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INTRODUCTION OF THE AMERICAN/ASIAN GENOTYPE OF DENGUE 2 VIRUS INTO THE YUCATAN STATE OF MEXICO

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Abstract. A dengue (DEN) outbreak occurred in the Yucatan State of Mexico in 2002. Three isolates were obtained from patients presenting with DEN-like symptoms, and examined by partial nucleotide sequencing and phylogenetic analysis. The isolates were identified as DEN-2 viruses of the American-Asian genotype; this is the first report of this genotype in the Yucatan State. The DEN-2 viruses of the American-Asian genotype have been associated with more severe disease outcomes. Thus, its introduction into the Yucatan State presents a serious problem to public health authorities. During this outbreak, DEN virus infection was confirmed in 18% (282 of 1,560) of the patients who presented with DEN-like symptoms. Of these, 87 (31%) patients met the World Health Organization criteria for dengue hemorrhagic fever, including two patients who died. The majority (77%) of the patients experienced secondary infections in this epidemic.

INTRODUCTION

Dengue (DEN) viruses (family Flaviviridae, genus *Flavivirus*) are the most important arthropod-borne viruses in terms of human morbidity and mortality. There are four serologic types, DEN-1, DEN-2, DEN-3, and DEN-4, and all can be transmitted to humans by *Aedes aegypti* and certain other *Aedes* spp. vectors. In humans, DEN virus infection is usually asymptomatic or characterized by a benign flu-like illness known as DEN fever (DF). Clinical symptoms of DF include fever, severe headache, myalgia, arthralgia, and rash. In some cases, DEN virus infection can result in DEN hemorrhagic fever (DHF), which is characterized by thrombocytopenia, bleeding, hemoconcentration, low blood albumin, and pleural effusions. It can progress to DEN shock syndrome (DSS) and death.[1,2]

Dengue fever and DHF were first observed in the Yucatan State of Mexico in 1979 and 1984, respectively.[3,4] Dengue epidemics have been reported in the Yucatan State in 1979–1982 (DEN-1),[3,5] 1984 (DEN-1 and DEN-4),[4] 1991 (DEN-2 and DEN-4), 1994 (DEN-1, DEN-2, and DEN-4), and 1995–1997 (all serotypes) (Farfán-Ale JA, unpublished data). Since 1983, the Laboratorio de Arbovirología of the Universidad Autónoma de Yucatán (UADY) has conducted DEN virus surveillance in the Yucatán State.

Studies on the envelope (E) gene sequences of many strains of DEN-2 virus have shown genetic variation worldwide. The maximum-likelihood trees constructed by Twiddy and others[6] with 142 isolates of DEN-2 virus showed that these strains can be classified into five clusters termed genotypes. The American genotype consists of recent isolates from Latin America, including Mexico, and older isolates from India, the Caribbean, and the Pacific Islands. The Asian genotype 1 cluster contains isolates from Thailand and Malaysia. The Asian genotype 2 is composed of isolates from China, the Philippines, Sri Lanka, Taiwan, and Vietnam. The American/Asian genotype consists of isolates from China, Thailand, Vietnam, Brazil, Venezuela, and the Caribbean. The Cosmopolitan genotype cluster contains isolates from Australia, the Pacific Islands, Southeast Asia, the Indian Sub-continent, the Middle East, Africa, and Mexico.

The incidence of epidemic dengue and DHF/DSS has increased dramatically in the last decade. The original emergence of DHF and DSS in the Americas was associated the introduction of a virulent genotype of DEN-2 virus from Southeast Asia.[7–9] Introduction of a new genotype of DEN-3 virus was also associated with increased incidence of DHF and DSS in many countries in Latin America.[10–12] Clearly, epidemics of DHF and DSS can result from the introduction of new viral genotypes or serotypes, which displace other viruses.[9,13,14] More information on the risk factors that condition epidemic dengue and DHF/DSS in Latin America and the clinical and epidemiologic consequences of such introductions is warranted and critical.

Here we report the introduction of a new DEN-2 virus of the American/Asian genotype into the Yucatan State. We also describe the clinical, virologic, and epidemiologic correlates of a DEN-2 virus outbreak in 2002.

MATERIAL AND METHODS

Study population and sample collection. The study population consisted of patients who presented with DEN-like illness at Instituto Mexicano del Seguro Social (IMSS) outpatient clinics and hospitals or at clinics of private physicians and who were referred to the Laboratorio de Arbovirología. In some cases, only the serum and clinical information were sent to the Laboratorio de Arbovirología. A patient was considered to have a DEN-like illness if he or she presented with at least one of the following symptoms: fever, severe headache, myalgia, arthralgia, rash, bleeding, hemoconcentration, thrombocytopenia, and pleural effusions.

A total of 1,804 serum samples were obtained from 1,560 patients who presented with symptoms consistent with a DEN-like illness. All patients were residents of the Yucatan State. The travel history of each study participant was recorded, and any patient who had traveled outside the Yucatan State in the 15 days prior to disease onset was ex-

cluded from the study. The majority of the samples were collected during routine diagnosis of DEN virus infection from January 1 to December 31, 2002. Samples were also collected as part of the human investigations protocol "Molecular Determinants of Dengue Epidemic Potential (A Pilot Study to Define Dengue Virus Variants Circulating in the Yucatan)," which has been reviewed and approved by the institutional review boards of the IMSS, UADY, Colorado State University, and the Division of Microbiology and Infectious Diseases of the National Institutes of Health. Upon presentation at the Laboratorio de Arbovirología, patients were invited to participate in a study to identify the infecting agent. Patients were informed of the clinical and epidemiologic aspects of dengue, and of the importance of providing both acute and convalescent blood samples for laboratory diagnosis.

