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Dengue 2 genotypes in the state of Oaxaca, Mexico

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Summary. To genetically characterize dengue 2 (DEN-2) viruses in Oaxaca, Mexico, the C protein, and a portion of the prM protein genes of 8 isolates from the 2001 DEN epidemic were sequenced. The sequences were compared to those of prototype DEN-2 viruses from various parts of the world. Phylogenetic analysis suggested that the 2001 isolates of DEN-2 were of the American/Asian genotype and were most similar to the Jamaica and Venezuelan isolates MARA3, LARD1996 and LARD1910. Molecular analyses confirmed the origin of the isolates. This study indicates that DEN-2 strains of American/Asian genotype probably from Southeast Asian are circulating in Oaxaca.

Introduction

Dengue fever (DF) and dengue hemorrhagic fever and shock syndrome (DHF/DSS) are mosquito-borne infectious diseases that have become major international public health concerns. DF and DHF/DSS occur in tropical and sub-tropical regions around the world, predominantly in urban and semi-urban areas. There are four dengue serotypes, which are transmitted to humans principally through the bites of *Aedes aegypti*. Recovery from infection by one serotype provides lifelong immunity against that serotype, but confers only partial and

transient protection against subsequent infection by the other three. Sequential infection increases the risk of DHF, and this may be also be associated with the order of serotypes infecting a patient [32]. DHF/DSS cases have also been associated with primary infections with virulent strains of DEN virus (DENV) [30].

Dengue virus (family *Flaviviridae*, genus *Flavivirus*, species *Dengue virus*) genotypes are defined in phylogenetic studies as having no more than 6% variation in the E/NS1 junction gene [27]. DEN-1 consists of five subtypes (I–V) [27] and DEN-2 virus contains six, although DEN-2 virus subtype III has been further divided into sublineages IIIa and IIIb [19]. Armstrong and Rico-Hesse [1] classified DEN-2 strains isolated from America in two major groups, Asian and American. Twiddy et al. [37] have proposed the following genotypes: American, Cosmopolitan, Asian Genotype 1, Asian Genotype 2, American/Asian, in addition to a Sylvatic lineage. DEN-3 and DEN-4 viruses are currently classified into four and two genotypes, respectively [16, 17].

Molecular epidemiological studies suggest that infections with viruses in the American DEN-2 genotype were not associated with DHF/DSS [27, 28, 40]. In contrast, DHF/DSS epidemics have been associated with the introduction of Southeast Asian DEN-2 genotypes, the first of which was the Cuban epidemic in 1981 [9, 28]. There were an estimated 10,000 cases of DHF/DSS [14]. Until recently virus isolates made during the Cuban epidemic were not generally available for study, however, isolates were made during that time in neighboring Jamaica, and phylogenetic analyses of these viruses revealed that their origin was Southeast Asia, presumably Vietnam, Malaysia or Thailand [6, 28]. Recently, Cuban isolates from the epidemic of 1997 were demonstrated to have a total conservation of the E gene sequence and have been classified as American/Asian genotype [29].

An epidemic of DHF in the New World took place in Venezuela in 1989; isolates of DEN-1, -2 and -4 were obtained during this epidemic, but the most severe cases were associated with DEN-2 infections [25, 26]. A DHF epidemic in Brazil in 1990, was also associated with DEN-2 virus [24, 26]. A major epidemic of DHF/DSS in Nicaragua in 1994 was associated with the introduction of DEN-3 virus, probably of Indian or Sri Lankan origin.

DHF was first reported in Mexico in 8 patients in 1985 and until 1994, only sporadic DHF cases were detected. However, in 1995, there were 358 DHF cases confirmed, and most virus isolates were DEN-2 [4]. In 1995–1996, 539 DHF cases and 30 deaths were reported. DHF cases were confirmed and reported in 14 countries in the Americas and DHF was endemic in many of these areas by 1995 [10]. Major epidemics of DEN-1 occurred in Mexico (Chiapas, Edo. De Mexico, Guerrero, Hidalgo, Jalisco, Michoacan Oaxaca, Puebla, Queretaro, Quintana roo, San Luis Potosi, Tabasco, Tamaulipas, Veracruz, Yucatán) from 1979–1983. DEN-2 appeared for the first time in Oaxaca and Tamaulipas in 1982, but was not associated with DHF/DSS. DEN-3 was introduced later in 1995. This serotype appeared in Chiapas, Puebla, San Luis Potosi, Tamaulipas and Veracruz. DEN-3 was reported in Oaxaca in 2002. Cases of DEN-4 were reported in 1982 in Tamaulipas, and Oaxaca and in the Yucatan in 1984. Clearly, the epidemiology of DEN in a geographic areas is complex and dynamic.

