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Molecular Epidemiological Study of Dengue Virus Type 1 in Taiwan

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Taiwan has experienced several major outbreaks of dengue (DEN) virus since 1981. The predominant virus type involved has been dengue virus type one (DEN-1), which first appeared in 1987. To understand the molecular epidemiology of this virus, 15 strains of DEN-1 isolated during 1987–1991 and 1994–1995, including 11 epidemic strains, two sporadic strains, and two imported strains have been studied. Fragments of 490 nucleotides (nt) from the E/NS1 junction were amplified by reverse transcription-polymerase chain reaction and the nt sequences were determined. Of the 490 nt of the E/NS1 junction, 240 nt (nt 2282–2521) were aligned and compared. Nucleotide substitutions were found at 54 positions among 15 isolates. Most nt changes were synonymous substitutions, and only three amino acid changes were found. A total of 61 strains isolated worldwide were analyzed by the Neighbor-joining method, and separated phylogenetically into three distinct genotypes, I–III. Genotype I comprised isolates from Japan and Hawaii collected in the 1940s. Genotype II included most strains isolated from Asia in 1977–1995. Genotype III consisted of isolates from three continents in 1964–1995: Asia, the Americas, and Africa. Genotype III was divided further into two subgenotypes, IIIA and IIIB. Most recent isolates from Taiwan, except for the sporadic strain isolated in 1995, were similar genetically and have been classified as Genotype II. ***J. Med. Virol. 70:404–409, 2003.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: DEN-1; genotype

INTRODUCTION

Dengue viruses (DEN) belong to the family *Flaviviridae* and the genus *Flavivirus*. They are responsible for an increase in health problems in tropical and subtropical countries, especially those of Southeast Asia and the western Pacific region [Rico-Hesse, 1990; Rico-Hesse et al., 1997; Fonseca and Fonseca, 2002]. Infection with this mosquito-borne virus varies from mild, flu-like symptoms (dengue fever, DF) to a fulminating dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Furthermore, unusual central nervous system manifestations and hepatic involvement associated with dengue infection have been reported [Thisyakorn and Thisyakorn, 1994; Thisyakorn et al., 1999]. In recent years, DF/DHF has emerged as a global health problem due to the greater convenience of air travel [Gubler and Clark, 1995; Guzman and Kouri, 2002].

Four closely related but antigenically distinct serotypes, Types 1–4 (DEN-1–4) have been described. The viral genome is a single-stranded positive sense RNA of about 11 Kb, which has a cap structure at its 5′ end and lacks a poly(A) tract at its 3′ extremity [Deubel et al., 1990]. The viral genome consists of structural and nonstructural proteins. The gene order is 5′-C-prM (M)-E-NS_1-NS_2A-NS_2B-NS_3-NS_4A-NS_4B-NS_5-3′ [Speight and Westaway, 1989; Wright et al., 1989]. Among the encoded proteins, the envelope protein (E) and nonstructural protein (NS1) are the most important.

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The E protein is associated with the neutralization and the hemagglutination of goose erythrocytes, and NS1 may be of immunological importance [Guirakhoo et al., 1989]. Thus, many monoclonal antibodies have been prepared by targeting these regions [Schlesinger et al., 1985, 1987]. Phylogenetic analyses based on these regions have been reported also [Rico-Hesse, 1990].

The extent of molecular evolution within DEN-1, DEN-2, DEN-3, and DEN-4 genotypes has been described previously [Blok et al., 1989, 1991; Lewis et al., 1993; Zulkarnain et al., 1994]. Based on the nucleotide (nt) sequence of the E and E/NS1 gene region, DEN-1 viruses have been classified into three to five genotypes [Rico-Hesse, 1990; Chungue et al., 1995], DEN-2 viruses into five genotypes [Rico-Hesse et al., 1997], DEN-3 viruses into four genotypes [Lanciotti et al., 1994], and DEN-4 viruses into two genotypes [Chungue et al., 1995].

In Taiwan, three outbreaks of dengue virus infection were reported in 1915, 1931, and 1942 [Wu, 1986]. From that time, no outbreak was reported until 1981, when an outbreak of DEN-2 occurred on the island of Liouchyou [Wu, 1986]. There was a small outbreak in 1987 and a large outbreak in 1988, followed by a small outbreak in Kaohsiung in 1991 [Wu et al., 1993]. Most of the viruses isolated were DEN-1, but some DEN-2, 3, and 4 cases also were found. Since 1991, only a few sporadic cases were reported until 1994. In 1995, DEN-1 was isolated for the first time in Taipei, indicating that the dengue viruses have moved northward and are now endemic throughout Taiwan. There have been two outbreaks in the last 5 years, one in Tainan (DEN-3) in 1998, and in Kaohsiung from 2001–02 (DEN-2). No DEN-1 outbreak has occurred in Taiwan since 1995.

