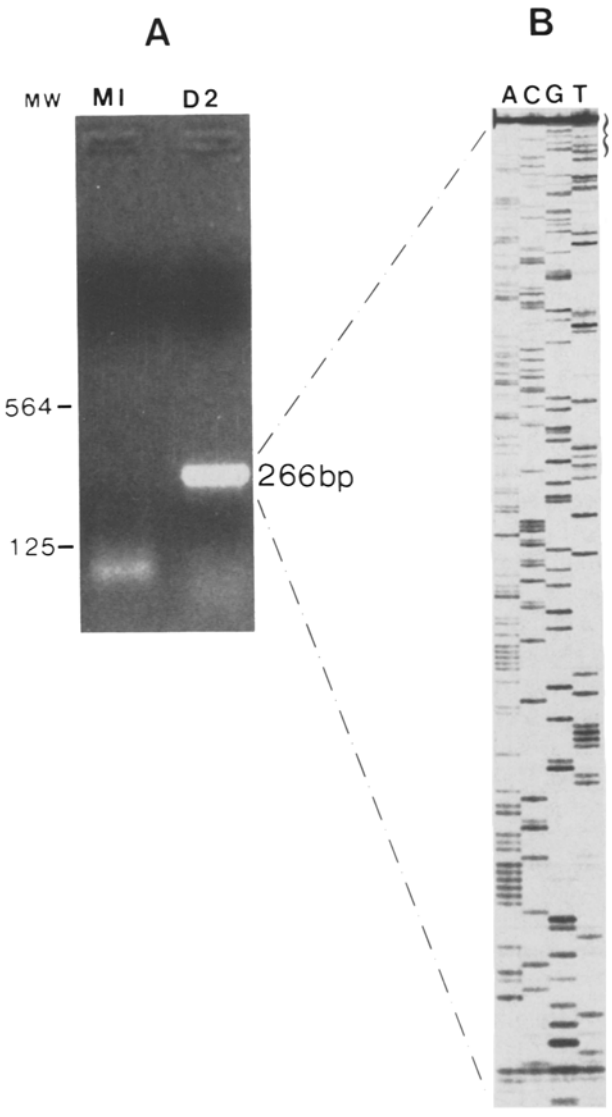

Fig. 1. Amplification and sequence analysis of a total dengue-2 gene primed cDNA fragment. **A** Ethidium bromide-stained 1.5% agarose gel. Arrow indicates the position of the expected amplified band (266 bp) by comparison with the molecular weight markers. cDNA was prepared from dengue-2-infected (*D2*) and mock-infected (*MI*) AP 61 cells. **B** Autoradiogram of a sequence reaction of the dengue-2 fragment using the genomic sense (39–65) primer. The position of the sequence corresponding to the antigenomic sense (305-285) primer is bracketed at the top of the gel

hydrogen bonding in the neosynthetised hybrid and consequently to prevent stops in the progression of the T 7 polymerase, dITP provided with the sequencing kit was used instead of dGTP in the sequencing reaction mixture. A short run of the samples in a single 6% polyacrylamide gel allowed the reading of more than 200 bases within the amplified E-gene fragment (Fig. 1 B).

Nucleotide and deduced amino acid sequences of the E-gene fragment of dengue-2 virus isolates

Sequences from 34 virus isolates were compared between nucleotides 85 and 282 in the dengue E-gene, using the fast/approximate pairwise alignment method [37]. Maximum divergence over this region was approximately 20% when the dengue-2 virus isolates were compared to the Jamaican 1409 prototype strain.

Moreover, the nucleotide changes were scattered throughout the gene fragment (data not shown). This percentage of divergence is similar to that found by comparing a different segment across the E/NS 1 junction of several dengue-2 isolates [25]. The majority of mutations occurred at the third position of the codon and were mostly silent. The encoded amino acids between 29 and 94 showed a low variability ($\leqslant 9\%$) when compared to the Jamaican prototype strain (Fig. 2). As expected from previous comparative amino acid sequence analyses derived from RNA sequencing, strains from Southeast Asia (i.e., Vietnam, Burma, Thailand, Malaysia) shared a high degree of amino acid identity ($> 95\%$) with the prototype strain while less similarity was observed among African isolates. Amino acid variability is mainly scattered within the half C-terminal fraction of the E-fragment which contains the unaffected glycosylation site NTT at position 67–69 (Fig. 2). Most of the amino acid changes were nonconservative.

