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GENETIC VARIATION WITHIN THE PREMEMBRANE CODING REGION OF DENGUE VIRUSES FROM THE YUCATAN PENINSULA OF MEXICO

FRANCISCO J. DÍAZ, JOSE A. FARFÁN-ALE, KEN E. OLSON, MARÍA A. LOROÑO-PINO, DUANE J. GUBLER, CAROL D. BLAIR, WILLIAM C. BLACK IV, AND BARRY J. BEATY

Arthropod Borne and Infectious Diseases Laboratory, Department of Microbiology, Colorado State University, Fort Collins, Colorado; Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatan, Merida, Yucatán, Mexico; Division of Vector Borne Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado

Abstract. Single-strand conformation polymorphism (SSCP) and sequence analyses were used to characterize genetic polymorphisms and phylogenetic relationships, respectively, among dengue (DEN) viruses isolated between 1980 and 1997 from Yucatan, Mexico and surrounding states. Amplified cDNAs from the premembrane (prM) coding region of the DEN viruses were characterized by SSCP. There were six distinct haplotypes of DEN-1 viruses, four haplotypes of DEN-2, four haplotypes of DEN-3, and eight haplotypes of DEN-4. The diversity index for DEN-3 isolates was significantly lower than that of the other serotypes, probably reflecting the recent introduction of this viral serotype into Mexico. The SSCP was a sensitive (84.5%) and specific (95.5%) technique for identifying nucleotide substitutions. Sequence analyses provided insight into the phylogenetic relationships of the DEN strains isolated in Yucatan. One DEN-2 isolate from 1996 was demonstrated to cluster with viruses of the Sri Lanka genotype, none of which have been detected before in the Americas.

INTRODUCTION

Epidemic dengue (DEN) and dengue hemorrhagic fever (DHF) have emerged as major public health problems in tropical and subtropical areas throughout the world.[1,2] Infection with DEN virus can result in clinical manifestations ranging from asymptomatic to fatal. Most cases are classified as dengue fever (DF), an incapacitating and painful but self-limiting condition. Dengue hemorrhagic fever and its extreme form, dengue shock syndrome (DSS), are potentially fatal presentations of the illness characterized by hemostatic disorders and plasma leakage.[1–6] The DEN viruses are mosquito-borne pathogens, and *Aedes aegypti*, a highly domesticated mosquito, is the most important vector. *Aedes aegypti* and DEN viruses have undergone dramatic expansions since the middle of the 20th century in most of the tropical and subtropical regions of the world.[1,6] In the Americas, epidemic DHF/DSS appeared for the first time in Cuba in 1981 during an explosive outbreak that lasted for four months.[6] It then occurred sporadically in several countries until 1989 when epidemic DHF emerged again in Venezuela. Other countries of South America, Central America, and the Caribbean islands were soon affected, and DHF became a permanent threat to this region.[1,5]

The DEN viruses (genus *Flavivirus*, family Flaviviridae) contain a positive-sense, single-stranded RNA approximately 11,000 nucleotides in length. The genome contains a single open reading frame that encodes a polyprotein. This polyprotein is cleaved co- and post-translationally to produce three structural proteins: capsid (C), premembrane (prM) and envelope (E), and seven nonstructural (NS) proteins.[7,8] There are four antigenically related DEN virus serotypes (DEN-1-4), all of which have similar transmission cycles and cause similar clinical manifestations in the human host. All four serotypes now circulate in the Americas.[5]

Molecular epidemiologic approaches have revealed extensive genetic variability within the DEN serotypes and have led to the recognition of two to five different genotypes within the respective serotypes.[9–16] Genotypes differ in their ability to cause severe DEN disease. For example in the Americas, infection with the native DEN-2 American genotype typically results in DF cases, whereas infection with recently introduced, Southeast Asian genotype more frequently results in DHF/DSS.[17,18]

The changes in the epidemiology of DEN in the Western Hemisphere are illustrated by events in Mexico. From 1947 to the 1960s, health authorities in Mexico conducted intensive campaigns to eradicate *Ae. aegypti*, and the mosquito was officially declared eradicated from the country in 1963.[19] However, *Ae. aegypti* was again detected in Mexico only two years later.[20] Major epidemics of DEN-1 occurred on the eastern coast of Mexico during 1979 and 1980. Approximately 17,000 cases of DEN were reported in Mexico in 1981. In 1984 and 1985, DEN was diagnosed in 25 of 32 states in Mexico and DEN-1, 2, and 4 were reported throughout Mexico. In 1995, DEN-3 was isolated for the first time and cases of DHF were confirmed.[21] In the Yucatan peninsula (Figure 1), from 1979 to 1981, thousands of cases were reported each year and DEN-1 virus was identified as the etiologic agent. The DEN-4 virus caused a major epidemic in 1984 with more than 5,000 reported cases but only one case met all World Health Organization criteria for DHF.[22] In 1991, DEN-2 was detected in Yucatan and 364 cases were confirmed. Dengue hemorrhagic fever appeared in epidemic form for the first time in 1996[23] and in 1997, 4,049 cases of DEN were reported in Yucatan with 163 cases of DHF (Sistema Nacional de Vigilancia Epidemiológica, Mexico, 1998). The DEN-3 virus was the predominant serotype in 1997, representing 92% of the DEN virus isolates. Thus, DF has been diagnosed in Mexico every year since 1978, but epidemic DHF/DSS occurred only after 1995, when the incidence of DEN infection increased abruptly.[21]

Sequence analyses of DEN-1 and DEN-2 isolates have suggested that at least two different genotypes in each serotype have circulated in Mexico.[9,18] Two DEN-4 isolates from 1984 were classified in a single genotype.[13,16] As far as we know, the RNA sequences of DEN-3 viruses from Mexico have never been studied. Knowledge of the phylogenetic relationships of DEN viruses in Yucatan is important to understand the trafficking and evolutionary potential of the viruses. Timely information on specific genotypes circulating in a region would also be of great value to public health authorities.