To understand the genetic relationship of DEN-1 collected in Taiwan from 1987–95, 15 isolates were studied. Fragments of 490 nt of the E/NS1 junction were amplified and the nt sequences were determined. Of the 240-nt sequences of the E/NS1 junction, 111 nt from the 3′ end of the E region and 129 nt from the 5′ end of the NS1 region, were analyzed. A global comparison was made together with 46 strains worldwide.

MATERIALS AND METHODS

Cells and Viruses

Fifteen virus strains isolated from outbreaks of DEN-1 during 1987–95 were obtained from the Division of Epidemiology, National Institute of Preventive Medicine, Department of Health. Strains No. 027001 and 157001 were imported from Thailand and the Philippines, respectively. Strains No. 366367 and 436134 were isolated from sporadic cases in Kaohsiung and Pingtung in 1994 and 1995, respectively. The viruses were grown on AP-61 (*Aedes pseudoscutellaris*) cells for 5 days in Mitsuhashi and Maramorosch insect culture (MM medium, Sigma, St. Louis, MO, M9257) and supplemented with either 10% fetal calf serum (FCS) for growth or 2% FCS for maintenance [Fu et al., 1992]. The serotypes were confirmed by indirect immunofluorescence assay with type-specific monoclonal antibodies, prepared in-house from hybridoma cell lines (HB-46–49, ATCC).

Reverse Transcription-Polymerase Chain Reaction

RNA was extracted from cell culture medium using a Micro RNA Isolation Kit (Stratagene). One primer pair set flanking the E/NS1 region was used in the reverse transcription-polymerase chain reaction (RT-PCR) [Morita et al., 1991]. The complementary primers used were: 5′-ATGGGTTGTGGCCTAATCAT-3′, (nt 2718–2699), and sense primer: 5′-GGACTGCGTAG-GAGTTTG-3′, (nt 2229–2248). All reagents and amplification conditions were identical to those described previously [Tung et al., 1995].

Sequencing and Genetic Analysis

RT-PCR products were purified as reported previously [Lin et al., 2001]. Briefly, the products were separated in 8% polyacrylamide gel. DNA bands were eluted from the gel and extracted with phenol-chloroform mixture (1:1), and DNA was recovered by ethanol precipitation. The purified PCR product (0.25–0.5 μg) was labeled with Ampli/Taq DNA Polymerase Fluorescent Sequencing kit (ABI PRISM, Foster City, CA) and the nt sequence was determined with an automated sequencer (ABI Model 373A). Sixty-one strains of DEN-1, including 15 strains in this study and 46 strains in the GenBank taken worldwide during the period from 1943–95, were analyzed. The nt sequences were aligned using the Sequence Analysis Package from Genetics Computer Group (Madison, WI). Distances were measured by Kimura two-parameter method. A phylogenetic tree was constructed by the modified Neighbor-Joining (NJ) method [Saitou and Imanishi, 1989; Rzhetsky and Nei, 1992]. One thousand times bootstrap [Felsenstein, 1985; Saitou and Nei, 1987] was used for confirmation of statistical significance of phylogenetic analysis by Mega [Kumar et al., 1993] and Phylip [Felsenstein, 1989] software. The dendrogram was displayed using TreeView [Page, 1996].

Accession Numbers of the Nucleotide Sequence

The nt sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nt sequence databases with the following accession numbers: 768180: AB008121; 768468: AB008122; 776669: AB008123; 776836:AB008124; 066244: AB008125; 006332: AB008126;027001: AB008127; 157001: AB0081218; 360023:AB008129; 360032: AB008130; 360068: AB008131; 366367: AB008132; 400124: AB008133.

RESULTS

Nucleotide Sequence Alignment and Pairwise Comparison

Nucleotide sequences of the E/NS1 junction of the 15 strains of DEN-1 genome were aligned together with those of 46 DEN-1 strains deposited in GenBank. Pairwise comparison of the 240 of 490 nt was analyzed. Neither insertion nor deletion was found. The nt sequence homologies among the 61 strains worldwide were 85–100% (data not shown). The nt sequence homologies of the 15 Taiwanese strains isolated during 1987–1991, as well as those of strains isolated during 1994–1995, were 97–99%. Homology between the strains in these two periods, however, was 95–97% (data not shown). By comparing the nt sequence among the 15 strains in this study, 11 epidemic and one sporadic Taiwanese isolates, as well as the imported Thai isolates, were found to be similar genetically. In contrast, the other sporadic Taiwanese isolate from Pingtung was different and genetically similar to the imported Philippine isolate. Over 1,000,000 Taiwanese people travel to countries in Southeast Asia , including Thailand, the Philippines, and Indonesia every year. The annual epidemic in Taiwan spread via imported cases.