Phylogenetic studies provide powerful approaches to monitor the introduction, movement and trafficking of viruses as well as to predict the potential epidemiological consequences of such events [12, 28, 36]. Many studies indicate that dengue viruses are evolving and diverging rapidly via intramolecular changes and perhaps recombination [38, 39, 41]. This could result in the generation of new, more virulent dengue viral genotypes in the future. A number of genetic markers have been proposed to condition the increased virulence of dengue viruses from Asia [18]. Identification of genotypic markers correlated with increased virulence would be of public health importance.

To provide more information on DEN epidemiology and virulence, we characterized a number of DEN-2 viruses isolated in Oaxaca, Mexico in 2001. The nucleotide sequence of the C and a portion of the prM protein genes of 8 DEN-2 viruses isolated from patients with DF and DHF were sequenced. Phylogenetic and genetic analyses revealed the putative geographic origin of the viruses and potential molecular determinants of virulence.

Materials and methods

Viruses

Isolates of DEN-2 viruses were obtained from acute-phase plasma collected from patients in the epidemiologic surveillance program of the Secretaria de Salud Oaxaca, Mexico during 2000–2001 [4]. Eight isolates were kindly provided by the State of Oaxaca Public Health Laboratory. These were anonymous samples and only information on the clinical disease associated with the respective infection was provided (Table 1). All viruses were passaged once in C6/36 cells, and the resultant viruses were analyzed in the present studies.

Antibodies

Murine hybridomas against human DENV antigens (anti-DEN-2 HB46) were obtained from the ATCC and grown in Dulbecco's modified Eagle medium (GIBCO-BRL) supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin G (100 U/ml), and

Table 1. Dengue virus isolates from Oaxaca, Mexico used in this study[a]

Strain	Location	Date	Clinical status[b]	Localization in map
SALC9	Salina Cruz, Oaxaca	06-2001	DF	1
JUCH5	Juchitan, Oaxaca	04-2001	DHF	2
TUX19	Tuxtepex, Oaxaca	06-2001	DHF	3
TON4	Tonala, Oaxaca	07-2001	DHF	4
HUAT2	Huatulco, Oaxaca	03-2001	DF	5
HUAT11	Huatulco, Oaxaca	07-2001	DHF	5
HUAT12	Huatulco, Oaxaca	07-2001	DF	5
HUAT17	Huatulco, Oaxaca	07-2001	DF	5

[a]Virus isolates are from one passage in C6-36 cells
[b]DF = dengue fever; DHF = dengue hemorrhagic fever

streptomycin (100 μg/ml) at 37 °C in 5% CO_2. Supernatants were collected from cell cultures that had reached >50% cell death, centrifuged, filtered, and stored at −20 or −70 °C.

Immunofluorescence

Infected and non-infected monolayers of C6/36 cells were fixed for 15 min with 1.85% formaldehyde and 0.125% glutaraldehyde at 37 °C, washed with PBS, and incubated with 1 ml glycine for 15 min at 28 °C. The cells were then stained as described previously [23].

RNA extraction

Total RNA was extracted from cell culture supernatant using Trizol LS (GIBCO BRL, Gaithersburg, Md.) according to the manufacturer's recommendations. Ethanol-precipitated RNA was recovered by centrifugation and air-dried. The RNA pellet was suspended in 50 μl of H_2O treated with diethylpyrocarbonate (DEPC, SIGMA-ALDRICH) and used as a template to obtain different DNA products by Reverse Transcription with the Polymerase Chain Reaction (RT-PCR).

RT-PCR (reverse transcription-polymerase chain reaction)

Synthetic oligonucleotide primer pairs were designed based on published sequence data for DENV strains: 16681, New Guinea C, and Jamaica 1409, and were optimized over the course of the study. A fragment with the expected size of 594 bp (23 bp of the UTR-5′ region, nucleotide 73–96, the structural C protein gene and nucleotides 438–572 of the prM gene) was amplified by RT-PCR using Super Script™ One-Step RT-PCR with Platinum® Taq (Ivitrogen, Life Technologies). A mixture of 5 μl of total RNA (0.1–1 μg), 50 pM of corresponding sense (CTACGTGGACCGACAAAGACAG) and antisense (TTGCACCAACAGTCAATGTCTTCAGGTTC) [15] PCR primers, and DEPC-treated water (in a total volume of 50 μl) was incubated at 85 °C for 5 min and chilled on ice. A one-tube reaction mixture containing 2 × PCR buffer, 0.4 mM of each deoxyribonucleotide triphosphates and stabilizers and 2.4 mM $MgSO_4$, and Super Script™ RT/platinum® Taq Mix (Invitrogen TM Life Technologies) was added. The RT reaction was performed at 50 °C for 30 min. Thermocycling began with a hot start at 94 °C for 2 min, and the PCR conditions differed according to primer pairs and the expected fragment size. The PCR performed for amplification of the 594 bp fragment was: 40 cycles of 94 °C for 15 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for one min.