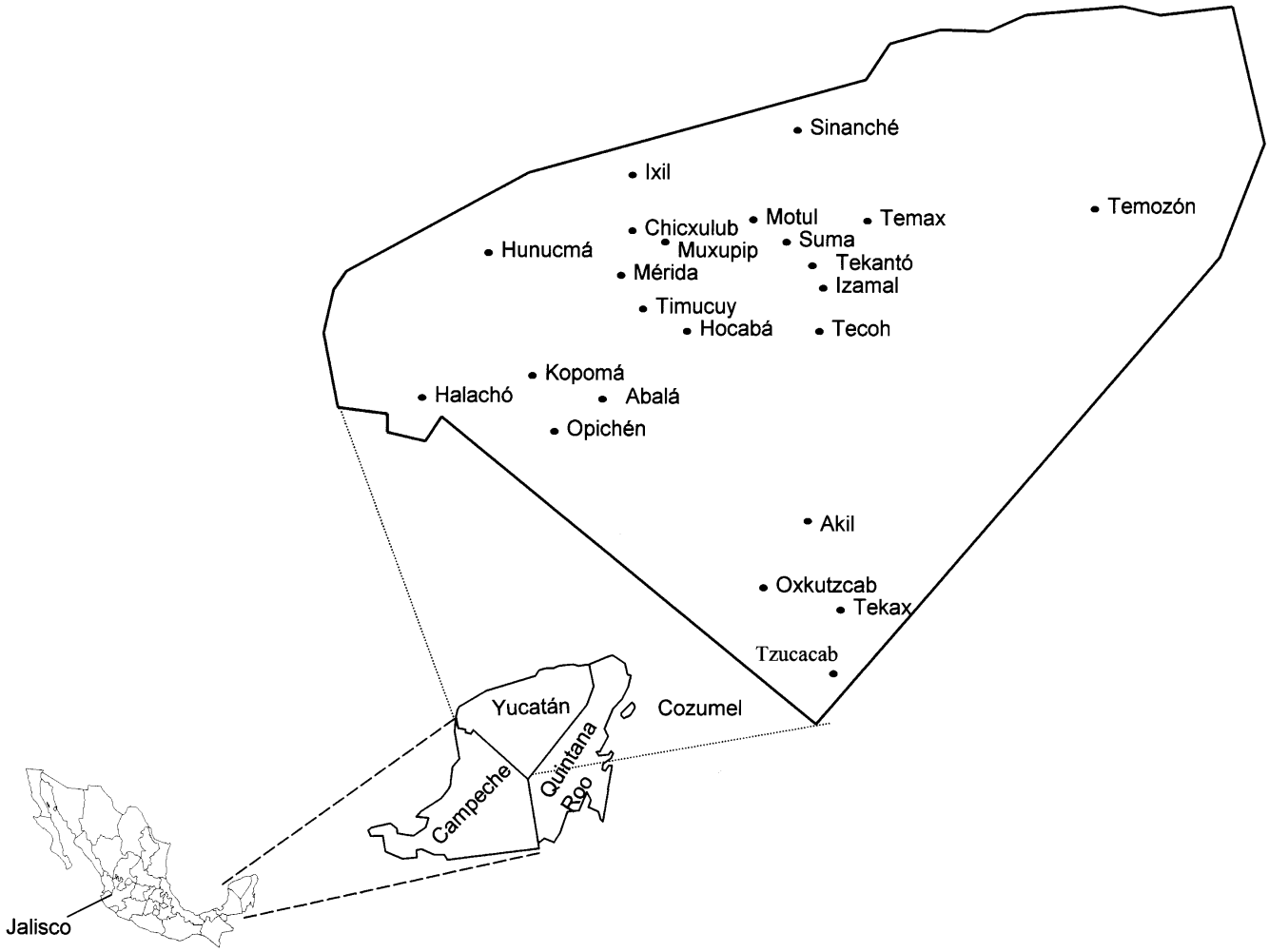

FIGURE 1. Map showing cities and states in Mexico from which dengue viruses originated for this study. Each location is a site where virus was transmitted to patients.

We previously used single-strand conformation polymorphism (SSCP) analysis to characterize rapidly genetic variation at three loci within the RNA genome of 12 DEN-2 viruses.[11] Comparison of SSCP patterns with E gene nucleotide sequences in GenBank demonstrated that SSCP analyses were reproducible and sensitive for detecting base changes within the virus RNA genome (Farfán JA, Ph.D Dissertation, Colorado State University, Fort Collins, CO, 2001). The prM locus proved to be more variable and presumably more informative for genetic analyses than loci at the E and NS5 genes.[11] In this work, molecular epidemiologic techniques (i.e., SSCP and sequence analyses) were used to genetically characterize the DEN viruses circulating in Yucatan, Mexico during an 18 year period. The sensitivity and specificity of the SSCP technique for detecting genetic polymorphisms in the prM region of the DEN genome were estimated and phylogenetic relationships of the viruses were established.

MATERIALS AND METHODS

Viruses. The DEN viruses from Yucatan, Mexico were obtained from a virus collection provided by the Centro de Investigaciones Regionales Dr. Hideyo Noguchi of the Universidad Autónoma de Yucatan and the Division of Vector Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC), San Juan Laboratories, (San Juan, PR). The DEN viruses in this collection were isolated from human serum, passed one to several times in C6/36 (*Ae. albopictus*) cells, and the serotype was determined by immunofluorescence analysis using serotype-specific antibodies.[24] Some of the isolates were obtained from patients that had hemorrhagic manifestations, but only one met the complete DHF definition.[3,22] The virus collection was comprised of 23 DEN-1, 10 DEN-2, 62 DEN-3 and 45 DEN-4 isolates (Table 1). Seventy-four (51%) of the viral isolates were obtained from patients from Merida, 51 (36%) were from other municipalities in the state of Yucatan, and 7 viruses (5%) were from patients from the states of Campeche and Quintana Roo in the Yucatan peninsula (Figure 1). Two viruses were from patients from the state of Jalisco and four (two from Yucatan and two from Quintana Roo) had no information regarding the specific municipality. The remaining four samples were obtained from CDC, and these viruses were designated only as originating from Mexico.