Standard epidemiologic information was obtained from all patients including age, sex, date of illness onset, date of sample collection, clinical information and results of tourniquet and laboratory tests. The DF and DHF cases were diagnosed by clinical, hematologic, serologic, and/or virologic observations. The DHF cases were graded for severity by using the World Health Organization (WHO) criteria.[15] Questionnaire data and clinical and laboratory results were entered in the database and statistical package Epi-Info version 6.04.[16]

Paired serum samples (acute and convalescent) were obtained from 244 patients and a single serum (acute only) was obtained from 1,316 patients. A signed consent form was obtained from all patients or legal guardians who presented at the Laboratorio de Arbovirología.

Serologic and virologic tests. A patient was considered to have a recent DEN infection if he or she met one or more of the following criteria: 1) IgM antibody was detected in either the acute or convalescent phase serum by antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA), 2) the antibody titer in the convalescent phase serum was at least four-fold greater than in the acute phase serum as determined by hemagglutination-inhibition (HI) assay, 3) DEN virus was isolated by cell culture and detected by indirect immunofluorescence assay (IFA), or 4) DEN virus RNA was amplified by a reverse transcription–polymerase chain reaction (RT-PCR).

Antibody-capture enzyme-linked immunosorbent assay. The MAC-ELISAs were performed as described by Kuno and others.[17] Briefly, sheep anti-human IgM (Kirekegaard and Perry Laboratories, Gaithersburg, MD) was used as the capture antibody and 6B6C-1 was used as the detector antibody.[18] The MAC-ELISA antigen was prepared from all four DEN virus serotypes and pooled before use.[17] A serum sample was considered positive for antibodies to DEN if the optical density value was ≥ 0.2, as recommended by the Centers for Disease Control and Prevention. All patients were tested for antibodies to DEN virus by MAC-ELISA.

Hemaglutination-inhibition assays. The HI assays were performed following the protocol of Clarke and Casals.[19] The assays were conducted typically with DEN-2 virus antigen, and sera were treated with kaolin and erythrocytes to eliminate nonspecific inhibitors. Hemagglutination-inhibition is a sensitive test for detecting antibodies to flaviviruses, but is not specific. However, the test does permit differentiation of primary or secondary DEN infections when acute and convalescent sera are separated by at least seven days. A patient was considered to have a recent flavivirus infection if the antibody titer of the convalescent serum was at least four times higher than the antibody titer of the acute serum. If antibodies to DEN virus were detected in the acute phase serum, but a convalescent phase serum was not available for testing, the patient could not be definitively diagnosed. The HI assays were performed on all patients negative for DEN virus infection by the MAC-ELISA. Generally, patients positive by the MAC-ELISA were not tested by the HI assay.

Virus isolation and IFA. For virus isolation, patient specimens were inoculated onto C6/36 mosquito cells and incubated at 28°C for at least 10 days. Cells were harvested, fixed in acetone, and isolates were serotyped by an indirect IFA.[20] The following monoclonal antibodies (MAbs) were used in the IFAs to identify the infecting DEN virus serotype: DEN-1 virus-specific MAb D2-1F1-3, DEN-2 virus-specific MAb 3H5-1-21, DEN-3 virus-specific MAb D6-8A1-12, and DEN-4 virus-specific MAb 1H-10-6-7.[21] The majority of sera collected within six days of illness onset were examined by virus isolation and IFA.

Isolation of RNA and RT-PCR. Dengue virus RNA was isolated from serum samples by using the QIAamp viral RNA kit (Qiagen, Valencia, CA). Seminested RT-PCR amplifications were performed following the protocol of Lanciotti and others.[22] Primers used were D1, D2, TS1, TS2, TS3 and TS4 (Table 1). Briefly, DEN virus RNA was RT-PCR amplified using forward D1 and reverse D2 primers that encompassed a region of the capsid (C) and premembrane (prM) genes of all DEN virus serotypes. This was followed by a nested PCR that used the same forward primer (D1) and a pooled suspension of reverse primers, each specific to one DEN virus serotype. The nested PCR reverse primers were as follows: TS1 (DEN-1 virus-specific), TS2 (DEN-2 virus-specific), TS3 (DEN-3 virus-specific), and TS4 (DEN-4 virus-specific). The expected sizes of the PCR products for DEN-1, DEN-2, DEN-3, and DEN-4 viruses were 482, 119, 290, and 392 basepairs, respectively. The RT-PCRs were performed on a subset of sera collected within six days of illness onset. However, the majority of sera collected at this time were examined only by virus isolation/IFA because this technique is considerably less expensive than the RT-PCR. No serum sample collected within six days of illness onset was examined by both techniques.

