Molecular Characterization of Dengue Viruses Imported Into Taiwan during 2003–2007: Geographic Distribution and Genotype Shift

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Abstract. We presented our surveillance results on imported dengue cases in Taiwan during 2003–2007. A total of 542 imported dengue patients were identified. The travelers were infected in 17 countries in Southeast Asia, the Indian subcontinent, East African islands, South Pacific islands, and Central America. Most of these imported cases were infected in Southeast Asian countries. Phylogenetic analyses were conducted to examine 288 imported dengue virus (DENV) strains introduced from 13 countries. The results provide an updated view on the geographic distribution and dynamic transmission of epidemic DENV strains circulated in Southeast Asian countries. Although the geographic distributions of genotypes of DENV-3 isolated from Southeast Asian countries remain unchanged, the introductions and local expansions of epidemic DENV-1, DENV-2, and DENV-4 strains into new areas in Asia were observed. These findings highlight the importance to strengthen laboratory-based dengue surveillance for better understanding of transmission dynamics and molecular evolution of DENVs.

INTRODUCTION

Dengue viruses (DENVs; serotypes 1–4) are mosquito-borne members of the genus *Flavivirus* in the family *Flaviviridae*. They are the most prevalent arboviruses in tropical and subtropical regions of the world, following the geographical distribution of two competent vectors *Aedes aegypti* and *Ae. albopictus*.1 In recent decades, the number of dengue cases reported worldwide and the number of countries with endemic dengue has increased dramatically because of the expanding habitat of the *Aedes* spp. mosquito vectors, growing numbers of susceptible human hosts, and increasing spread of DENVs through rapid and frequent global travel.2,3

With the worldwide increase in travel, the rapid expansion of DENV strains to different part of the world has been well documented.4–9 Studies on returned travelers have provided useful insights into the geographic distribution and global movement of DENV strains. The risk of acquiring dengue is highest when traveling in Southeast Asia.10,11 Phylogenetic analyses of DENV strains isolated from imported cases were used to determine the country origins where infections occurred.3,12 Furthermore, the discovery of novel DENV strains and lineages were reported.13,14

Major dengue outbreaks and characteristics of dengue epidemics in Taiwan have been reported recently.15 Molecular epidemiologic studies analyzing a wide variety of DENV strains isolated from imported and indigenous dengue cases during 1981–2006 showed that different serotypes, genotypes, and/or strains were responsible for the yearly outbreaks, and the epidemic strains disappeared with the ending of each local outbreak. The results suggested that constant importation of multiple DENVs from the neighboring Southeast Asian countries through close commercial links and air travel was responsible for local outbreaks that occur each year. To reduce the introduction of DENV strains and prevent their local outbreaks, the Taiwan Centers for Disease Control has implemented various passive and active surveillance systems to detect imported dengue cases.7 Phylogenetic analyses based on gene sequences obtained from a database of DENV strains imported from a wide variety of countries are presented in this study.

MATERIALS AND METHODS

**Human serum samples.** Dengue (dengue fever and dengue hemorrhagic fever) are Category 2 reportable infectious diseases in Taiwan, and suspected cases must be reported within 24 hours of clinical diagnosis. To provide effective surveillance, both passive (hospital-based reporting system) and active (such as fever screening at airports, self-reporting, expanded screening for contacts of confirmed cases, patients with fever of unknown origin, school-based reporting) surveillance systems were implemented by central and local health departments in Taiwan. Human serum samples of suspected dengue cases were submitted to the Research and Diagnostic Center, Centers for Disease Control, Taiwan (Taiwan CDC), for confirmation of DENV infection. Human serum samples used in this study were derived from confirmed dengue cases submitted to Taiwan CDC during 2003–2007. An imported dengue case was defined as an infected patient who had been traveling abroad > 2 weeks before the onset of illness, because it will be considered to have been infected abroad and imported into Taiwan. When overseas travel is not indicated, it will be recorded as an indigenous case.