Extraction of RNA and amplification of cDNA. Viruses were propagated in C6/36 cells once prior to isolation of viral RNA. Total RNA was extracted from the infected cells by a

TABLE 1
Place of origin and year of isolation of 150 dengue (DEN) virus isolates used in single strand conformation polymorphism analysis*

Serotype	State	City	Year	Isolate designation
Dengue-1	Yucatan	Merida	1980	**BC159**
		Merida	1994	**BC136, BC137,** BC138, BC120, **BC131,** BC132, **BC119, BC121,** BC125, **BC126,** BC127, **BC128,** BC129, BC140, BC141, BC260, BC261, BC262, BC263
	Campeche	Campeche	1995	BC3, BC264,
	Quintana Roo	Cozumel	1995	BC7
Dengue-2	Yucatan	Merida	1994	**BC134**
		Merida	1996	**BC17**
	Quintana Roo	Unknown	1994	**BC133, BC139**
	Jalisco	Unknown	1992	**BC122,** BC123
	Unknown	Unknown	1983	**SJL1421**
	Unknown	Unknown	1984	SJL1481, SJL1479, **SJL1482**
Dengue-3	Yucatan	Merida	1995	**BC4**
		Merida	1996	**BC21, BC22,** BC23, BC24, BC28, **BC29,** BC35, BC205
		Merida	1997	BC158, BC159, BC186, BC187, BC188, BC189, BC289, BC294, BC298, BC299
		Temax	1996	BC19, BC20, BC2
		Motul	1996	BC25, BC179, BC181, BC203, BC200
		Hunucma	1996	BC30, BC36, **BC285**
		Oxkutzcab	1996	BC37
		Oxkutzcab	1997	BC288
		Izamal	1996	BC38, BC284
		Sinanche	1996	BC180
		Akil	1996	BC182, BC183, BC184
		Tekax	1996	BC206
		Ixil	1996	BC208
		Muxupip	1996	BC283
		Chicxulub	1996	BC286
		Hocaba	1997	BC156, **BC192,** BC194, **BC196,** BC199, BC291, BC292, BC296, BC297
		Timucuy	1997	**BC178**
		Suma	1997	BC190
		Tecoh	1997	**BC195**
		Opichen	1997	BC290
		Temozon	1997	BC295
		Kopoma	1997	BC300
		Halacho	1997	BC301
		Unknown	1997	BC157, BC191
	Quintana Roo	Cancun	1997	**BC177**
		Unknown	1997	**BC197**
Dengue-4	Yucatan	Merida	1984	**BC146, BC147,** BC148, **BC149,** BC155, BC156, BC157, **BC158,** BC142, **BC143,** BC144, BC145, BC150, BC180, BC187, **BC195, BC196,** BC197, BC201, BC202, BC203, BC204, BC205, BC206, **BC207,** BC208, BC209, BC210
		Merida	1994	BC135
		Merida	1995	**BC9**
		Merida	1996	**BC18**
		Oxkutzcab	1995	BC2, **BC5, BC10, BC24**
		Chicxulub	1996	BC201, BC204, BC207, **BC293**
		Abala	1995	**BC6**
		Izamal	1996	BC26
		Tekanto	1996	BC185
		Tzucacab	1997	**BC287**
	Quintana Roo	Benito Juarez	1995	**BC8**
		Unknown	1995	BC11

* Isolates sequenced in the premembrane gene are shown in **bold.**

guanidine thiocyanate/phenol-chloroform method[25] or with the QIAamp® system according to the manufacturer's instructions (Qiagen, Inc., Valencia, CA). Primers were designed to amplify cDNA segments from the prM region that were approximately 300 nucleotides for SSCP or 400–500 nucleotides for sequencing (Table 2). The nucleotide sequences used in primer design were from the following strains: DEN-1 S275/Singapore90,[26] DEN-2 NGC/New Guinea44,[27] DEN-3 H-87/Philippines56,[28] and DEN-4 814669/Dominica81.[29]

Viral RNA was reverse transcribed to cDNA using the SuperScript II RNaseH-reverse transcriptase (Life Technologies, Gaithersburg, MD) or in some instances RAV-2 reverse transcriptase (Amersham, Buckinghamshire, United Kingdom) following the instructions of the manufacturers. Two microliters of first-strand cDNA reaction were amplified in a total volume of 25 μL containing 0.5mM of each forward and reverse primers in 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.05 units/μL of *Taq* polymerase (Promega, Madison, WI). The polymerase chain reaction (PCR) was performed as follows: an initial denaturation of one minute at 94°C, 30 cycles of 94°C for one minute, annealing at 53–58°C (Table 2) for one minute, and extension at 72°C for two minutes. The samples were then incubated at 72°C for 10 minutes. The expected sizes of the amplified products were verified by electrophoresis in 2% agarose.