Sequencing and phylogenetic analysis. Three DEN-2 virus isolates from the 2002 outbreak, as well as three isolates from 2001 from Santa Elena, Oxkutzcab, and Tekax in the Yucatan State, were propagated in C6/36 cells for seven days. Three additional DEN-2 virus isolates from previous outbreaks in Mexico, including two from the Yucatan State in 1994 and 1996, were also propagated (Table 2). Total RNA was then extracted from the cells using a silica-based extraction method (RNeasy; Qiagen). The cDNAs were prepared using primer D2-2588 and Rous-associated virus reverse transcriptase (Amersham, Piscataway, NJ). A 2,474-basepair segment that encompassed that the entire prM and E genes was amplified by PCR using the Expand Long Template PCR System (Roche Diagnostics, Penzburg, Germany) and primers D1 and D2-2588 (Table 1). The PCR program consisted of 40 cycles of amplification (94°C for 10 seconds, 53°C for 30 seconds, and 68°C for 3 minutes). The PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) and both strands of the entire E gene were sequenced using

TABLE 1
Primers used for reverse transcription–polymerase chain reaction (RT-PCR) and DNA sequencing

Primer	Sequence 5′ → 3′	Use
D1	TCAATATGCTGAAACGCGCGAGAAACCG	PCR, Semi-nested PCR
D2	TTGCACCAACAGTCAATGTCTTCAGGTTC	RT-PCR
TS1	CGTCTCAGTGATCCGGGGG	Semi-nested PCR
TS2	CGCCACAAGGGCCATGAACAG	Semi-nested PCR
TS3	TAACATCATCATGAGACAGAGC	Semi-nested PCR
TS4	CTCTGTTGTCTTAAACAAGAGA	Semi-nested PCR
D2-739	ATGGGATTGGAGACACGAACTGAA	Sequencing
D2-1184	ATGAAGAGCAGGACAAAAGGTT	Sequencing
D2-1225	CCATTTCCCCATCCTCTGTCTAC	Sequencing
D2-1540	GAAGACAAAGCTTGGCTGGTG	Sequencing
D2-1648	GCATGGGGATTTTTGAARGTGAC	Sequencing
D2-2028	GGAGGTTCTGCTTCTATGTTGACT	Sequencing
D2-2050	CCATTCGGAGACAGCTACATCAT	Sequencing
D2-2588	TCTTGTTACTGAGCGATTC	RT-PCR, sequencing

TABLE 2
Dengue-2 viruses included in the phylogenetic analysis*

Name of strain	GenBank accession no.	Country	Year of isolation	Disease	Genotype
13381-Chocholá	**AY449683**	**Mexico**	**2002**	**DHF**	**American/Asian**
13382-Tizimín	**AY449684**	**Mexico**	**2002**	**DF**	**American/Asian**
13404-Opichén	**AY449685**	**Mexico**	**2002**	**DF**	**American/Asian**
11936-Santa Elena	**AY449681**	**Mexico**	**2001**	**DF**	**American/Asian**
12021-Oxkutzcab	**AY449682**	**Mexico**	**2001**	**DF**	**American/Asian**
12914-Tekax	**AY449680**	**Mexico**	**2001**	**DF**	**American/Asian**
BC134-Mérida	**AY466449**	**Mexico**	**1994**	**DF**	**American**
BC17-Mérida	**AY449677**	**Mexico**	**1996**	**DF**	**Cosmopolitan**
Oax468-Juchitán	AY158341	Mexico	2000	NA	American/Asian
328298-Reynosa	AY158338	Mexico	1995	NA	American
131-Sonora	AF100469	Mexico	1992	DF	American
SJL1482 (city unknown)	**AY449675**	**Mexico**	**1984**	**DF**	**American**
India-57	L10043	India	1957	NA	American
IQT2913	AF100468	Peru	1996	DF	American
PR159	L10046	Puerto Rico	1969	NA	American
TR1751	L10053	Trinidad	1953	NA	American
Ven2	AF100465	Venezuela	1987	DF	American
CAMR5	AF410370	Australia	1992	NA	Cosmopolitan
CAMR10	AF410374	India	1994	NA	Cosmopolitan
CAMR14	AF410377	Thailand	1998	NA	Cosmopolitan
CAMR16	AF410378	Saudi Arabia	1992	NA	Cosmopolitan
INDON	L10044	Indonesia	1976	DSS	Cosmopolitan
SEY-42	L10047	Seychelles	1977	DF	Cosmopolitan
SL714	L10055	Sri Lanka	1989	NA	Cosmopolitan
Somalia 10	L10051	Somalia	1984	DF	Cosmopolitan
M1	X15434	Malaysia	1987	DHF	Asian 1
PUO-218	D00345	Thailand	1989	DF	Asian 1
ThNH-7/93	AF022434	Thailand	1993	DSS	Asian 1
16681	U87411	Thailand	1964	NA	Asian 1
CTD113	AF410358	Vietnam	1997	DHF	Asian 2
43	AF204178	China	1987	DF	Asian 2
DOH-078	AF295700	Philippines	NA	NA	Asian 2
New Guinea C	AF038403	New Guinea	1944	NA	Asian 2
Philip	L10045	Philippines	1983	NA	Asian 2
TAIWAN	L10052	Taiwan	1987	NA	Asian 2
CTD29	AF410346	Vietnam	1998	DHF	American/Asian
Brazil	L10041	Brazil	1990	NA	American/Asian
China-04	AF119661	China	1985	NA	American/Asian
D80-038	M24448	Thailand	1980	DHF	American/Asian
1409	M20558	Jamaica	1983	NA	American/Asian
Mara4	AF100466	Venezuela	1990	DHF	American/Asian
Mart/98-703	AF208496	Martinique	1998	NA	American/Asian
PTCOL96	AF163096	Colombia	1996	NA	American/Asian
LARD2213	AF363084	Venezuela	1998	DHF	American/Asian
LARD3146	AF398106	Venezuela	1998	DHF	American/Asian
Guinea-81-PM33974	AF231719	Guinea	1981	NA	Sylvatic
Mal70-P8-1407	AF231717	Malaysia	1970	NA	Sylvatic
IC80-DAKAr578	AF231718	Ivory Coast	1980	NA	Sylvatic
Sen70-DAKHD10674	AF231720	Senegal	1970	NA	Sylvatic