The protocol of Seah et al. [33] was followed to confirm the DENV serotype with the thermocycler set to: 10 cycles of 95 °C for 30 sec, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min with a ramp time of 1 min and 25 cycles of 95 °C for 30 sec, 50 °C for 30 sec and 72 °C for 5 min with a ramp time of 30 sec and a final extension at 72 °C for 7 min. Reaction mixtures were stored at 4 °C until further processing.

Sequencing of PCR fragments

For automated sequencing, spin column-purified (Quiagen, Chatsworth, Calif.) DNA fragments were analyzed by the cycle-sequencing dye terminator method. The Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Applied Biosystems, Foster City, Calif) was chosen. Cycle sequencing parameters used were as described in the manufacturer's protocol (25 cycles of 96 °C for 30 sec, 50 °C for 60 sec, and 60 °C for 4 min). The reaction mixture was column purified (Centri-Sep, Princeton Separations, Adelfia, N.J.) and the DNA was dried in a vacuum centrifuge for 20 min. The pellet was suspended in 16 μl of template suppression reagent, heated for 2 min at 95 °C, and kept on ice until sequenced using an

Applied Biosystems Prism 310, a short capillary (47 cm by 50 um [inside diameter]), and Performance Optimized Polymer 6 (Perkin-Elmer, Applied Biosystems).

Nucleotide and amino acid sequence analysis

Prior to phylogenetic analysis, DEN-2 virus nucleic acid sequences of the C protein gene and nucleotides 438–572 of the prM protein were aligned with prototype DEN-2 strains (Table 2) by using the multiple sequence alignment methods CLUSTAl and JOTUN-HEIN, within the MEGAALIGN program (DNASTAR Inc.). The predicted amino acid sequences were analyzed by using algorithms within the PROTEAN software package (DNASTAR, Inc.).

Phylogenetic analyses

Distance/neighbor-joining, maximum parsimony, and maximum likelihood methods were used in phylogeny reconstruction. In all analyses, DEN-1 was used as an outgroup. Distances were estimated using the method of Tamura and Nei [35] because of unequal rates in the number and types of transitions and transversion. Neighbor-joining analysis (NJ) was performed [31] and support for the derived phylogenies was examined with bootstrapping over 1000 replications. Maximum parsimony analysis was included a bootstrap analysis with 1000 replications was performed to test support for the derived phylogeny. A maximum likelihood tree (ML) was derived using the method of Felsenstein [8]. All of these analyses were performed using PAUP 4.0 [34].

Table 2. Dengue virus reference strains used in the phylogenetic analyses[a]

Strain	Location	Year	Genotype [18]	Genotype [39]	Clinical status
JAM/1409	Jamaica	1983	III	American/Asian	DHF
MARA3	Aragua, Venezuela	1990	II	American/Asian	DHF
LARD1701	Marino, Venezuela	1997	I	American/Asian	DF
LARD1910	Marino, Venezuela	1997	II	American/Asian	DF
LARD1996	Marino, Venezuela	1997	II	American/Asian	DF
MARA4*	Aragua, Venezuela	1990	II	American/Asian	DHF
NGC	New Guinea	1944	I	Asian 1	DHF
THNH7/93	Nakhon Phanom, Thailand	1993	III	Asian 1	DHF
16681	Thailand	1964	III	Asian 1	DHF
16681PDK53	Vacunal strain	1964	–	Asian 1	–
IQT2913	Iquitos, Loreto, Peru	1996	NC	American	DF
PR159	Puerto Rico	1969	V	American	DF
MEX/200787	Mexico	1983	NC	NC	DHF
SON/0131	Navojoa, Sonora, Mexico	1992	NC	American	DF
CHNFJ10	China	NR	NC	NC	NR
VEN2	Venezuela	1987	NC	American	DF

[a]Genotypic classification reference was taken from: Lewis et al. [20] and Uzcategui et al. [39]

*MARA is considered a recombinant, although it has been classified as American/Asian

NC Not classified

Nucleotide sequence accession number

The nucleotide sequences reported in this study have been deposited in the GenBank database under the accession numbers: AY692465 (SALC9), AY692466 (JUCH5), AY692467 (TUX19), AY692468 (TON4), AY692469 (HUAT2), AY692470 (HUAT11), AY692471 (HUAT12), DQ070873 (HUAT17).