TABLE 2
Primer sequences, length of the amplified cDNAs, and annealing temperatures used in the polymerase chain reaction of each dengue virus serotype*

Primer name	Sequence (5′ → 3′)	Product length (basepairs)	Annealing temperature (°C)
DN1PRM1F	CGTTCCATTTGACTACACGAG	311	54.7
DN1PRM1R	TAGACCAAGTCCCACGTGTGG		
DN2PRM1F	ACCACACGTAACGGAGAACCA	291	54.7
DN2PRM1R	TCCCACATGTGGAACGAGTGC		
DN3PRM1F	GCAACACTTGCTTTCCACTTA	287	53.4
DN3PRM1R	GATCTCTTATCGCGTCTATGC		
DN4PRM1F	GTCAACAAGAGATGGCGAACC	284	53.4
DN4PRM1R	GTGGTGTTAAAGCTACTGAGC		
DEN1F-314	CAAAGTGCTACGGGGTTTCAA	445	56.4
DEN1R-738	GGACATCCACGTTTCGGTTCT		
DEN2F-367	ATGCTGAACATCTTGAACAGG	504	53.9
DEN2R-850	TATGGTGTATGCCAGGATTGC		
DEN3F-385	ACGGAAAAAGACATCGCTCTG	392	56.2
DEN3R-758	CAGCCGACATCCAGGTTTG		
DEN4F-357	GAGATAGGCCGCATGCTGAAC	486	54.3
DEN4R-822	GTGGTGTTAAAGCTACTGAGC		

* The first four pairs were used for single strand conformation polymorphism and the last four for sequencing.

SSCP analysis. The original SSCP procedure described by Orita and others[30] was modified as described in detail.[11,31] Briefly, 1 μL of the PCR product was mixed with 9 μL of denaturing solution, heated to 95°C for three minutes, and then immediately placed on ice for five minutes. Two microliters of each mixture were loaded directly onto an 8% polyacrylamide gel, subjected to electrophoresis, fixed, and stained with silver nitrate. When the cDNA from a virus exhibited a unique SSCP banding pattern, it was designated as a new haplotype. The term genotype was reserved for the groups produced in the phylogenetic analysis of sequences.

Statistical analysis of SSCP diversity. The diversity of SSCP haplotypes of each DEN serotype was analyzed using the T diversity index of Keefe and Bergersen[32] where $T = 1 - \Sigma p_i^2$ and p_i is the proportion of the haplotype i among all haplotypes in the group. For this analysis, the unbiased estimator $TU = 1 - (n/(n - 1))\{\Sigma(p_i)^2 - (1/n)\}$ was calculated and the Z score used to assess the significance of the differences between diversity indexes: $Z = (TU_1 - TU_2)/\{\sigma_1^2/n_1 + \sigma_2^2/n_2\}^{1/2}$, where σ_1^2 and σ_2^2 denote the estimated variance of TU_1 and TU_2, respectively.

Sequence analysis. The extraction of RNA, reverse transcription, and PCR were done as described earlier. Direct sequencing of both strands of the amplified products was performed with the same primers used for the PCR in ABI 377 DNA sequencers (Perkin-Elmer Inc., Foster City, CA). At least one isolate in each SSCP haplotype was selected for sequencing. The 43 sequenced isolates are noted in bold in Table 1. Sequence data were used as the gold standard to evaluate the sensitivity and specificity of SSCP for detecting single nucleotide differences. For the phylogenetic analysis, cDNA sequences of isolates of the same serotype were aligned using Seqman software (Dnastar, Inc., Madison, WI). Sequences were trimmed to the length that produced the longest possible alignment with clear sequencing results. Sequences of relevant reference strains available in GenBank (Table 3) were trimmed accordingly and included in the alignment. Sequences of DEN-1 Mexican isolates 1298/Mexico80 and 1378/Mexico83 were kindly provided by Dr. F. Liprandi (Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela). Two additional DEN-2 reference strains were sequenced in the prM locus as part of this work (10/Somalia84 and 1592/Sri Lanka85). A phylogenetic tree was constructed for each DEN virus serotype using the maximum likelihood algorithm in the Phylogenetic Analysis Using Parsimony (PAUP) program version 4.04[33] with 500 replicates for the bootstrapping.

RESULTS

SSCP analysis. The SSCP banding patterns for DEN-1 are shown in Figure 2 and are illustrative of the SSCP results for all serotypes. The SSCP analysis allowed us to distinguish six SSCP haplotypes from the 23 DEN-1 isolates obtained from states comprising the Yucatan peninsula (Figure 1). The DEN-1 isolated in 1980 comprised haplotype 1. Most (68%) DEN-1 viruses isolated during the 1994-1995 epidemic in Yucatan displayed an identical SSCP pattern that was designated haplotype 3. Three isolates from Merida 1994 were classified as haplotype 2 and exhibited minor differences in the SSCP pattern. These were later confirmed as identical by sequencing. The other four isolates comprised haplotypes 4, 5 and 6, respectively (Figure 2). The DEN-2-4 isolates were similarly analyzed. Four, four, and eight distinct haplotypes were observed among 10, 62, and 45 isolates of DEN-2, DEN-3, and DEN-4, respectively. Diversity indexes for each serotype and comparison among them (Z-score) are shown in Table 4. The DEN-3 viruses were significantly less diverse than the others.

Nucleotide sequences of the same segments used in SSCP were used to evaluate its ability of SSCP to detect nucleotide substitutions. The results revealed a sensitivity of 84.5% and a specificity of 95.5%. The predictive positive value was 99.5% and the negative predictive value was 37.0%. The overall efficacy of the test was 85.4%. In most of the cases (56.8%), SSCP detected single nucleotide differences; however DEN-2 isolates SJL1421/Mexico83 and BC134/Merida94, which differ by five nucleotides (1.7% divergence), yielded identical SSCP patterns.