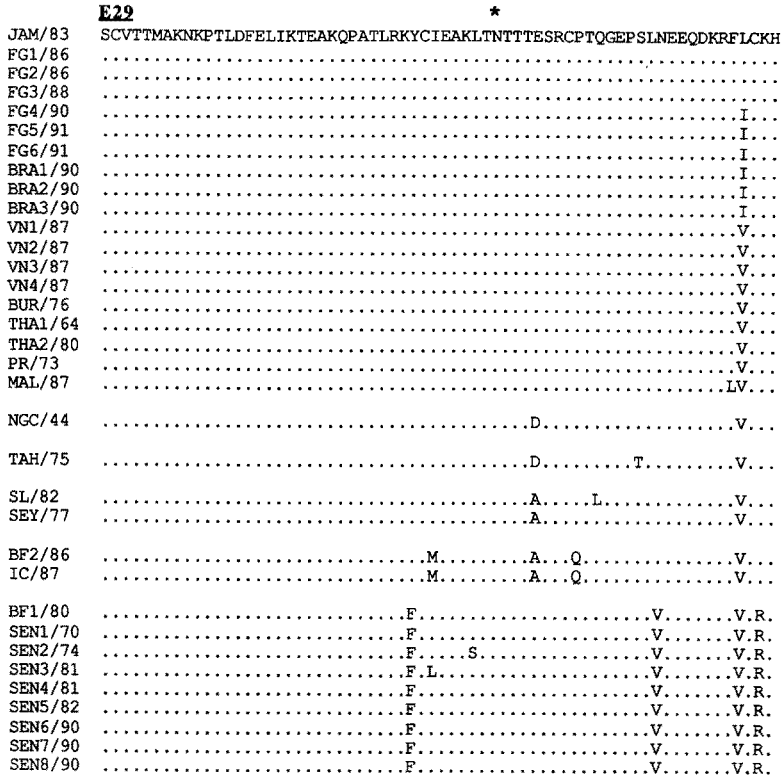

Fig. 2. Amino acid sequences deduced from the 198 nucleotides used to determine genetic relatedness among dengue type 2 isolates. The first amino acid 29 characterized in the dengue E-protein sequence is mentioned. Dots indicate amino acids homologous to those of the Jamaican 1983 (JAM/83) prototype. The potential site of N-glycosylation is indicated by an asterisk

Genetic relationships among dengue-2 isolates

A dendrogram was constructed by calculating the similarity score obtained by sequence pair comparison (Fig. 3) using the UPGMA cluster analysis method [33]. The limit of unweighted pair group method using arithmetic averages for drawing dendrograms is the diminution of the resolution between genotypic groups according to the degree of divergence. Nevertheless, dendrograms accurately reflect closely-related strains, but do not affect broad genetic relationships (data not shown). This method has already been shown useful for dengue sequence comparative studies [3, 25].

Five clusters of viruses were defined corresponding to genotypes with less than 6% nucleotide divergence within the E-gene fragment. Figure 3 clearly