Among the 15 strains in this study, only three amino acid changes (amino acid residues 3, 26, and 27) were found in 54 nt substitutions of the 240 nt analyzed. These were two transitions at amino acid residues 3 (I → V) and 27 (V → A) and one transversion at residue 26 (M → L) (Fig. 1). The amino acid change at residue 3 (I → V) was unique to most of the Taiwanese strains and the strain from the Bahamas. The amino acid change at residue 26 (M → L) was unique to 13 of 15 strains, except for one each of the sporadic and imported strains. Most of the mutations in the nt sequences were located at the third position of the codon and were therefore silent.

Phylogenetic Analysis

The genetic relationships among 61 isolates worldwide were inferred by the Neighbor-Joining method based on the 240 nts (nt 2282–2521) in the GenBank. Three genotypes could be discerned in the tree (Fig. 2). Genotype I was comprised of isolates from Japan in 1943 and isolates from Hawaii in 1945. Genotype II included isolates from Taiwan collected from 1987–1995, and isolates from Thailand collected in 1981 and 1991, as well as an isolate from the Bahamas in 1977. Genotype III was divided further into subgenotypes IIIA and IIIB. IIIA consisted of isolates collected from 1974–1994 in Australia, the western Pacific area, Indonesia, and the Philippines, from Mexico in 1980, and from Taiwan in 1995. IIIB was comprised of strains gathered from the Americas, Africa, Southeast Asia, and Sri Lanka from 1964–1990.

DISCUSSION

In Taiwan, there were DEN-1 epidemics in Kaohsiung from 1987 to 1991, but only sporadic DEN-1 cases were found in 1994. Intriguingly, outbreaks of DEN-1 were then found beyond Kaohsiung, in Tainan as well as in Taipei, in 1994 and 1995, respectively; however, DEN-3 then became the major causative agent of the 1994 outbreak in Kaohsiung. In this study, the 240 nt of the E/NS1 junction of the viral genome of 15 strains isolated in Taiwan and 46 strains isolated worldwide were chosen as the best sequence of nts for comparison among these strains, because this gene region has a uniform rate of random mutation and no hypervariable regions [Rico-Hesse, 1990]. The maximum sequence variation of the 240 nts of the E/NS1 junction among the 15 strains, except for the two imported strains and the sporadic strain (436134) isolated in 1995, was 5.28% (data not shown). This finding was similar to that reported in 1995 for DEN-1 and DEN-4 (6.9% and 4.9%, respectively) [Chungue et al., 1995] but lower than that for DEN-2 (20%) [Deubel et al., 1993] and DEN-3 (12.3%) [Chungue et al., 1993].

In this study, phylogenetic analysis of 240 nts of the E/NS1 region of the 61 strains isolated worldwide revealed three genotypes for DEN-1. This finding differs from the five genotypes reported by Rico-Hesse [1990]. Differences in classification may be due to the method used for the genetic analysis. In the present study, the largest genotype was III, which consisted of DEN-1 viruses from three continents: the Americas, Africa, and Asia. Most recent isolates from Taiwan (1987–1995) were similar genetically and classified as Genotype II. The sporadic strain (436134) isolated in southern Taiwan in 1995 and the imported Philippine strain isolated in 1992, however, were included in subgenotype IIIA. This implies that the Taiwanese strains evolved earlier and formed a separate genotype. Intriguingly, strains of Genotype I have not been found since 1945.

It has been reported that nt sequence analysis of dengue viruses can be used to define genetic variation between strains of the same serotype and follow geographical movement of the strains, facilitating identification of the source of virus strains in new outbreaks [Rico-Hesse, 1990; Chungue et al., 1993; Lanciotti et al., 1994, 1997]. The analysis of nt variation in Taiwanese strains of Genotype II suggested that most of the Taiwanese DEN-1 strains had evolved chronologically during 1987–1995, with strains from 1994 and 1995 clustered together. This suggests a high level of dissemination of DEN-1 isolates during recent years.