Phylogenetic analysis of DEN viruses from Yucatan. A number of isolates that exhibited identical or very similar sequences were excluded. The sequences remaining in the

TABLE 3
Dengue (DEN) virus reference strains used in the phylogenetic analyses*

Type	Strain	Country of origin	Year of isolation	Genotype	Genbank accession no.
DEN-1	Mochizuki	Japan	1943	Not classified	S75335
DEN-1	17646	Nauru	1974	1	M23027
DEN-1	S275-90	Singapore	1990	1	M87512
DEN-1	AHF82-80	Thailand	1980	2	D00502
DEN-1	Abidjan/1056	Cotê d' Ivoire	1998	Not classified	AF298807
DEN-1	DJIB/98/606	Djibouti	1998	Not classified	AF298808
DEN-1	FGA/89	French Guyana	1989	1	AF226687
DEN-1	GZ80	China	Not reported	Not classified	AF350498
DEN-1	836-1	Philippines	1984	3	D00503
DEN-1	CV1636-77	Jamaica	1977	2	D00501
DEN-1	40514	Brazil	1990	2	S64849
DEN-1	1298	Mexico	1980	Not classified	Not available
DEN-1	1378	Mexico	1983	Not classified	Not available
DEN-2	PR159	Puerto Rico	1969	V	M19197
DEN-2	200787	Mexico	1983	Not classified	L04561
DEN-2	VEN2	Venezuela	1987	Not classified	AF100465
DEN-2	FJ-10	China	Not reported	Not classified	AF276619
DEN-2	1592	Sri Lanka	1985	IV	Not available
DEN-2	#10	Somalia	1984	IV	Not available
DEN-2	NGC	New Guinea	1944	I	M29095
DEN-2	16681	Thailand	1964	IIIa	M84727
DEN-2	1409	Jamaica	1983	IIIb	M20558
DEN-2	PUO218	Thailand	1980	IIIa	D00345
DEN-3	H-87	Philippines	1956	I	L11423
DEN-3	29472	Fiji	1992	I	L11422
DEN-3	85-159	Indonesia	1985	I	L11425
DEN-3	29586	Malaysia	1981	I	L11427
DEN-3	MK315	Thailand	1987	II	L11442
DEN-3	CH3489D	Thailand	1973	II	L11620
DEN-3	5987	Thailand	1962	II	L11440
DEN-3	1416	India	1984	III	L11424
DEN-3	2783	Sri Lanka	1991	III	L11438
DEN-3	1558	Mozambique	1985	III	L11430
DEN-3	1696	Samoa	1986	III	L11435
DEN-3	1327	Tahiti	1965	IV	L11439
DEN-3	1340	Puerto Rico	1977	IV	L11434
DEN-3	PR6	Puerto Rico	1963	IV	L11433
DEN-4	H241-P	Philippines	1956	I	S66064
DEN-4	B5	China	Not reported	Not classified	AF289029
DEN-4	814669	Dominica	1981	II	M14931

* The genotype classification was taken from the following references: for DEN-1, Chunge and others;[13] for DEN-2, Lewis and others;[14] for DEN-3, Lanciotti and others;[15] for DEN-4, Lanciotti and others.[16]

analysis were submitted to GenBank (Accession numbers AF459605 to AF459627).

DEN-1. The 324 basepairs analyzed (nucleotides 437-760 in the DEN-1 genome) contained 103 phylogenetically informative characters. The six isolates from Yucatan and the two other Mexican isolates diverged by 1% or less in the prM region and clustered together in the phylogenetic tree with the isolates 0514/Brazil90 and FGA/French Guyana89. They were more distantly related to CV1636/Jamaica77 and 836-1/Philippines84 (Figure 3A). Two sequences from 1980 (BC159/Merida80 and 1298/Mexico80) were identical. These could represent the same isolate present in two different collections.

DEN-2. The 357 basepairs analyzed (nucleotides 439-795) contained 104 phylogenetically informative characters. Six viruses in the collection that were isolated before 1995 diverged by less than 2% and clustered with older viruses of the Americas (genotype V in Lewis and others[14]), such as PR159/Puerto Rico69, VEN2/Venezuela87, and 200787/Mexico83 (Figure 3B). The SJL1421/Mexico83 isolate was identical in the prM sequence to VEN2/Venezuela87. The most recent Mexican isolate, BC17/Merida96, diverged between 8.9% and 10.2% from the other Mexican isolates and clustered with strains such as 1592/Sri Lanka85, FJ-10/China, and 10/Somalia84 in the genotype Sri Lanka (genotype IV[14]).

DEN-3. The 330 basepairs analyzed (nucleotides 437-766) contained 93 phylogenetically informative characters. None of eleven sequenced Mexican isolates differed by more than one nucleotide (0.3%) from the consensus sequence of the group. In the phylogenetic tree (Figure 3C), all of these isolates clustered with those in the Sri Lanka/India genotype (group III in Lanciotti and others[15]). The greatest similarity was found with the 1558/Mozambique85 isolate, which diverged by less than 1% from the Mexican viruses.

DEN-4. The 393 basepairs analyzed (nucleotides 441-833) contained 93 phylogenetically informative characters. The Mexican isolates clustered in a single branch of the phylogenetic tree (Figure 3D). Those from 1984 were closer to the reference strain 814669/Dominica81. Viruses isolated from 1995 to 1997 formed a separated clade within the same branch and diverged between 1.8% and 3.2% from the earlier isolates. The prototype strain H241-P/Philippines56 diverged between 6.0% and 7.8% from the Mexican isolates and clustered with B5/China.