Results

Dengue distribution

Dengue virus infections were evaluated in 12 Oaxaca sub-districts (Fig. 1) by Mac-ELISA (IgM Antibody Capture Enzyme Linked Immunosorbent Assay) and RT-PCR in an epidemiologic surveillance program of the Secretaria de Salud Oaxaca, Mexico during 2000–2001. Isolates of samples that were positive for both assays were obtained and tested by immunofluorescence. The Mac-ELISA test identified primary infections; consequently, some of the samples that were negative by this assay may have been positive for dengue secondary infection. Some of the negative samples were also tested by RT-PCR. Of the 95 samples tested by Mac ELISA; 32 were positive by RT-PCR, forty two were positive for DENV serotype 2, three for serotype 3 and four for serotype 1. Twenty four Mac-ELISA negative samples were positive by RT-PCR. DEN2 isolates were obtained from the positive samples and used in these studies (Table 1).

Fig. 1. Map of Mexico showing the state of Oaxaca. The 5 cities from which dengue viruses originated are noted. Each location is a site where virus was transmitted to patients

Phylogenetic analysis of Oaxaca isolates

In the 8 single passage isolates (Table 1), we amplified and sequenced the C protein gene and nucleotides 438–572 of the prM protein. A phylogenetic analysis

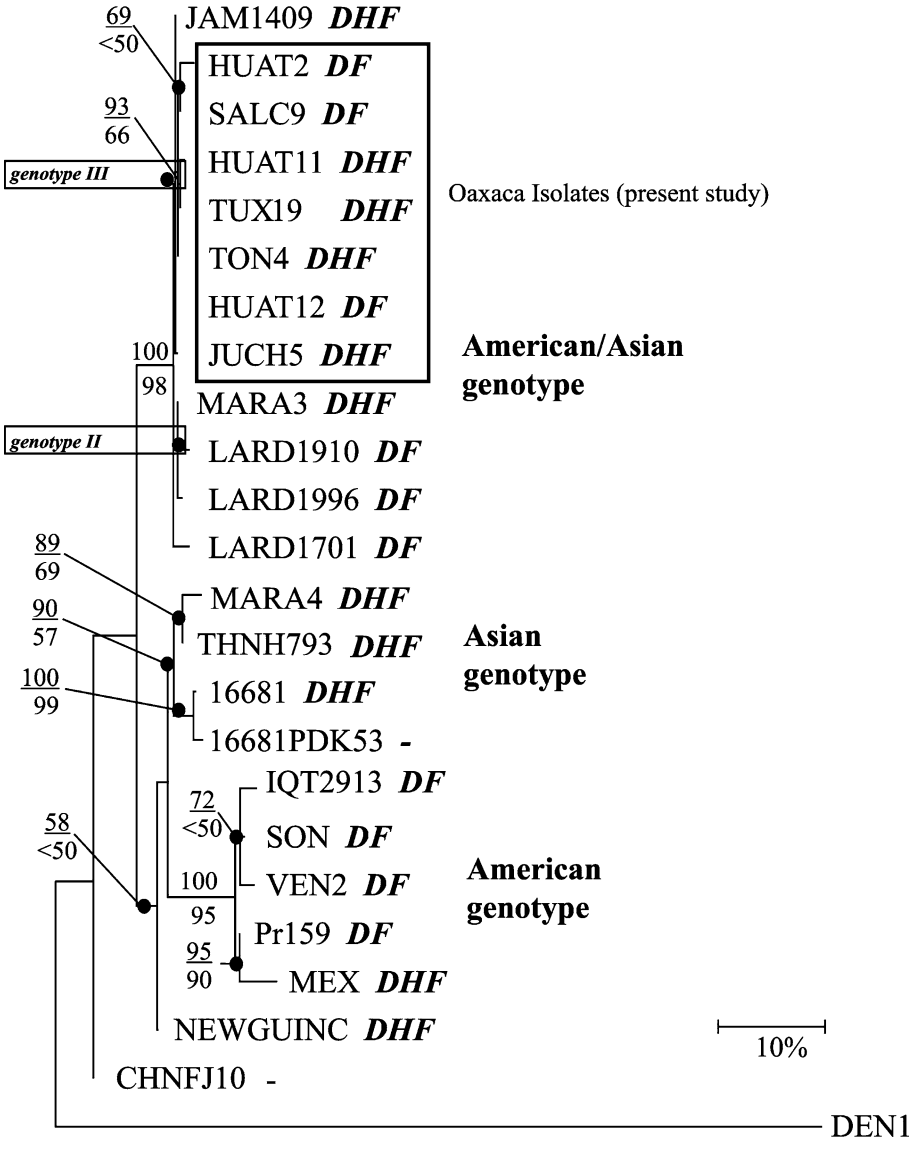

Fig. 2. Maximum likelihood tree derived using the method of Felsenstein [8]. DEN1 was used as an outgroup. Branch lengths are proportional to percentage divergence. For both distance/neighbor joining and maximum parsimony analyses, 1000 bootstrap replications were performed with PAUP 4.0 [33]. The percentage frequency with which each branch was supported using Tamura and Nei's [34] distance with gamma correction and neighbor joining appears above each branch. The percentage of replications supporting each branch when performing parsimony analysis with gaps included appear below each branch. Oaxaca isolates appear in the box

was then performed on these sequences and the sequences of other prototype characterized isolates. HUAT17 and TON4 were identical in sequence.