* Isolates sequenced in the present study are in **bold**. DHF = dengue hemorrhagic fever; DF = dengue fever; NA = not available; DSS = dengue shock syndrome.

the Big Dye terminator cycle sequencing system (Applied Biosystems, Foster City, CA) using eight different primers (Table 1). Maximum parsimony phylogenetic analysis was performed using the Phylogenetic Analysis Using Parsimony (PAUP) 4.10 package.[23]

RESULTS

Serologic results. A total of 1,804 serum samples were obtained from 1,560 patients with suspected DEN infection. Acute and convalescent phase specimens were obtained from 244 patients. Of these, 101 (41.4%) patients were positive for a recent DEN infection and 143 (58.6%) were negative. Only acute phase specimens were obtained from 1,316 patients. Of these, 181 (13.8%) patients were positive for a recent DEN infection and 77 (5.8%) were DEN negative. The remaining 1,058 (80.4%) patients had HI antibodies, but because convalescent phase sera were not available for testing we could not differentiate between previous and recent DEN virus infections. Thus, these patients were not definitively diagnosed. Sera may have been obtained from these patients before detectable IgM appeared. Alternatively, these patients may have been infected with influenza, measles, rubella, leptospira or rickettsia; these illnesses cause similar symptoms to dengue and are also found in Mexico.

Patients were tested for evidence of recent DEN virus infection by MAC-ELISA, HI assay, virus isolation/IFA, and/or RT-PCR. Overall, 282 (18.1%) patients were positive by at least one diagnostic technique, 220 (14.1%) were negative, and diagnostic results were not obtained for 1,058 (67.8%) patients. Of the 1,560 patients tested by MAC-ELISA, 165 (10.6%) were DEN positive. All patients negative by MAC-ELISA were further tested by HI assay. Seventy-six (31.1%) of 244 patients from which paired sera were obtained were positive by HI assay for DEN virus. Sera were obtained from 317 patients within six days of illness onset, and thus examined by RT-PCR or virus isolation/IFA. Eleven (14.1%) of 78 patients were DEN positive by RT-PCR, and 32 (13.4%) of 239 were DEN positive by virus isolation/IFA. Of these, 42 patients were positive for DEN-2 virus and one was positive for DEN-1 virus. A subset of patients were tested for secondary infection using the HI test; 43 (76.8%) of 56 were diagnosed as having a secondary DEN infection.

TABLE 3
Final diagnosis of confirmed dengue cases*

Clinical diagnosis	No.	%
Dengue fever without hemorrhagic manifestations	120	42.6
Dengue fever with hemorrhagic manifestations	75	26.6
DHF grade I	42	14.9
DHF grade II	32	11.3
DHF grade III	7	2.5
DHF grade IV	6	2.1
Total	282	100

* DHF = dengue hemorrhagic fever.

Dengue disease severity. Of the 282 DEN-positive cases, 120 (42.6%) were diagnosed as classic DF (without hemorrhagic manifestations), and an additional 75 (26.6%) of the 282 patients were diagnosed as DF with hemorrhagic manifestations (Table 3). Using the WHO criteria, DHF was diagnosed in 87 (30.8%) DEN virus-infected patients. Six DHF cases were considered to be severe (grade IV). Two DHF cases were fatal. The most frequent hemorrhagic clinical manifestations among DHF cases were the presence of petechia (64.4%), a positive tourniquet test result (43.7%), and epistaxis (28.7%) (Table 4). Ninety-seven (34.4%) of the 282 patients with DEN virus infection, including 84 with DHF, were hospitalized. Forty-four (45.4%) of the 97 DEN-infected patients admitted to hospitals were female; 53 (54.6%) were male.

The greatest number of DEN infections was observed among 10–14-year-old patients (n = 58), followed by 15–19- (n = 45) and 5–9- (n = 37) year-old patients (Table 5). When we compared the number of DEN patients with and without hemorrhagic manifestations by chi-square analysis, we observed that 10–14-year-old patients were significantly less likely to develop hemorrhagic symptoms than other age groups ($\chi^2 = 10.704$) (Table 6). In contrast, 20–25-year-old patients had a significantly greater chance of developing hemorrhagic manifestations ($\chi^2 = 5.04$). The P value obtained in the goodness of fit chi-square test was < 0.005.

Fatal cases. Two patients died, a 15-year-old girl and an 8-year-old boy. The female, from Valladolid, first developed symptoms on August 26, 2002, including fever, general malaise, myalgia, severe headache, intense abdominal pain, and vomiting with alimentary content. She was taken to the

TABLE 4
Signs and symptoms among the dengue cases studied in the Yucatan State in 2002*