**Laboratory diagnosis.** DENV infection was defined as a febrile illness associated with the detection of DENV-specific IgM and IgG antibodies, the isolation of DENV, or the detection of DENV RNA by reverse transcriptase-polymerase chain reaction (RT-PCR).15 One-step SYBR Green I real-time RT-PCR (QuantiTect SYBR Green RT-PCR kit; Qiagen, Hildén, Germany) was performed in the Mx4000 quantitative PCR system (Stratagene) to detect and differentiate DENV serotypes in the acute phase serum samples as previously described.16 Real-time RT-PCR was performed using two sets of consensus primers: one primer set targeting a region of the nonstructural protein 5 (NS5) genes to detect all of the flaviviruses and the other primer set targeting a region of the capsid (C) gene to detect all of the DENV serotypes. Positive samples were confirmed by DENV serotyping using four sets of serotype-specific primers targeting the C gene to differentiate the DENV serotypes. For RT-PCR–positive cases, partial NS5...
gene sequencing (153 nucleotides in length) was routinely performed using RT-PCR products of the one-step SYBR Green I real-time RT-PCR to determine the DENV serotypes and genotypes. For the detection of DENV-specific IgM and IgG antibodies, Envelope (E)/Membrane (M)-specific capture IgM and IgG ELISA were used to detect and differentiate primary and secondary DENV infection on both acute- and convalescent-phase serum samples as previously described. The isolation of DENV was performed using a mosquito cell line (clone C6/36 of *Ae. albopictus*). For each acute phase sample, 50 µL of 1:20, 1:40, 1:80, and 1:160 diluted serum were added to a 96-well samples (diluted with RPMI, Gibco/BRL, Life Technologies, serum sample, 50 µL of 1:20, 1:40, 1:80, and 1:160 diluted serum were combined for analysis and edited with the Lasergene software package (DNASTAR, Madison, WI). Nucleotide sequences were determined by the ABI Prism automated DNA sequencing kit and the ABI Prism 3700 DNA sequencer (Applied Biosystems) according to the manufacturer’s protocols. Overlapping nucleotide sequences were combined for analysis and edited using the Lasergene software package (DNASTAR, Madison, WI). Nucleotide sequences of complete E genes of DENV strains described in this study were submitted to GenBank and their accession numbers (EU448386–EU448464) are given in Figures 2–5.

**Phylogenetic analysis.** A total of 288 DENV strains imported into Taiwan were analyzed together with various global reference sequences of different genotypes available from GenBank. The nucleotide sequences of complete E genes of DENV strains were aligned, edited, and analyzed using Clustal W software. The phylogenetic analysis was performed using MEGA version 4 (http://www.megasoftware.net/). For the construction of phylogenetic trees, the Neighbor-joining algorithm and the Kimura two-parameter distance model were used. The reliability of the analysis was evaluated by a bootstrap test with 1,000 replications. Phylogenetic trees were also generated using maximum likelihood (PHYLIP v3.66) and Bayesian (MrBayes v3.1.2; http://mrbayes.csit.fsu.edu/) models. Because very similar topologies were generated in these trees, differing only within a few terminal groupings, only the phylogenetic trees constructed by the Neighbor-joining method were presented (Figures 2–5). Sequences

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### Table 1

Primers used for RT-PCR and DNA sequencing of DENV-1 and DENV-4

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Genomic region</th>
</tr>
</thead>
<tbody>
<tr>
<td>DN5URF</td>
<td>AGT TTG TCT CTG TGC TTA GAG ACC G</td>
<td>5’UTR (1–22)*</td>
</tr>
<tr>
<td>D1-545F</td>
<td>ATT GGG AGT GAT TTT GGA GAG</td>
<td>PrM (545–565)*</td>
</tr>
<tr>
<td>D1-631R</td>
<td>GTC AAC ATC TTC TGG TTC CG</td>
<td>PrM (612–631)*</td>
</tr>
<tr>
<td>D1-1125F</td>
<td>AAA ATC ACA ACA CCA CGG</td>
<td>(1,125–1,145)*</td>
</tr>
<tr>
<td>D1-1217R</td>
<td>TTC GTC GRC CAA ACA AGT TCG</td>
<td>(1,197–1,217)*</td>
</tr>
<tr>
<td>D1-1546F</td>
<td>ATC ATG GCT TGT CCA AAC</td>
<td>(1,546–1,565)*</td>
</tr>
<tr>
<td>D1-1629R</td>
<td>TTC CAA GTC GAT GT</td>
<td>(1,610–1,629)*</td>
</tr>
<tr>
<td>D1-2044F</td>
<td>CCA CCA TTT GTG GAG AGC TAC</td>
<td>(2,044–2,065)*</td>
</tr>
<tr>
<td>D1-2123R</td>
<td>TGTC TTC YCT TCT TGA ACC AGC</td>
<td>(2,103–2,123)*</td>
</tr>
<tr>
<td>D1-2583R</td>
<td>CAC ACA CCC TCC TCC CAT GCC</td>
<td>NS1 (2,563–2,583)*</td>
</tr>
<tr>
<td>D1-2630R</td>
<td>TTT GYT TCC ACA TGA TGT TCT C</td>
<td>NS1 (2,609–2,630)*</td>
</tr>
</tbody>
</table>

*Numbering from GenBank accession number AF188088.
†Numbering from GenBank accession number AF326573.

RT-PCR = reverse transcription-polymerase chain reaction; DENV = dengue virus; C = capsid; PrM = premembrane; E = envelope; UTR = untranslated region.