The Oaxaca isolates were most closely related to the Jamaica strain, subtype III (Fig. 2, Table) and occurred in a common clade with the 3 Venezuelan isolates, LARD (1996, 1910, 1701) and MARA 3. These strains previously reported have been classified as American/Asian genotype [12, 39].

The Mara 4 strain segregates with Asian genotype viruses in our study (Fig. 2). However, others classify it as an American/Asian genotype virus; this may be due to the fact that is a recombinant virus [39]. The American genotype strains cluster in a separate branch with a consistency of 100%. The relationships among the American/Asian strains are further revealed using MARA4 as an outgroup (Fig. 3). All Oaxaca isolates occur in the same branch as in Fig. 2. Our results support the view that these Asian/American strains are phylogenetically distinct from the main Asian and American genotypes [27].

Molecular analysis of C-pM sequence of Oaxaca isolates

We observed minor changes in nucleotide sequences of the C protein. The largest number of amino acid differences were associated with a change in nucleotide of the prM fragment (6.4%, 3/44 aa). Nucleotide changes in the sequence resulted in 3

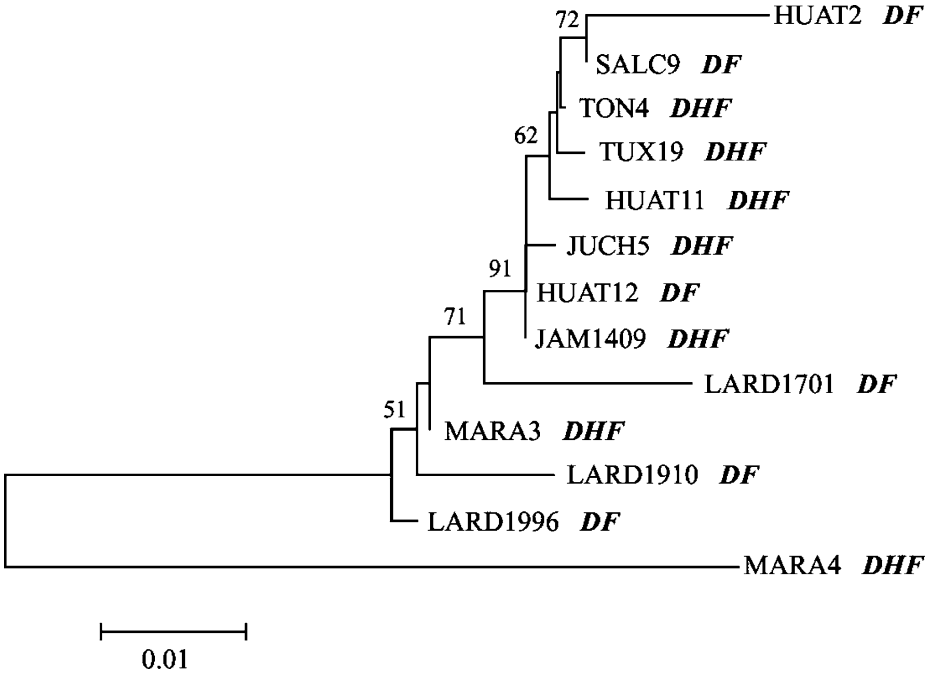

Fig. 3. UPGMA phylogenetic tree for the C and premembrane locus of DEN-2 viruses from Oaxaca, Mexico. DEN2 MARA4 was used as an outgroup The numbers displayed next to the nodes correspond to the bootstrap values (1000 replicates) supporting that clade. Tree was constructed as mentioned in Methods

Table 3. Summary of consistent amino acid change among Southeast Asian, American and American/Asian genotype viruses