	DF without hemorrhagic manifestations (n = 120)			DF with hemorrhagic manifestations (n = 75)			DHF cases (n = 87)		
Symptom	Yes	No	Unknown	Yes	No	Unknown	Yes	No	Unknown
Fever	119 (99.2%)	1 (0.8%)	0	71 (94.6%)	2 (2.7%)	2 (2.7%)	84 (96.6%)	3 (3.4%)	0
Myalgia	99 (82.5%)	17 (14.2%)	4 (3.3%)	65 (86.7%)	6 (8.0%)	4 (5.3%)	78 (89.7%)	8 (9.2%)	1 (1.1%)
Headache	105 (87.5%)	11 (9.2%)	4 (3.3%)	63 (84%)	8 (10.7%)	4 (5.3%)	79 (90.8%)	7 (8.1%)	1 (1.1%)
Arthralgia	98 (81.7%)	17 (14.1%)	5 (4.2%)	66 (88%)	6 (8.0%)	3 (4%)	79 (90.8%)	7 (8.1%)	1 (1.1%)
Petechiae	–	–	–	45 (60%)	23 (30.7%)	7 (9.3%)	56 (64.4%)	21 (24.1%)	10 (11.5%)
Tourniquet positive test result	–	–	–	38 (50.7%)	26 (34.7%)	11 (14.7%)	38 (43.7%)	25 (28.7%)	24 (27.6%)
Ecchymosis	–	–	–	8 (10.7%)	61 (81.3%)	6 (8.0%)	17 (19.5%)	60 (69.0%)	10 (11.5%)
Epistaxis	–	–	–	11 (14.7%)	58 (77.3%)	6 (8.0%)	25 (28.7%)	52 (59.8%)	10 (11.5%)
Gingival bleeding	–	–	–	11 (14.7%)	57 (76%)	7 (9.3%)	11 (12.6%)	66 (75.8%)	10 (11.5%)
Melena	–	–	–	5 (6.7%)	63 (84.0%)	7 (9.3%)	8 (9.2%)	68 (78.1%)	11 (12.6%)
Hematuria	–	–	–	2 (2.7%)	65 (86.6%)	8 (10.7%)	7 (8.1%)	67 (77.0%)	13 (14.9%)
Hematemesis	–	–	–	0	68 (90.7%)	7 (9.3%)	6 (6.9%)	70 (80.5%)	11 (12.6%)

* DF = dengue fever; DHF = dengue hemorrhagic fever.

TABLE 5
Age distribution and diagnosis of patients*

Age (years)	DF without HM	DF with MH	DHF (grade I)	DHF (grade II)	DHF (grade III)	DHF (grade IV)	DF/DHF (total)	DEN negative
0–4	4	3	0	2	0	0	9	9
5–9	11	11	8	1	3	3	37	29
10–14	37	8	5	6	1	1	58	44
15–19	17	17	8	2	0	1	45	33
20–25	8	9	7	9	1	0	34	19
26–30	10	6	6	1	0	0	23	19
31–35	4	5	1	2	1	0	13	11
36–40	4	3	0	1	0	1	9	16
41–45	5	5	6	4	1	0	21	13
46–50	7	5	0	0	0	0	12	7
≥ 51	13	3	1	4	0	0	21	20
Total	120	75	42	32	7	6	282	220

* DF = dengue fever; HM = hemorrhagic manifestations; DHF = dengue hemorrhagic fever; DEN = dengue.

Health Center in her community and to the Health Center in nearby Valladolid City, where analgesics and antibiotics were prescribed. At day 5 of illness, she was hospitalized. Symptoms included pale, cold, and clammy skin and rapid and weak pulse. She had no detectable arterial blood pressure and no fever. She had abundant bleeding from the nose, mouth, and conjunctiva. She developed DSS and died on August 31. The cause of death was reported as multiple organ failure with irreversible hypovolemic shock. A serum sample was taken on August 31. The serum was positive for IgM antibodies to DEN virus by MAC-ELISA. The patient was diagnosed as having had a secondary DEN virus infection. Unfortunately there was insufficient sample for an RT-PCR, and attempts at virus isolation in cell culture were not successful.

The eight-year-old male patient was a resident of Merida. Clinical manifestations began on October 24, 2002 with general malaise, myalgia, fever, intense headache, severe abdominal pain, and vomiting. Because of fever and uncontrollable vomiting, he was hospitalized. At the time of referral, the patient was severely dehydrated, and had generalized pale, cold, and clammy skin. His pulse became weak and rapid, and he received parenteral solutions. He developed abundant bleeding from mouth, conjunctiva, urethra, and rectum. A blood sample was taken on October 29, and the patient died of DSS later that day. The serum was positive for IgM antibodies to DEN virus by MAC-ELISA. He had experienced a secondary infection as shown by the HI test (antibody titer to DEN-1 virus = 10,240). Dengue 2 virus RNA was amplified from the serum sample by an RT-PCR. Virus was not isolated in cell culture. This patient had the concurrent diagnosis of idiopathic thrombocytopenic purpura.

TABLE 6
Observed and expected numbers of dengue patients with and without hemorrhagic manifestations by age*

Age (years)	DF without HM (observed and expected)	DF with HM (observed and expected)	Total	Chi-square test value
0–4	4 (3.83)	5 (5.17)	9	0.013
5–9	11 (15.74)	26 (21.26)	37	2.489
10–14	37 (24.68)	21 (33.32)	58	**10.704**
15–19	17 (19.15)	28 (25.85)	45	0.420
20–25	8 (14.47)	26 (19.53)	34	**5.034**
26–30	10 (9.79)	13 (13.21)	23	0.008
31–35	4 (5.53)	9 (7.47)	13	0.738
36–40	4 (3.83)	5 (5.17)	9	0.013
41–45	5 (8.94)	16 (12.06)	21	3.018
46–50	7 (5.11)	5 (6.89)	12	1.222
≥ 51	13 (8.94)	8 (12.06)	21	3.217
Total	120	162	282	

* The chi-square test was performed using SAS software version 8.02 (SAS Institute, Cary, NC). Degrees of freedom used = 10; $\chi^2 = 26.883$; $P < 0.005$.
Statistically significant values are denoted in **bold.**
DF = dengue fever; HM = hemorrhagic manifestations.