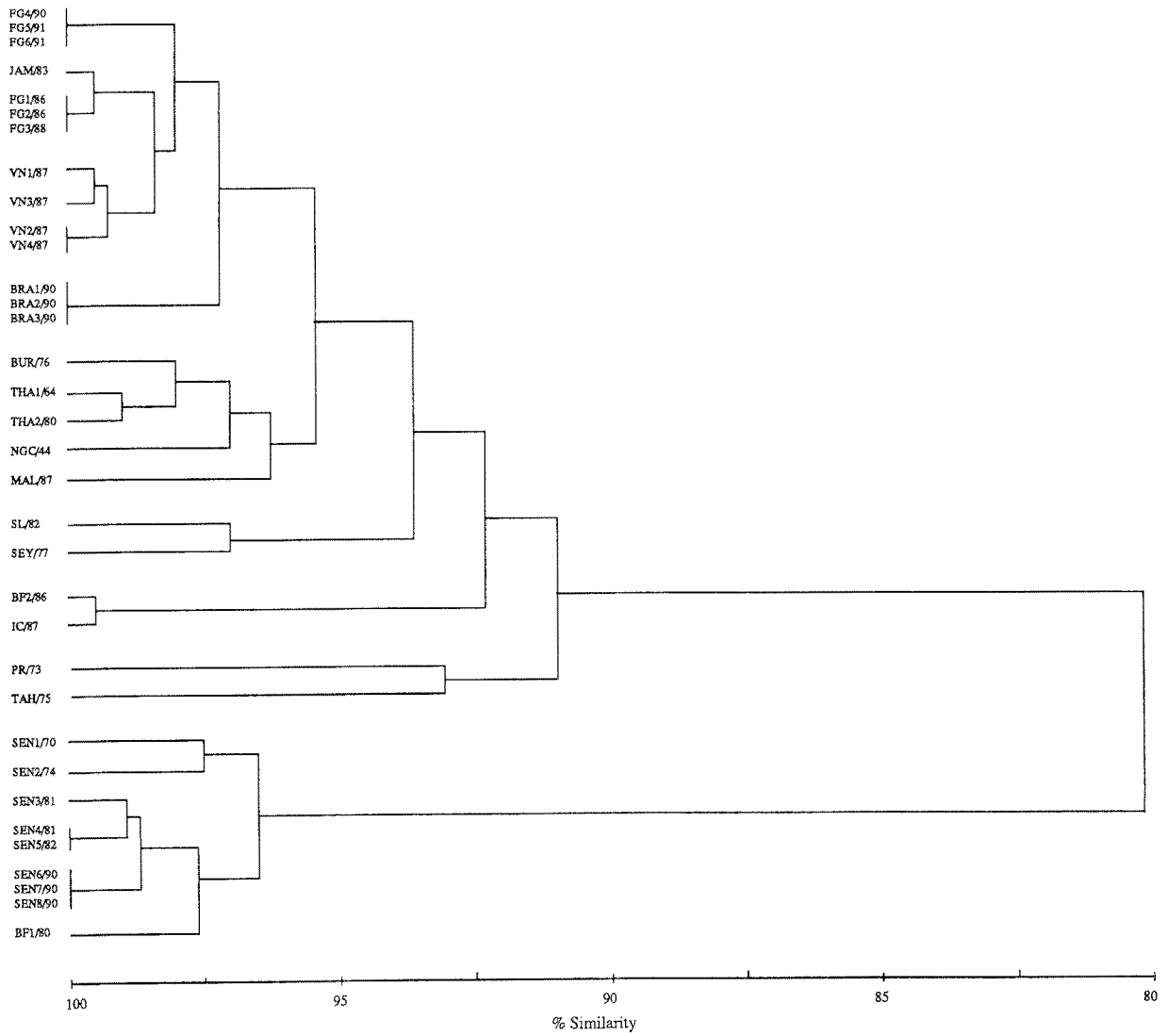

Fig. 3. Tree presenting the extent of base sequence similarity

shows that the genetic relationship within the gene does not necessarily correlate with the geographic origin of the strain. For example, it is obvious that a close relationship exists between Southeast Asian (Thailand 1964–1980, Burma 1976, Vietnam 1987, Malaysia 1987) isolates and recent Carribean (Jamaica 1983) and South American (Brazil 1990, French Guyana 1986–1991) isolates. Surprisingly, these strains revealed a relatively close identity (95.5 to 98%) to the time-distant 1944 New Guinea C reference strain. On the other hand, two distinct genotypes were observed in the Carribean (Jamaica 1983 and Puerto Rico 1973 prototypes) and two in West Africa (Burkina Faso 1986 and Senegalese 1970 prototypes). A Tahitian strain, 1975, clustered independently from the other strains.