Gene		Amino acid positions[a]													
C and prM	Genotype	3	9	10	47	101	104	112	129	130	142	143	145	153	Accession number
Gene prM										15	28	29	31	39	
IQT-2913	American	N	R	N	F	T	M	V	S	R	K	D	T	M	AF100468
SON	American	N	R	N	F	T	M	V	S	R	K	D	T	M	AF100469
MEX	American	D	R	N	F	T	M	V	I	R	K	D	T	M	L04561
PR159	American	D	R	N	F	T	M	V	S	R	K	D	T	M	M19197
VEN2	American	N	R	N	F	T	M	V	S	R	K	D	T	L	AF100465
16681-PDK53	Asian	D	K	N	Y	S	M	V	S	R	E	V	V	M	M84728
16681	Asian	D	K	N	Y	S	M	V	S	R	E	D	V	M	M84727
ThNH793	Asian	N	K	N	F	S	M	V	S	I	E	D	V	M	AF022434
NEWGUINC	Asian	N	R	N	F	T	M	V	S	R	E	D	V	M	M29095
CHNFJ-10	NC	N	R	N	F	T	M	A	S	R	E	D	V	M	AF276619
MARA4*	American/Asian	N	K	S	F	S	M	V	S	I	E	D	V	M	AF100466
JAM	American/Asian	N	R	S	F	T	V	A	G	R	E	D	V	I	M20558
LARD1701	American/Asian	N	R	S	F	T	V	A	G	R	E	D	V	I	AF360861
LARD1910	American/Asian	N	R	S	F	T	V	A	G	R	E	D	V	I	AF360862
LARD1996	American/Asian	N	R	G	F	T	V	A	G	R	E	D	V	I	AF360863
MARA3	American/Asian	N	R	S	F	T	V	A	G	R	E	D	V	I	AY044442
TON4	American/Asian	N	R	S	F	T	V	A	G	R	E	D	V	I	AY692468
JUCH5	American/Asian	N	R	S	F	T	V	A	G	R	E	D	V	I	AY692466
TUX-19	American/Asian	N	R	S	F	T	V	A	G	R	E	D	V	I	AY692467
SALC-9	American/Asian	N	R	S	F	T	V	A	G	R	K	D	V	I	AY692465
HUAT12	American/Asian	N	R	S	F	T	V	A	G	R	E	D	V	I	AY692471
HUAT2	American/Asian	N	R	S	F	T	V	A	G	R	K	V	V	L	AY692469
HUAT11	American/Asian	N	R	S	F	T	V	A	G	R	K	D	V	I	AY692470

[a]Amino acid sequence was numbered starting from protein C or prM to the end of the last codon sequenced
*Recombinant strain [39]

amino acid alterations. Table 3 summarizes amino acid changes among Southeast Asian, American and American/Asian Genotype viruses.

We compared amino acid composition (C and prM) of the Southeast Asian genotype viruses with American genotypes (Table 3). The former have greater potential to cause DHF and the latter are associated with DF [18, 28]. The Oaxaca isolates contain Valine at position 145 in the prM gene (prM-31). In contrast, the American genotype has a Threonine residue at this position. Furthermore Oaxaca isolates SALC9, HUAT2 and HUAT11 possessed a Glutamic acid at position 142 in the C-prM coding region (prM-28) (Table 3). In contrast JUCH5, TUX19, TON4, HUAT12 have a Lysine residue at this position, similar to the American genotype.

All American/Asian group viruses with the exception of HUAT2 had an Isoleucine at position 153 (prM-38). In contrast, the Asian and American genotype viruses encode a Methionine at this position.

To see the separation among the American/Asian strains an additional tree was constructed (Fig. 3) and strain MARA4 was selected as outgroup. All Oaxaca isolates occurs in the same branch as in Fig. 2.

Discussion

The public health importance of DF and DHF/DSS is increasing worldwide and in areas where they were previously unreported. In Mexico, DHF/DSS emerged in 1995–1996. The global evolution of DEN-2 viruses has been studied by a number of investigators [3, 5, 6, 12, 19, 27, 28, 37, 39]. To investigate virus determinants of the emergence of epidemic DF and DHF/DSS in Mexico, we characterized 8 DEN-2 isolates obtained in Oaxaca, Mexico. The isolates appear to be descendants of an American/Asian genotype of DEN-2 virus [37], represented by the isolate Jam83, that was introduced into Latin America during the early 1980s, presumably from Vietnam, Malaysia or Thailand [6, 19, 27]. Closely related American/Asian genotype viruses were also recently detected in the Yucatan [21]. The close phylogenetic relationship between the Oaxaca isolates and Venezuelan viruses (LARD1910, LARD1996, and Mara3) suggests that our viruses may have been introduced into Mexico from Venezuela.

The more severe form of DHF/DSS has been associated with immune enhancement caused by infection by a second DENV serotype [11] and/or by infection with a more virulent DENV genotype or strain [30]. The appearance of DHF/DSS in the New World was associated with the introduction of a South East Asian strain of DEN-2 virus into the Caribbean region [28]. Indigenous Latin American strains of DEN-2 virus were not associated with DHF. These findings promoted studies to identify the viral genetic determinants of DENV virulence. A number of different nucleotide and amino acid substitutions have been associated with DHF or DSS [2, 13, 18, 22]. Previous studies have shown that the prM gene region has a greater genetic variation than E or NS5 gene regions [7]. In particular, Leitmeyer et al. [18] identified nucleotide substitutions that distinguish the American genotype from the Southeast Asian genotype as determinants of disease severity. For example, the prM-28 and prM-31 amino acids may be determinants of DHF. In addition, all American genotypes, which are not associated with DHF, possess K and T residues at position prM-28 and prM-31, respectively. All Asian strains, which have induced DHF have E and V residues in these positions respectively.