Epidemiologic results. At least one confirmed DEN case was reported in 42 (39.6%) of the 106 municipalities in Yucatan State. The municipality with the highest number of DEN infections was Merida (n = 144). For most of the confirmed DF and DHF cases (59 and 42 patients, respectively), onset of symptoms occurred during August.

Molecular epidemiology. Nucleotide sequences representative of previously described genotypes of DEN-2 virus[6,8] were obtained from the Genbank database and aligned to the eight Yucatan sequences determined in our studies (Table 2 and Figure 1). The phylogenetic tree constructed from the E gene sequences showed that Mexican DEN-2 virus isolates from the 1980s and early 1990s belong to the American genotype and isolates from the mid 1990s cluster either in the Cosmopolitan or the American genotypes (Figure 1). Isolates from both 2001 and 2002 clustered in the American-Asian genotype. The fact that recent DEN-2 virus isolates are in a different clade from any previous Mexican isolates suggests that the latter are not descendent from the former.

DISCUSSION

Díaz and others[24] previously reported the circulation of two DEN-2 virus genotypes in the Yucatan: the American genotype and the Sri-Lanka genotype (now designated the Cosmopolitan genotype[6]). Our data suggest that DEN-2 viruses of the American-Asian genotype have also been introduced into this region. The three DEN-2 viruses isolated in Yucatan State in 2002 were most similar to strains isolated in Venezuela in 1998 and Martinique in 1988.[25] In contrast, the three isolates from 2001 appear to be derived from a strain isolated in Juchitán in the southern Mexican state of Oaxaca in 2000. These data suggest that there have been at least two separate introductions of the American-Asian genotype in the Yucatan State. This topologic arrangement was strongly supported by the bootstrap analysis. In addition, four nucle-

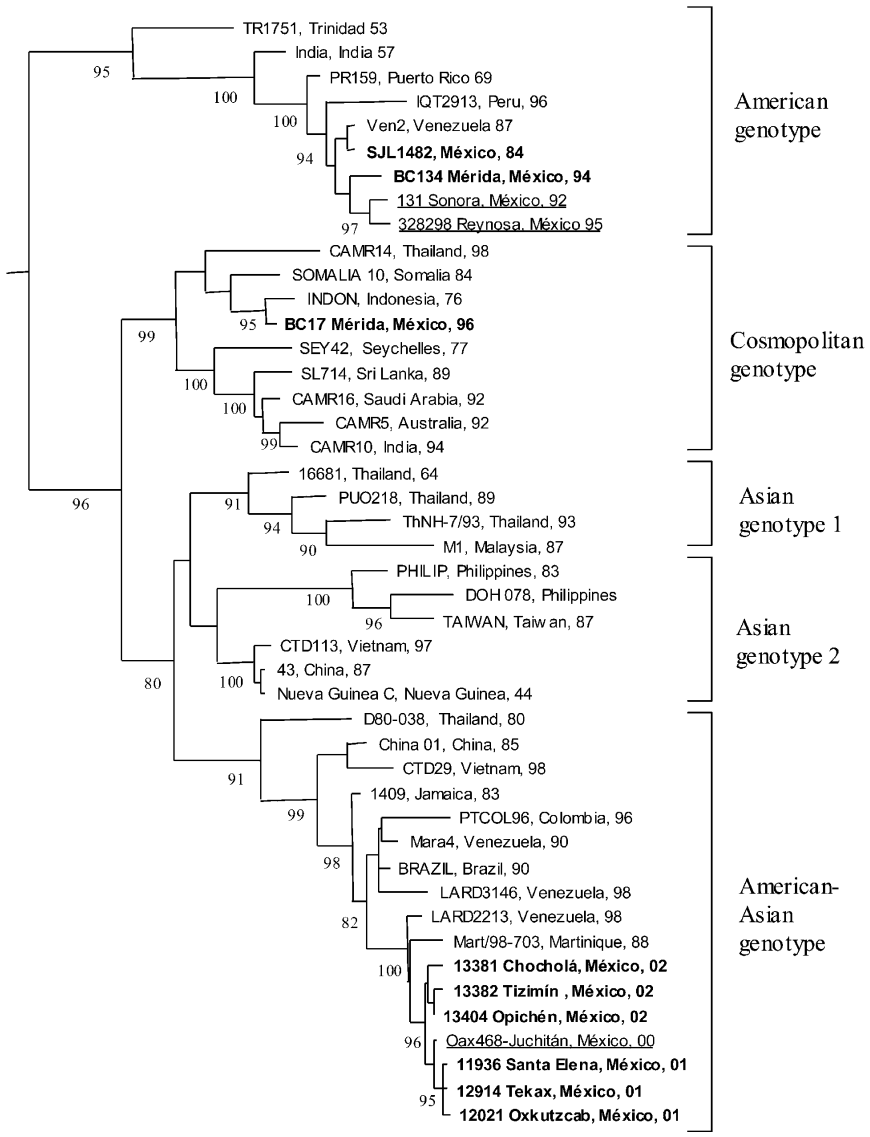

FIGURE 1. Maximum parsimony phylogenetic analysis of the envelope gene of dengue 2 (DEN 2) viruses. Numbers near branches are bootstrap values (1,000 replicates). Values < 70 are not shown. Genotypes are according to Twiddy and others.[6] The tree was rooted with four strains of the DEN virus sylvatic genotype (not shown). The Mexican strains of 2001 and 2002 are at the lower branches of the tree. The DEN-2 viruses from Mexico that were sequenced in the present study are shown in **bold**. The DEN-2 viruses from Mexico that were not sequenced in this study are underlined.

otide substitutions (at positions 121, 137, 630, and 753) in the E gene of all strains isolated in 2001 were not present in any of the 2002 viruses. The possibility of a reversion to the ancestral sequence in all four positions is unlikely.