The results of this study have shown that most of the Taiwanese DEN-1 isolates belong to Genotype II and may have originated in Thailand. The sporadic strain isolated from southern Taiwan (436134) in 1995, however, showed more similarity to the imported strain from the Philippines isolated in 1992 and they were clustered together into subgenotype IIIA. This implies that a DEN-1 strain of different geographic origin might have entered Taiwan and evolved independently. The sporadic strain (436134) showed a much closer relationship with the strains obtained from Indonesia in 1977 and 1978 than with the imported strain from the Philippines

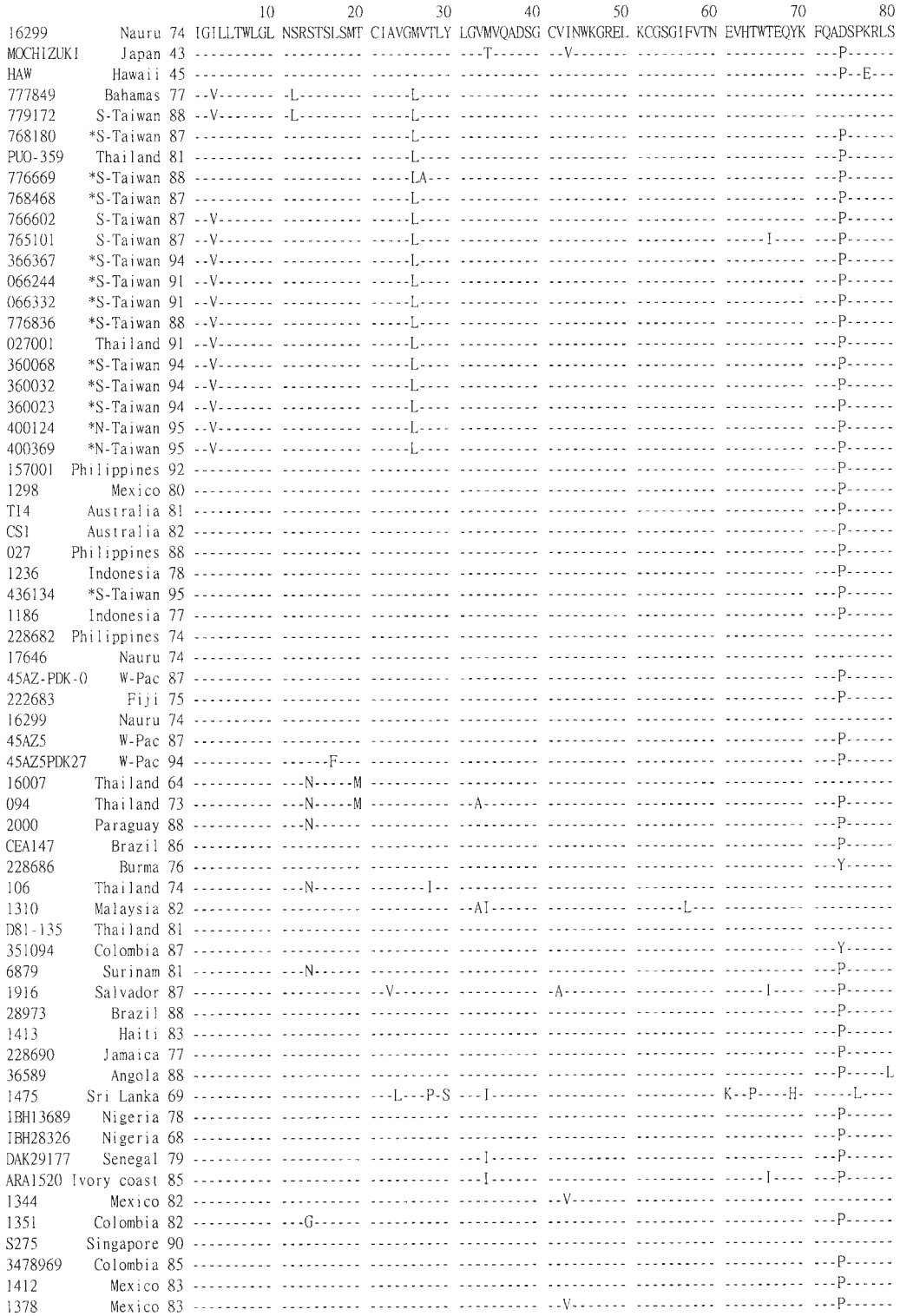

Fig. 1. Comparison of 80 amino acids of the E/NS1 junction among 61 DEN-1 isolates worldwide. The last two numbers of the strain name indicates the year of isolation. S-TW, southern Taiwan; N-TW, northern Taiwan; W-Pac, west Pacific. *Strains studied in this report.