All of the strains from Oaxaca, Mexico sequenced possessed the typical Asian V residue at position 31. This amino acid substitution in the prM protein has been reported to be typical of Asian genotype DEN-2 viruses [18]. Furthermore, all American/Asian genotypes and all of the Oaxaca isolates except HUAT2 have an I at position prM-38 that may be typical for this genotype. All of this hints at the Asian ancestry of these Oaxaca strains similar to the Venezuelan isolates studied by Uzcategui et al. [39]. These results also suggests that all of

these genotypes have the potential to cause DHF, independently of the host or environment.

The same evolutionary relationships among DEN-2 viruses that we found by analyzing a portion of the C and prM genes are revealed when other regions of the viral genome were used (e.g., E or NS1) [1–3, 20], or portions of genes (e.g., 198 nucleotides of the E gene) [7]. This is interesting because previous studies have also shown that the prM gene region have a greater genetic variation than E or NS5 gene regions [8].

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References

1. Armstrong PM, Rico-Hesse R (2003) Efficiency of dengue serotype 2 virus strains to infect and disseminate in *Aedes aegypti*. Am J Trop Med Hyg 68: 539–544
2. Blok J, Gibbs AJ, McWilliam SM, Vitarana UT (1991) NS1 gene sequences from eight dengue-2 viruses and their evolutionary relationships with other dengue-2 viruses. Arch Virol 119: 209–223
3. Blok J, Samuel S, Gibbs AJ, Vitarana UT (1989) Variation of the nucleotide and encoded amino acid sequences of the envelope gene from eight dengue-2 viruses. Arch Virol 105: 39–53
4. Cisneros-Solano A, Moreno-Altamirano MMB, Martinez-Soriano U, Jiménez-Rojas F, Diaz-Badillo A, Muñoz ML (2004) Sero-epidemiological and Virological investigation of Dengue infection in Oaxaca, México, during 2000–2001. Dengue Bull 28: 28–34
5. Briseno-Garcia B, Gomez-Dantes H, Argott-Ramirez E, Montesano R, Vazquez-Martinez AL, Ibanez-Bernal S, Madrigal-Ayala G, Ruiz-Matus C, Flisser A, Tapia-Conyer R (1996) Potential risk for dengue hemorrhagic fever: the isolation of serotype dengue-3 in Mexico. Emerg Infect Dis 2: 133–135
6. Deubel V, Kinney RM, Trent DW (1988) Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus Jamaica genotype: comparative analysis of the full length genome. Virology 165: 234–244
7. Deubel V, Nogueira RM, Drouet MT, Zeller H, Reynes JM, Ha DQ (1993) Direct sequencing of genomic cDNA fragments amplified by the polymerase chain reaction for molecular epidemiology of dengue-2. Arch Virol 129: 197–210
8. Farfan JA, Olson KE, Black WC 4th, Gubler DJ, Beaty BJ (1997) Rapid characterization of genetic diversity among twelve dengue-2 virus isolates by single-strand conformation polymorphism analysis. Am J Trop Med Hyg 57(4): 416–422
9. Felsenstein J (1981) Evolutionary trees from DNA sequences: A maximum likelihood approach. J Mol Evol 17: 368–376
10. Guzman MG, Deubel V, Pelegrino JL, Rosario D, Marrero M, Sariol C, Kouri G (1995) Partial nucleotide and amino acid sequences of the envelope and the envelope/nonstructural protein-1 gene junction of four dengue-2 virus strains isolated during the 1981 Cuban epidemic. Am J Trop Med Hyg 52: 241–246
11. Gubler DJ, Clark GG (1995) Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg Infect Dis 1: 55–57