The DEN-2 viruses belonging to the Southeast Asian genotype appear to be better adapted to mosquito transmission, are associated with more severe clinical outcomes, and are apparently displacing the American genotype of DEN-2 virus in some regions of Latin American.[8,9,26] For instance, DEN-2 viruses of the American-Asian genotype were responsible for the first epidemic of DHF in the Americas, which occurred in Cuba in 1981.[27,28] Shortly afterwards, viruses of the American-Asian genotype were responsible for the first DHF epidemics in Venezuela (in 1989) and Brazil (in 1990), where they potentially displaced the native American genotype.[9,29–31] Thus, the detection of this genotype in the Yucatan State is a major concern to public health authorities. We did not isolate DEN-2 viruses belonging to the American or Cosmopolitan genotypes during the 2002 outbreak. However, additional research is required to determine whether these genotypes have been displaced by the newly-introduced American-Asian genotype. Furthermore, enhanced DEN virus surveillance is needed to determine whether the recent introduction of the American-Asian genotype will coincide with an increase in the incidence and severity of dengue cases in this region.

Indeed, the number of hemorrhagic cases in the Yucatan State since 1984 has increased dramatically. During the 1984 outbreak of DEN-4, there were nine hemorrhagic cases reported, but only one met the WHO criteria for DHF.[4] In 2002, 282 DF cases were confirmed. Among these confirmed cases, 162 (57.5%) patients had hemorrhagic manifestations,

including 87 cases of DHF, all with severe clinical manifestations.

The procurement of acute and convalescent phase specimens contributed greatly to the diagnosis of DEN infections. The low rate of laboratory confirmation of DEN infections in this study was attributable to a number of factors, but the failure of the patients to return to the laboratory to provide the convalescent phase blood specimen was a major problem. When the acute and convalescent phase specimens were available, DEN infection was confirmed in 41.4% of the patients. These findings are in concordance with other DEN virus outbreaks; typically 30–60% of clinically suspect cases are confirmed to have a DEN virus infection.[4,32–34] However, when only the acute phase specimen was available, the confirmation rate decreased to 13.8%. The acute phase sample taken upon presentation (typically five or six days post onset) maximizes potential detection of DEN virus by RT-PCR or virus isolation techniques. The convalescent phase specimen enhances the serologic diagnosis of DEN infections by providing additional time for synthesis of specific IgM for assay by ELISA.

We believe that the total number of cases in this outbreak was underestimated. On September 23, 2002, Hurricane Isidore severely damaged the electrical services, water supplies, and general services in the Yucatán State. Movement and communication between communities were slowed due to extensive road damage and downed power lines. Even when patients were able to access their clinics, samples were not forwarded to the Laboratorio de Arbovirología for diagnosis. For several months following Hurricane Isidore, the only blood samples received at the Laboratorio de Arbovirología for DEN diagnosis were from Merida.

The onset of symptoms for most of the confirmed DF and DHF cases was during August. Presumably rains from April to June contributed to mosquito proliferation, resulting in the increase in DEN virus infections during the following months. Indeed, since 1985, the Yucatan State health authorities have organized campaigns to eliminate mosquito breeding sites and to apply larvicides and adulticides in cities and small rural towns. The most commonly used larvicide is temephos (Abate™; American Cyanamid, Princeton, NJ) and a pyrethroid adulticide (Aqua Res™; Bayer Environmental Science, Long Lake [Minneapolis], MN). These activities are conducted before the rainy season, but unfortunately the number of DEN virus infections and more severe disease continue to increase.

Clearly, there are needs to develop less expensive and more specific and sensitive diagnostic techniques. The RT-PCR is too expensive to use as a diagnostic test for all of the suspected cases, including those with classic DF. Therefore, the RT-PCR was only used for diagnosis with hospitalized cases, severe symptoms, and/or hemorrhagic manifestations. New tests, such as the use of an ELISA to detect specific IgA and IgM in sera and saliva of dengue cases,[35] offer great potential in this regard for both diagnosis and surveillance. Hopefully, new approaches and techniques will also allow the detection of other medically important flaviviruses such as West Nile, Saint Louis encephalitis, and yellow fever viruses, as well as determination of DEN viruses causing secondary infections. Such tests would be a great addition to the armamentarium of public health practitioners trying to cope with the resurgence of flavivirus diseases in many parts of the world.

In summary, we report the introduction of a new DEN-2 virus of the American/Asian genotype into the Yucatan State. Certainly, expanded surveillance for dengue virus is required in this region to better understand the clinical, virologic, and epidemiologic consequences of this introduction.