Discussion

A gene fragment encoding an antigenic domain in dengue-2 E protein targetted by immunological responsiveness [20, 21, 26] and showing sufficient gene variability for direct comparison [3] has been amplified through RT/PCR procedure and directly sequenced. In addition to time saved, the direct sequencing of the dominant population in PCR product reduced the recording of misincorporation errors by the *Taq* polymerase on the sequencing ladder. We have demonstrated in this study that the size of the sample was sufficient for reliable measures and relatedness among dengue-2 viruses and confirmed the suitability of this method to understand dengue virus epidemiology. Using genomic RNA of several dengue viruses for T 1 oligonucleotide mapping [13, 24, 34–36] and the direct sequencing of either E genes [3, 30–32] or limited gene fragments [25] allowed the distinction of genetic variants among serologically identical viruses. Antigenic differences in dengue-2 virus strains were observed using a panel of six selected monoclonal antibodies [22] directed against conformational structures in E protein [21]. This signature analysis allowed a grouping of dengue viruses similar to that determined by oligonucleotide mapping or nucleotide sequencing. These approaches required large amounts of virulent supernatant fluid for virus and RNA purification.

Topotypes are defined as viruses sharing more than 80% of large T 1 oligonucleotides corresponding to about 4–5% divergence in nucleotide sequence [2]. Therefore, the classification of dengue variants obtained by T 1 mapping is more diversified compared to nucleotide sequencing, because of its high sensitivity to point mutation revealed by either charge or mass variation in the oligonucleotide profile. However, limited nucleotide sequence allows an examination of derivation within genes of interest.

Rico-Hesse [25] by E/NS 1 gene junction analysis grouped dengue-2 viruses in five distinct genotypic varieties having less than 6% nucleotide sequence divergence, according to geographic origin: (1) the Carribean (1954–1981), Central America (1983–1988) and South Pacific (1971); (2) Taiwan (1981–1987), the Philippines (1988), New Guinea C (1944), and Thailand (1964); (3) Vietnam (1987), Jamaica (1981–1982), and Thailand (1983); (4) Indonesia (1973–1978),

the Seychelles (1977), Burkina Faso (1982–1986), and Sri Lanka (1981–1985); and (5) rural Africa (1970–1980). We confirmed this genetic classification and relationships between virus isolates when less than 6% sequence divergence was taken within the chosen E-gene interval. We demonstrated the Jamaican genotype was related to virus strains from Southeast Asia: the nucleotide sequence of a Jamaican isolate was similar to Vietnamese strains (98.5% similarity) and to a lesser extent to strains from Burma, Thailand and Malaysia (95.5% similarity). Dengue-2 isolates from French Guyana 1986–1988 are the most closely related to Jamaican genotype showing only 0.5% divergence in nucleotide sequence and identical amino acid sequence. This suggests that the virus spread from the Carribean to South America. This genotype seems to have evolved within few years. The 1990–1991 strains of French Guyana share 98% similarity with the former strains. These results revealed an evolution in the viruses higher than expected from T1 oligonucleotide mapping, which was evaluated at about 1% nucleotide change every two years [35]. This discrepancy cannot be correlated with the immunogenic nature of the gene product chosen for the comparative study because most of the changes are silent. This may also suggest the presence of a mixed population which evolves separately. Brazilian strains show identical amino acid sequence with those from French Guyana, although they share only 97% similarity in their nucleotide sequence. Whether these viruses exchanged between closely related geographic regions or evolved in parallel is presently unknown. Additional strains should be analyzed to accurately determine these relationships.