12. Halstead SB (1992) The XXth century dengue pandemic: need for surveillance and research. World Health Stat Q 45: 292–298
13. Holmes EC (2004) The phylogeography of human viruses. Mol Ecol 13: 745–756
14. Kinney RM, Butrapet S, Chang GJ, Tsuchiya KR, Roehrig JT, Bhamarapravati N, Gubler DJ (1997) Construction of infectious cDNA clones for dengue 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. Virology 230: 300–308
15. Kouri GP, Guzman MG, Bravo JR, Triana C (1989) Dengue haemorrhagic fever/dengue shock syndrome: lessons from the Cuban epidemic, 1981. Bull World Health Organ 67: 375–380
16. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 30: 545–551
17. Lanciotti RS, Gubler DJ, Trent DW (1997) Molecular evolution and phylogeny of dengue-4 viruses. J Gen Virol 78: 2279–2284
18. Lanciotti RS, Lewis JG, Gubler DJ, Trent DW (1994) Molecular evolution and epidemiology of dengue-3 viruses. J Gen Virol 75: 65–75
19. Leitmeyer KC, Vaughn DW, Watts DM, Salas R, Villalobos I, de Chacon, Ramos C, Rico-Hesse R (1999) Dengue virus structural differences that correlate with pathogenesis. J Virol 73: 4738–4747
20. Lewis JA, Chang GJ, Lanciotti RS, Kinney RM, Mayer LW, Trent DW (1993) Phylogenetic relationships of dengue-2 viruses. Virology 197: 216–224
21. Lorono-Pino MA, Farfan-Ale JA, Zapata-Peraza AL, Rosado-Paredes EP, Flores-Flores LF, Garcia-Rejon JE, Diaz FJ, Blitvich BJ, Andrade-Narvaez M, Jimenez-Rios E, Blair CD, Olson KE, Black WC, Beaty BJ (2004) Introduction of a new genotype of Dengue-2 virus in Yucatan State, Mexico, 2002. Am J Trop Med Hyg 71: 485–492
22. Mangada MN, Igarashi A (1998) Molecular and in vitro analysis of eight dengue type 2 viruses isolated from patients exhibiting different disease severities. Virology 244: 458–466
23. Munoz ML, Cisneros A, Cruz J, Das P, Tovar R, Ortega A (1998) Putative dengue virus receptors from mosquito cells. FEMS Microbiol Lett 168: 251–258
24. Nogueira RM, Miagostovich MP, Lampe E, Souza RW, Zagne SM, Schatzmayr HG (1993) Dengue epidemic in the stage of Rio de Janeiro, Brazil, 1990–1: co-circulation of dengue 1 and dengue 2 serotypes. Epidemiol Infect 111: 163–170
25. Pan American Health Organization (1990) Dengue hemorrhagic fever in Venezuela. Epidemiol Bull 11: 7–9
26. Pan American Health Organization (1992) Dengue and dengue haemorrhagic fever in the Americas: an overview of the problem. Epidemiol Bull 13: 9–10
27. Rico-Hesse R (1990) Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology 174: 479–493
28. Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, Boshell J, de Mesa MT, Nogueira RM, da Rosa AT (1997) Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230: 244–251
29. Rodriguez-Roche R, Alvarez M, Gritsun T, Rosario D, Halstead S, Kouri G, Gould EA, Guzman MG (2005) Dengue virus type 2 in Cuba, 1997: conservation of E gene sequence in isolates obtained at different times during the epidemic. Arch Virol 50: 415–425
30. Rosen L (1977) The Emperor's New Clothes revisited, or reflections on the pathogenesis of dengue hemorrhagic fever. Am J Trop Med Hyg 26: 337–343
31. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425

32. Sangkawibha N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, Salitul V, Phanthumachinda B, Halstead SB (1984) Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 120: 653–669
33. Seah CLK, Chow VTK, Tan HC, Chan YC (1995) Rapid, single-step RT-PCR typing of dengue viruses using five NS3 gene primers. J Virol Methods 51: 193–200
34. Swofford DL (1996) "PAUP Phylogenetic Analysis Using Parsimony," Version 4.0. Smithsonian Institution, Washington, D.C.
35. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of the mitochondria1 DNA in humans and chimpanzees. Mol Biol Evol 10: 512–526
36. Trent DW, Grant JA, Rosen L, Monath TP (1983) Genetic variation among dengue 2 viruses of different geographic origin. Virology 128L: 271–284
37. Twiddy SS, Farrar JJ, Vinh CN, Wills B, Gould E, Gritsun T, Lloyd G, Holmes EC (2002) Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. Virology 298: 63–72
38. Twiddy SS, Holmes EC (2003) The extent of homologous recombination in members of the genus Flavivirus. J Gen Virol 84: 429–440
39. Uzcategui NY, Camacho D, Comach G, Cuello de Uzcategui R, Holmes EC, Gould EA (2001) Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for in situ virus evolution and recombination. J Gen Virol 82: 2945–2953
40. Watts DM, Porter KR, Putvatana P, Vasquez B, Calampa C, Hayes CG, Halstead SB (1999) Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever. Lancet 354: 1431–1434
41. Worobey M, Rambaut A, Holmes EC (1999) Widespread intra-serotype recombination in natural populations of dengue virus. Proc Natl Acad Sci USA 96: 7352–7357

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