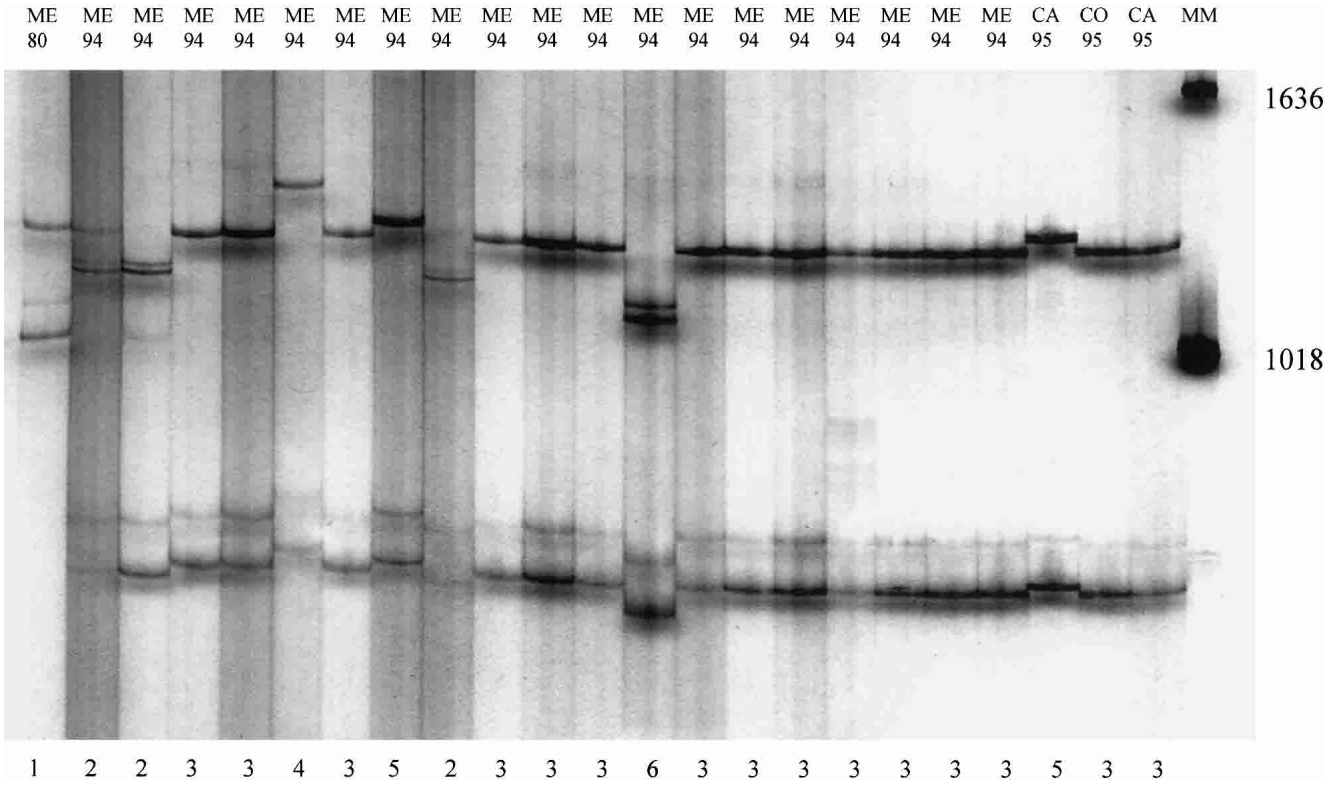

FIGURE 2. Single strand conformation polymorphism (SSCP) analysis of dengue-1(DEN-1) virus isolates from the Yucatan Peninsula. ME = Merida; CA = Campeche; CO = Cozumel; MM = Molecular marker. See Figure 1 for location of transmission sites. The year of transmission is given below the site of transmission. The numbers at the bottom of the SSCP gel indicate the assigned haplotype based on the SSCP pattern.

DISCUSSION

SSCP is an easy, rapid, and comparatively inexpensive means of detecting genetic variation at specific loci within the genomes of arboviruses.[11,31] Previous studies had determined that SSCP could detect 99% of the point mutations in DNA segments of less than 300 basepairs.[35] Here we report a sensitivity of 84.5% for sequences ranging in size between 284 to 311 bases. This sensitivity, although somewhat lower, is still enough for detecting sequence differences greater than 2%. A threshold of 6% divergence is currently used to separate different genotypes within a DEN serotype.[9,14] Our previous work showed that SSCP analysis of the prM locus consistently differentiated isolates from different genotypes.[11] Thus, SSCP can be used to screen large collections of isolates to select those that could be more informative in phylogenetic studies or to detect the introduction of new viruses in prospective molecular epidemiologic studies. However, some of the sequences that produce a distinct SSCP pattern differ in just one or a few nucleotides and may not represent different strains.

The low SSCP diversity index of DEN-3 haplotypes could be the result of the history of this serotype in Yucatan. The DEN-3 virus was isolated for the first time in Yucatan in September 1995. In contrast, DEN-1, DEN-2, and DEN-4 were originally identified in 1979, 1991, and 1984, respectively. It could be speculated that DEN-3 haplotypes originated from a reduced number of parent genotypes that were introduced into the peninsula and are still in an early time in the process of diversification. The others serotypes could have been introduced several times and/or persisted in Yucatan for a longer time and thereby diversified more than DEN-3.

Sequence data were used to determine phylogenetic relationships of the viruses within each serotype. Two phylogenetic studies analyzing a large number of DEN-1 genomic sequences have been published.[9,13] They analyzed either a 240 nucleotide sequence in the E-NS1 junction region of the genome[9] or a 180 nucleotide sequence in the E gene[13] and suggested that there were potentially five or three DEN-1 genotypes, respectively. The phylogenetic relationships in those studies were not comparable. Our analysis of a 324

TABLE 4

Keefe-Bergerson diversity index (TU) within dengue (DEN) virus serotypes and significance of differences among diversity indexes of DEN serotypes in Mexican isolates*

Diversity within DEN serotypes			
Serotypes	No of isolations	No. of haplotypes	TU
1	23	6	0.5691
2	10	4	0.5691
3	62	4	0.0951
4	45	8	0.5771

Significance of differences among DEN serotypes		
Serotypes	Z-score	*P*
1 vs. 3	3.8338	<0.05
1 vs. 4	0.0601	NS
3 vs. 4	5.5278	<0.05

* TU for DEN-2 was not compared with others serotypes because of the small number of the isolates available, some of which were not from the Yucatan. NS = not significant.