Whether the recent dengue-2 outbreak in Brazil associated with severe cases (R. M. Nogueira, unpubl. results) is related to the incursion of the new variant derived from the Jamaican genotype cannot be demonstrated. Furthermore, the presence of this dengue-2 genotype in French Guyana since 1986 without related severe manifestation of disease before 1990 (J. M. Reynes, unpubl. obs.) is intriguing. The number of severe cases may be related to the increased level of transmission. Another point is that dengue-1 virus was introduced into the Americas in 1977 and spread as single topotype throughout the region. This genotype preceded the dengue-2 outbreak in Cuba four years later. It has been hypothesized that DHF/DSS cases were predominantly observed in secondary infected patients [10]. Therefore, a seroepidemiological study should be carried out in dengue-infected patients in Brazil and in French Guyana to determine if previous outbreak of dengue-1 serotype in Brazil in 1986–1987 [23] and its isolation in French Guyana in 1989 (J. P. Moreau, pers. comm.) may account for the antibody-dependent enhancement of dengue-2 virus.

We analyzed recent strains isolated in 1990 in the sylvatic area of Senegal (Kedougou) from a wild mosquito and from two febrile patients. Their genotype showed a high rate of similarity (96.5% to 98.5%) with the major endemic topotype isolated in West Africa during the last 20 years. We confirmed the existence of an outbreak virus in West Africa genetically distinct from the sylvatic virus. A close relationship between virus isolates from Burkina Faso

(1986) and Ivory Coast (1987) was demonstrated. Only 92.5% similarity was observed between the outbreak viruses in Africa and strains from Sri Lanka and the Seychelles although it was previously suggested a closer relationship of epidemic strains from West Africa with strains from islands of the Indian Ocean [25]. On the other hand, Puerto Rican strains were previously shown to be transmitted in the Carribean, Central and South America and South Pacific [25], but in the present study a Puerto Rico strain (1973) clustered differently from a Polynesian strain (1975). Small variations in the phylogenic tree in our study and that of Rico-Hesse [25] may be explained either by the presence of genetic variants at a single location and within a short period of time [34–36], or by the different gene regions targetted for sequence analysis. This discrepancy may reflect the limit of sequencing short gene fragment (corresponding to about 4–5% of the genome) compared to mapping T1 oligonucleotides which allows the examination of approximately 10% of the whole genome.

Few evolutionary relationships could be established by comparing short amino acid sequences, mainly because they were conserved within a serotype (less than 10% divergence). Amino acid divergences were not randomly located in the gene fragment between genotypes since changes occurred in a region which seems to be affected by the immune selection of epitopes. It has been shown that an amino acid substitution occurred at position 78 and 72 on the E protein of tick-borne encephalitis [19] and yellow fever [18] variants, respectively, which were selected in the presence of neutralizing monoclonal antibodies. No mutation occurred between amino acids 29 and 58 of dengue-2 virus E protein which contains a linear epitope hidden in the native protein [21]. On the other hand, fragment 59 to 95 may be part of conformational neutralizing epitope [21] and of the putative fusion domain [26]. Therefore a sequence comparison of this fragment of E protein among numerous dengue isolates may help in an attempt to correlate genetic markers with biological functions of the E protein. Indeed, mismatches were recognized either at a crucial proline position ($P_{75} \rightarrow Q$) or at a glutamic acid position ($E_{71} \rightarrow A$) in the epidemic strains of West Africa. Mutations at position 59 ($Y \rightarrow F$) and 83 ($N \rightarrow V$) have been observed in endemic strains. Whether these molecular differences may be associated to biological features and to pathogenesis is unknown. Point mutations into linear amino acid sequences 72–78 and 380–384, respectively, have been suggested to be correlated to attenuation in an animal model [18, 19]. These sequences are organized within a steric arrangement on the virion surface resulting from an overlapping of the disulfide-stabilized domains 60–120 and 300–397 in the E protein [21, 26]. Therefore, it is expected that virulence determinants may be defined by three-dimensional structures on the surface of the virion. Thus, an extended study including a detailed pattern of the distribution and evolution of virus strains and additional sequencing of the entire E-gene region is required to correlate genetic and phenotypic changes with virulence.

Data such as those presented in this report should be extended in order to determine their usefulness for epidemiologic surveillance and disease prevention. An exhaustive analysis of the variability of targetted antigenic epitopes may be valuable in designing efficient vaccines directed against epidemic strains.

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