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**Preparation of viral RNA, RT-PCR amplification, and nucleotide sequencing.** Viral RNA was extracted from either cell C6/36 of *Ae. albopictus* cells. For each acute phase serum sample, 50 µL of 1:20, 1:40, 1:80, and 1:160 diluted serum samples (diluted with RPMI, Gibco/BRL, Life Technologies, containing 1% fetal calf serum [FCS]) were added to a 96-well microtiter plate, and then 10^5 cells/100 µL/well of C6/36 were added to a 96-well microtiter plate after incubation for 7 days at 30°C. Cells were harvested, and infection was confirmed by an immunofluorescence assay using dengue serotype-specific monoclonal antibodies. The viruses were subcultured in C6/36 cells and harvested for nucleotide sequencing after the first or second passage. Viruses were identified using the nomenclature of DENV strains were aligned, edited, and analyzed using the QIAamp Viral RNA Mini kit (Qiagen). Primers used for RT-PCR and nucleotide sequencing of DENV-2 and DENV-3 were described previously. Primers used for RT-PCR and nucleotide sequencing of DENV-1 and DENV-4 are listed in Table 1. Primers were designed to amplify and sequence C, premembrane (PrM) and E gene sequences. Two sets of primers, DN5URF/D1-1217R and D1-1125F/D1-2630R, were used for RT-PCR to amplify DENV-1 sequences. Primer sets, D1-5UTRF/D1-1234F and D1-1108F/D1-2609F, were used for RT-PCR to amplify DENV-4 sequences. All of these primers were used for sequencing. Titan one tube RT-PCR system (Roche, Mannheim, Germany) was used for RT-PCR. After an initial denaturation at 90°C for 3 minutes, RT was carried out at 50°C for 45 minutes followed by PCR at 94°C for 3 minutes, 35 cycles of 94°C for 20 seconds, 50°C for 30 seconds, and 68°C for 2 minutes, with 5 s/cycle added to the elongation step after the first 15 cycles, and a prolonged elongation at 68°C for 7 minutes. PCR products were purified using the Qiagen QIA quick Gel Extraction kit (Qiagen). Nucleotide sequences were determined by the ABI Prism automated DNA sequencing kit and the ABI Prism 3700 DNA sequencer (Applied Biosystems) according to the manufacturer’s protocols. Overlapping nucleotide sequences were combined for analysis and edited using the Lasergene software package (DNASTAR, Madison, WI). Nucleotide sequences of complete E genes of DENV strains described in this study were submitted to GenBank and their accession numbers (EU448386–EU448464) are given in Figures 2–5.

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of D2/NewGuinea/NGC/1944 strain (M29095), D1/USA/Hawaii/1945 strain (AF425619), and D2/NewGuinea/NGC/1944 strain (M29095) were used as outgroup to root the tree of the DENV-1, DENV-2, DENV-3, and DENV-4, respectively.

RESULTS

Imported dengue cases in Taiwan during 2003–2007. A total of 542 imported dengue cases were identified in Taiwan during 2003–2007. Among them, 17 (28.8%), 57 (62.6%), 46 (44.2%), 48 (44.0%), and 75 (41.9%) cases were identified by fever screening at airports from a total of 59, 91, 104, 109, and 179 imported cases for 2003, 2004, 2005, 2006, and 2007, respectively (Table 2).

<table>
<thead>
<tr>
<th>Country origin</th>
<th>Vietnam</th>
<th>Indonesia</th>
<th>The Philippines</th>
<th>Thailand</th>
<th>Cambodia</th>
<th>Malaysia</th>
<th>Myanmar</th>
<th>Singapore</th>
<th>China</th>
<th>India</th>
<th>Bangladesh</th>
<th>Solomon Islands</th>
<th>Sri Lanka</th>
<th>Laos</th>
<th>Sri Lanka</th>
<th>Madagascar</th>
<th>El Salvador</th>
<th>Belize</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case number</td>
<td>9</td>
<td>15</td>
<td>12</td>
<td>11</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>542</td>
<td>133</td>
<td>74</td>
<td>31</td>
<td>16</td>
<td>28</td>
<td>46</td>
<td>25</td>
<td>11</td>
<td>19</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

The main purposes for travel were associated with family visit of new immigrants (31.9%), business trip (26.0%), and tourism (25.3%) during 2005–2007 (Table 3). With the increasing trend of imported dengue cases, we also witnessed larger dengue outbreaks in Taiwan resulting in 965 and 2,000 indigenous dengue cases in 2006 and 2007, respectively (Table 2).