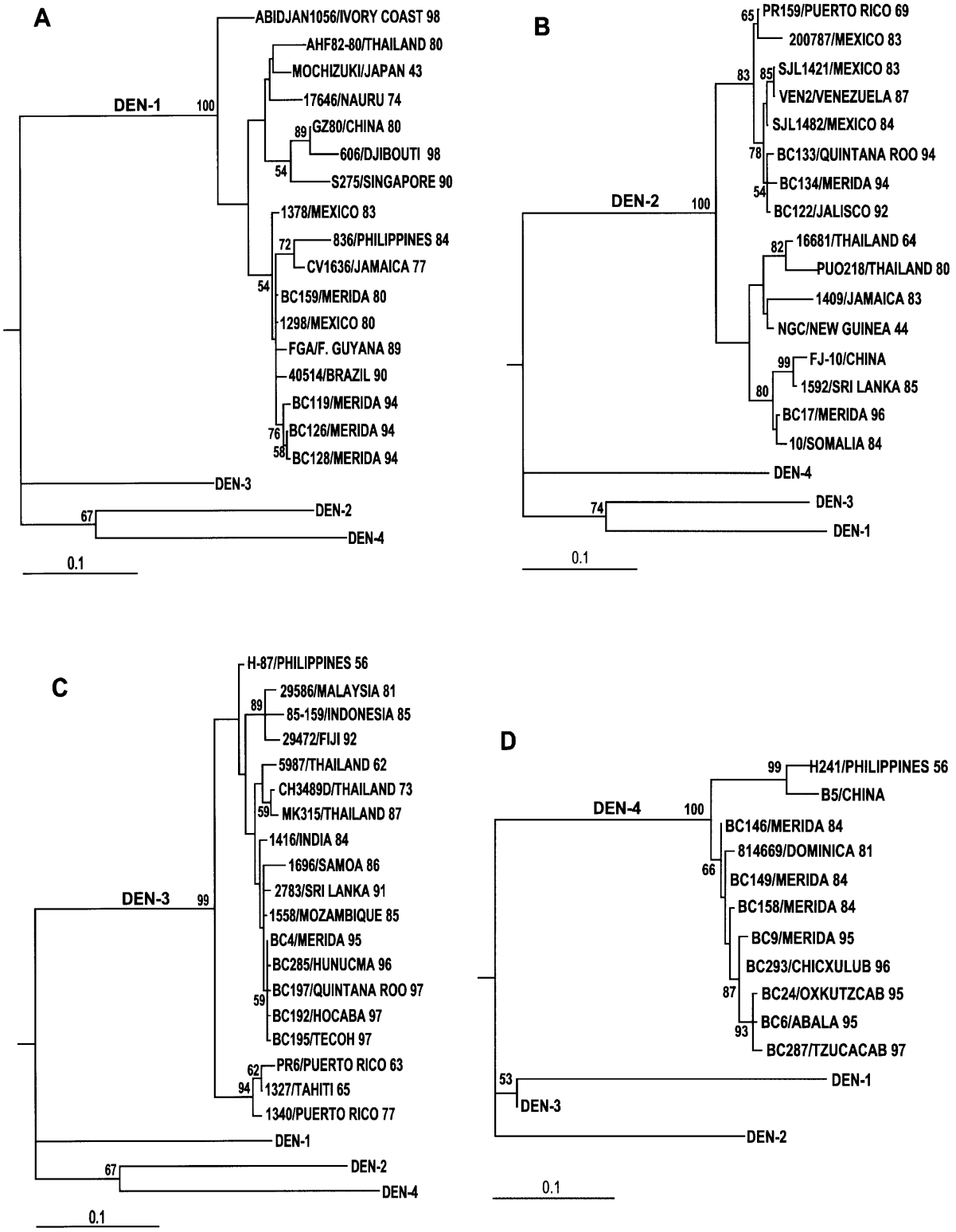

FIGURE 3. Maximum likelihood phylogenetic trees for the premembrane locus of **A**, dengue-1 (DEN-1), **B**, DEN-2, **C**, DEN-3, and **D**, DEN-4 viruses. The numbers displayed next to the nodes correspond to the bootstrap values (500 replicates) supporting that clade. Strains used as outgroups were DEN-1 17646/Nauru 74, DEN-2 PR159/Puerto Rico 69, DEN-3 H-87/Philippines 56, and DEN-4 814669/Dominica 81. Trees were constructed with Phylogenetic Analysis Using Parsimony (Swofford[33]) and drawn using Drawgram (Felsenstein[34]).

nucleotide sequence in the prM region of DEN-1 viruses generated a different tree with three major branches (Figure 3A) The different outcomes in this and the other studies may be partially attributable to the different isolates studied and different genomic segments analyzed. All the DEN-1 Mexican isolates that we analyzed clustered in a single branch. The possibility that a different DEN-1 genotype had circulated there in 1980, as suggested by the location of the isolate 1298/

Mexico80 in the phylogenetic tree of the E-NS1 junction,[9] was not supported in this work since this isolate appeared in the same branch containing isolates of 1983 and thereafter.

The DEN-2 viruses are genetically the most diverse among the DEN serotypes. At least four different genotypes have been identified in previous studies.[9,14,18,36] They are the sylvatic, Americas, Sri Lanka, and Southeast Asia genotypes. Most cases of DHF/DSS have been associated with this latter genotype. It includes not only viruses isolated in Southeast Asia, but also many of the DEN-2 isolates found in the Americas since 1981. Previous studies of the E-NS1 gene junction have shown that DEN-2 Mexican isolates belong either to the Americas or to the Southeast Asia genotypes depending on whether they were isolated before 1994 or thereafter.[18] In this study, all the Mexican viruses isolated until 1994 were classified in the Americas genotype, but the BC17/Meida 96 isolate of 1996 surprisingly clustered in the DEN-2 Sri Lanka genotype. This finding was confirmed by sequencing and phylogenetic analysis of the E-NS1 region. This is the first DEN-2 isolate from the Western Hemisphere found grouping in this genotype. This isolate was most closely related to the 10/Somalia84 isolate, from which it differed in only four nucleotides (1.1%); it diverged from the viruses in the Southeast Asia genotype by 4.6–8.1%. Since five Mexican DEN-2 viruses of 1995 studied by Rico-Hesse and others,[18] including four from the neighbor state of Chiapas, belong to the Southeast Asia genotype, it seems that two different DEN-2 genotypes arrived in southern Mexico at the same time.