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REFERENCES

1. Burke DS, Monath TP, 2001. Flaviviruses. Knipe DM, Howley PM, eds. *Fields Virology*. Fourth edition. Philadelphia, PA: Lippincott Williams and Wilkins, 1043–1126.
2. Gubler DJ, 1988. Dengue. Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Volume II. Boca Raton, FL: CRC Press, 223–260.
3. Pan American Health Organization, 1981. Dengue in Mexico and in the United States. *Epidemiol Bull 2:* 9–11.
4. Loroño Pino MA, Farfán Ale JA, Rosado Paredes EP, Kuno G, Gubler DJ, 1993. Epidemic dengue 4 in the Yucatán, México, 1984. *Rev Inst Med Trop Sao Paulo 35:* 449–455.
5. Centers for Disease Control and Prevention, 1982. Dengue type 1 in Mexico. *MMWR Morb Mortal Wkly Rep 31:* 468, 473–474.
6. Twiddy SS, Farrar JJ, Vinh Chau N, 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.
7. 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.
8. Rico-Hesse R, 1990. Molecular distribution of dengue viruses type 1 and 2 in nature. *Virology 174:* 479–493.
9. Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, Boshell J, de Mesa MT, Nogueira RN, da Rosa AT, 1997. Origins of dengue type 2 viruses associated with increased pathogenesis in the Americas. *Virology 230:* 244–251.
10. Centers for Disease Control and Prevention, 1994. Dengue type 3 infection, Nicaragua and Panama, October-November 1994. *JAMA 273:* 840–841.
11. Guzman MG, Vazquez S, Martinez E, Alvarez M, Rodriguez R, Kouri G, de los Reyes J, Acevedo F, 1996. Dengue in Nicara-

gua: reintroduction of serotype 3 in the Americas. *Bol Oficina Sanit Panam 121:* 102–110.
12. Usuku S, Castillo L, Sugimoto C, Noguchi Y, Yogo Y, Kobayashi N, 2001. Phylogenetic analysis of dengue-3 viruses prevalent in Guatemala during 1996-1998. *Arch Virol 146:* 1381–1390.
13. Wittke V, Robb TE, Thu HM, Nisalak A, Nimmannitya S, Kalayanrooj S, Vaughn DW, Endy TP, Holmes EC, Aaskov JG, 2002. Extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period. *Virology 301:* 148–156.
14. Thu HM, Lowry K, Myint TT, Shwe TN, Han AM, Khin KK, Thant KZ, Thein S, Aaskov J, 2004. Myanmar dengue outbreak associated with displacement of serotypes 2, 3, and 4 by dengue 1. *Emerg Infect Dis 10:* 593–597.
15. WHO, 1997. *Dengue Hemorrhagic Fever: Diagnosis, Treatment and Control.* Second edition. Geneva: World Health Organization.
16. Dean AG, Dean JA, Coulombier D, Brendel KA, Smith DC, Burton AH, Dicker RC, Sullivan K, Fagan RF, Arner TG, 1996. *Epi Info, Version 6: A Word Processing, Database, and Statistics Program for Public Health on IBM-Compatible Microcomputers*. Atlanta, GA: Centers for Disease Control and Prevention.
17. Kuno G, Gomez I, Gubler DJ, 1991. An ELISA procedure for the diagnosis of dengue infections. *J Virol Methods 33:* 101–113.
18. Roehrig JT, Mathews JH, Trent DW, 1983. Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. *Virology 128:* 118–126.
19. Clarke DH, Casals J, 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg 7:* 561–577.
20. Henchal EA, McCown JM, Seguin MC, Gentry MK, Brandt WE, 1983. Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay. *Am J Trop Med Hyg 32:* 164–169.
21. Gubler DJ, Kuno G, Sather GE, Velez M, Oliver A, 1984. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. *Am J Trop Med Hyg 33:* 158–165.
22. Lanciotti RS, Calisher CH, Gubler DJ, Chang G-J, Vorndam V, 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol 30:* 545–551.
23. Swofford DL, 1998. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods).* Version 4. Sunderland, MA: Sinauer Associates.
24. Díaz FJ, Farfán-Ale JA, Olson KE, Loroño-Pino MA, Gubler DJ, Blair CD, Black WC IV, Beaty BJ, 2002. Genetic variation within the premembrane coding region of dengue viruses from the Yucatan Península of Mexico. *Am J Trop Med Hyg 67:* 93–101.
25. Tolou H, Coussinier-Paris P, Mercier V, Pisano MR, de Lamballeie X, de Micco P, Durand J-P, 2000. Complete genomic sequence of a dengue type 2 virus from the French West Indies. *Biochem Biophys Res Commun 277:* 89–92.
26. 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.
27. 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.
28. Sariol CA, Pelegrino JL, Martinez A, Arteaga E, Kouri G, Guzman MG, 1999. Detection and genetic relationship of dengue virus sequences in seventeen-year-old paraffin-embedded samples from Cuba. *Am J Trop Med Hyg 61:* 994–1000.
29. Pan American Health Organization, 1990. Dengue hemorrhagic fever in Venezuela. *Epidemiol Bull 11:* 7–9.
30. Pan American Health Organization, 1992. Dengue and dengue haemorrhagic fever in the Americas: an overview of the problem. *Epidemiol Bull 13:* 9–10.
31. 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.
32. Aviles G, Rangeon G, Baroni P, Paz V, Monteros M, Sartini JL, Enria D, 2000. Outbreak of dengue-2 virus in Salta, Argentina, 1988. *Medicina (B Aires) 60:* 875–879.
33. 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.
34. Guzman MG, Kouri G, Valdes L, Bravo J, Alvarez M, Vazques S, Delgado I, Halstead SB, 2000. Epidemiologic studies on Dengue in Santiago de Cuba, 1997. *Am J Epidemiol 152:* 793–799.
35. Balmaseda A, Guzmán MG, Hammond S, Robleto G, Flores C, Tellez Y, Videa E, Saborio S, Pérez L, Sandoval E, Rodríguez Y, Harris E, 2003. Diagnosis of dengue virus infection by detection of specific immunoglobulin M (IgM) and IgA antibodies in serum and saliva. *Clin Diagn Lab Immunol 10:* 317–322.