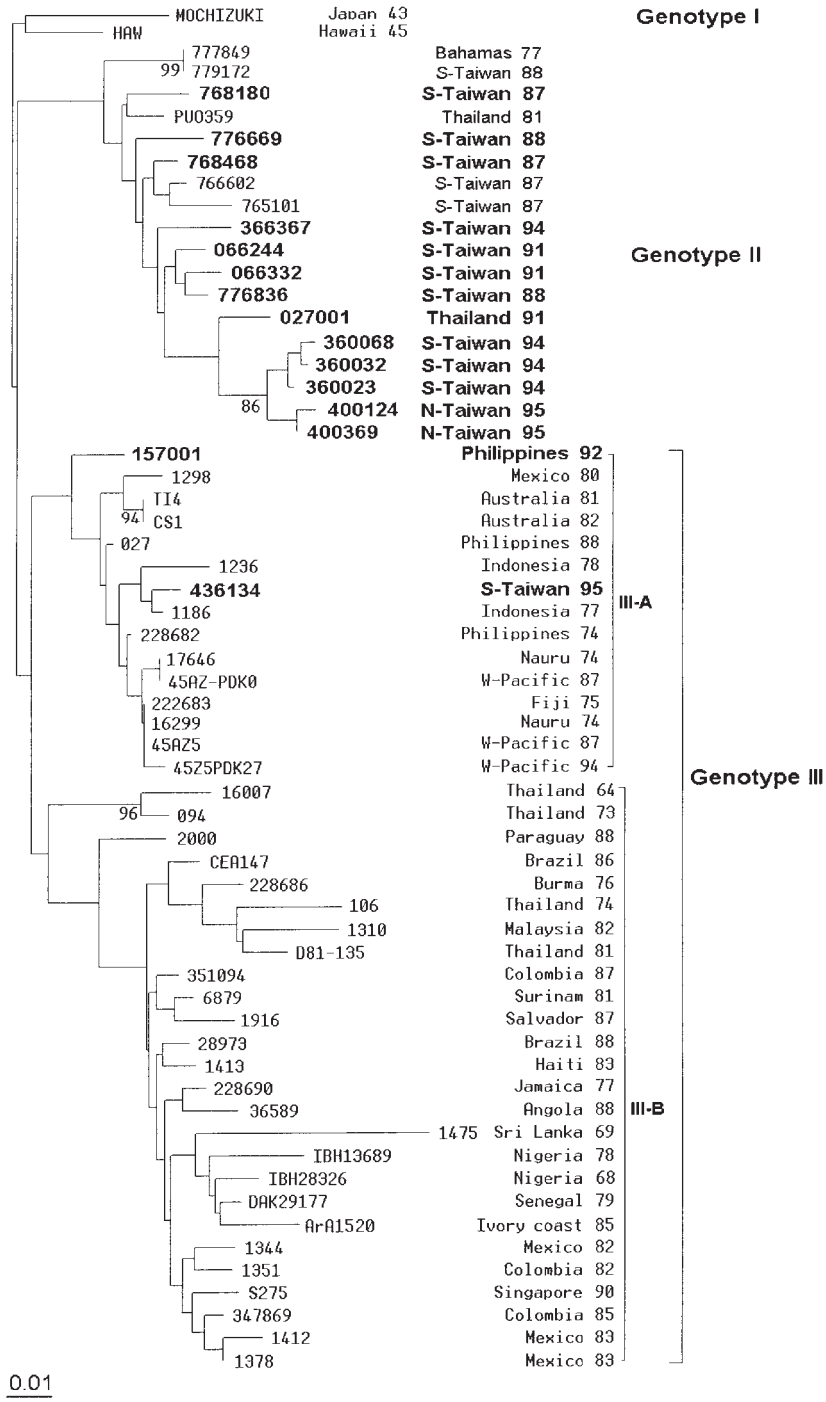

Fig. 2. Phylogenetic analysis of the 61 strains of DEN-1 worldwide, based on the E/NS1 junction (240 nts). Tree topology was inferred by the Neighbor-joining method. The upper numbers show the branch length denoted as percentage of difference. The lower numbers indicate the bootstrap values. Three genotypes (G I–III) are indicated. Strains used in this study are highlighted. S-TW, southern Taiwan; N-TW, northern Taiwan; W-Pacific, west Pacific.

isolated in 1992. This implies that the sporadic Taiwanese strain (436134) obtained in 1995 might have a different origin. The evolution of the viruses will continue to be monitored.

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