Phylogenetic relationships of DEN-3 viruses have also been investigated. Chungue and others[12] compared 194 bases of the E gene of 27 isolates mostly from the Pacific and Southeast Asia and found four genotypes diverging by 6% or more. Lanciotti and others[15] compared the entire sequence of prM/M/E genes of 23 viruses, and also found four genotypes. The two studies only concurred in genotype IV, which contained old American and old Polynesian isolates. Differences in the selected isolates and length of the segments studied might account for the differences. Our DEN-3 phylogenetic tree, based on 330 bases of the prM gene, resembles that of latter study.[15]

The DEN-3 virus was absent from the Western Hemisphere for 16 years (1978–1993) and reappeared simultaneously in Nicaragua and Panama in 1994.[37,38] It was soon dispersed to other countries in Central America and Mexico and later to the Antilles and South America. An early isolate from these outbreaks (Nicaragua in 1994) was partially sequenced and included in the phylogenetic tree of Chungue and others,[12] where it clustered with viruses of genotype 2, which includes isolates from Southeast Asia.[37] Another early DEN-3 isolate in 1994 from Panama was genotyped at CDC and classified in the Sri Lanka/India genotype.[38] This analysis of isolates from Yucatan, as well as the analysis of isolates from Nicaragua in 1998,[39] supports the grouping of the new DEN-3 strain now circulating in the Americas in the DEN-3 Sri Lanka/India genotype (Figure 3C).

Two phylogenetic analyses of a significant number of DEN-4 isolates produced similar results;[13,16] both studies showed two well-defined groups of DEN-4 isolates. The first included viruses from Southeast Asia, The Philippines, Sri Lanka, and Senegal, and the second comprised viruses from the South Pacific, the Americas, and some from Indonesia. Although our phylogenetic analysis of DEN-4 was significantly limited because there were only three sequences of the DEN-4 prM region in GenBank, it seems to resemble the published phylogenies. Both the earlier (1984) and the later (1994–1997) Mexican DEN-4 viruses, cluster in the second genotype with the 814669/Dominica81 isolate (Figure 3D). The low divergence between the two subgroups of Mexican DEN-4 isolates (1.8-3.2%) is compatible with the rate of nucleotide substitution reported for this virus,[16] which suggests that the strain introduced in Yucatan in 1994 is the same virus that caused the 1984 outbreak,[22] and that both are derived from the original DEN-4 virus that appeared for the first time in the Americas in Dominica in 1981.

The most important change in the epidemiology of DEN in Mexico in the last two decades is the abrupt increase in the number of cases of DHF/DSS in 1995.[21,40] In that year, all four DEN serotypes were found simultaneously circulating, DEN-3 was detected for the first time in the country, and the Southeast Asian genotype of DEN-2 also appeared,[18] closely followed by the DEN-2 Sri Lanka genotype. Which of these changes in DEN circulation conditioned the worsening of the epidemiologic situation? The data available are limited but provide some hints. Twenty of 25 isolates obtained from DHF/DSS cases were DEN-2.[21] Some of them were classified as DEN-2 genotype Southeast Asia,[18] which is well known to contain virulent viruses. The role of the DEN-2 genotype Sri Lanka described in this study is difficult to assess since the only virus available (BC17/Merida96) was isolated from a 10-year-old patient with a primary infection and who clinically presented as a DF case (Farfán JA, unpublished data). However, it is provocative that the year of introduction (1996) coincided with the emergence of DHF in Yucatan.[23] The eventual contribution of the new DEN-3 strain to DHF in the Yucatan remains to be determined. This DEN-3 Sri Lanka/India genotype had been associated with mild disease in the early 1980s, but it was identified later in outbreaks of severe cases in Mozambique in 1984–1985,[39] Sri Lanka in 1989, and India in 1990.[15] Its emergence in 1994 in the Americas was temporally associated with cases of DHF in Nicaragua, but data are lacking about its role in more recent DHF outbreaks in the Americas.

A final issue to consider is the route of arrival of the new DEN-2 and DEN-3 viruses found in Yucatan. It is noteworthy that both viruses are phylogenetically related to viruses that have circulated in similar geographic regions. The DEN-3 Sri Lanka/India genotype viruses have been reported in Sri Lanka in 1981–1991, India in 1984, Mozambique in 1985, and Samoa in 1986.[15] The DEN-2 Sri Lanka genotype viruses have been found in Malaysia in 1969, Indonesia in 1973–1978, the Seychelles Islands in 1997, Sri Lanka in 1981–1990, Burkina Faso in 1982–1986 and Somalia in 1984.[9,14,36] It is also interesting that both new viruses exhibit the closest relationship in the prM gene with strains from East Africa (DEN-3 from Mozambique in 1985 and DEN-2 from Somalia in 1984), and that they were introduced to Mexico about the same time (1995–1996). This suggests trafficking of viruses from the Indian Ocean or Africa to the Americas. Commerce and travel between India and eastern African countries and Pacific Islands have been implicated in the dispersal of DEN viruses to Africa and Polynesia, respectively.[9,15] To understand the origin of the new DEN-2 virus in Yucatan, more information is needed about the presence of the Sri Lanka genotype viruses in recent years in Africa, Southeast Asia, and the Pacific Islands, as well as a more detailed study of the

DEN-2 viruses prevalent in Mexico and Central America in recent years.

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Authors' addresses: Francisco J. Díaz, Ken E. Olson, Carol D. Blair, William Black IV, and Barry J. Beaty, Arthropod Borne and Infectious Diseases Laboratory, Department of Microbiology, Colorado State University, Foothills Research Campus, 3107 Rampart Road, Fort Collins, CO 80523. Jose A. Farfán-Ale and María A. Loroño-Pino, Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatan, Merida, Yucatan 97000, Mexico. Duane J. Gubler, Division of Vector Borne Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO 80523.

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