Serotype distributions of imported DENV strains during 2003–2007. From the 542 imported dengue cases, 288 DENV strains were isolated from the acute-phase serum samples of patients infected in 13 countries (Table 4). The serotype distributions of these strains imported in each year from the seven most frequent importing countries during 2003–2007 are summarized in Figure 1. The results showed that the main DENV serotype identified in imported cases from Vietnam had shifted from DENV-2 during 2004–2006 to DENV-1 in 2007. In the Philippines, the main serotypes had shifted from DENV-1 and DENV-4 during 2003–2005 to DENV-2 and DENV-3 during 2006–2007. In Thailand, the significant DENV-4 serotype was observed during 2004–2007, especially in 2005, which was not observed in other countries. These findings provided interesting insight regarding the turnover of circulating DENV serotypes in these countries.

Molecular characterization of imported DENV strains. Phylogenetic analyses of the E gene sequences of DENV strains isolated from imported cases were conducted to determine the genetic relationship of these viruses. Table 4 summarized the serotype and genotype distributions of 288 DENV isolates in each of these countries during 2003–2007.
The designations of DENV genotypes are based on the classification of A-Nuegoonpipat and others, Twiddy and others, Lanciotti and others, and Klunthong and others for DENV-1, DENV-2, DENV-3, and DENV-4, respectively. The phylogenetic trees of the representative E gene sequences covering the full range of genetic diversity of 288 DENV strains observed in the original trees (data not shown) are shown in Figures 2–5. The data contained 29 DENV-1, 27 DENV-2, 27 DENV-3, and 17 DENV-4 sequences of Taiwan isolates together with 12 DENV-1, 7 DENV-2, 4 DENV-3, and 4 DENV-4 global reference sequences of different genotypes available from GenBank.

Phylogenetic analysis of DENV-1 isolates. DENV-1 viruses isolated from imported dengue cases showed that most of the viruses belong to genotypes I (62 strains) and II (31 strains) (Table 4; Figure 2). Genotype I contains viruses isolated from cases imported from Vietnam, Thailand, Indonesia, Malaysia, Cambodia, Myanmar, and Singapore. Genotype II comprises viruses from Indonesia, the Philippines, Malaysia, Vietnam, and Madagascar. The Madagascar strain was closely related to the strain from Reunion. Genotype III contains two isolates, one imported from India and another from El Salvador, which clustered with strains from Central America.

Phylogenetic analysis of DENV-2 isolates. DENV-2 viruses isolated from imported cases fell into four genotypes (Table 4; Figure 3). Asian genotype 1 (43 strains) contains viruses from Vietnam, Thailand, Myanmar, and Cambodia. Asian genotype 2 contains a virus strain isolated in 2003 from the Philippines. Asian/American genotype (six strains) comprises viruses from Vietnam and Cambodia. The cosmopolitan genotype (46 strains) comprises viruses from Indonesia, the Philippines, Malaysia, Singapore, India, Bangladesh, and Vietnam.

Phylogenetic analysis of DENV-3 isolates. DENV-3 viruses isolated from imported cases fell into two genotypes (Table 4; Figure 4). Genotype I (36 strains) contains viruses from Indonesia, the Philippines, Malaysia, and Singapore. Genotype II (28 strains) comprises viruses from Vietnam, Myanmar, Cambodia, Bangladesh, and Thailand.

Phylogenetic analysis of DENV-4 isolates. DENV-4 is the least frequently sampled serotype, and the isolates can be grouped into two genotypes (Table 4; Figure 5). Genotype I (25 strains) contains viruses from Thailand, the Philippines, Vietnam, and Cambodia. Genotype II (eight strains) comprises viruses mainly from Indonesia but also contain two strains, one from the Philippines and the other from the Solomon Islands, which are closely related to strains from Indonesia.

DISCUSSION

Through increased international trade and tourism, the number of annually imported cases has increased dramatically worldwide. In Taiwan, increasing numbers of imported dengue cases were identified through laboratory-based dengue surveillance during 2003–2007. The results provide us with an excellent opportunity to isolate a wide variety of DENV strains circulating globally, especially in Southeast Asia countries, because most (> 95%) of the imported patients detected by fever screening at airports are in their viremic stages with positive real-time RT-PCR and negative IgM and IgG results. Among these imported cases, 74% cases were identified on days 1–3 after onset of illness. In contrast, the imported cases reported from passive (hospital) surveillance systems were evenly distributed 1–20 days after the onset of illness. During 2003–2007, a total of 288 DENV strains introduced from 13 countries were successfully isolated, and their sequence database containing full-length structural genes was generated. Admittedly, these DENV strains we collected may only represent the most dominant epidemic strains circulated in the countries of origin. However, similar strains clustered in the same clade of a specific genotype were often repeatedly isolated from the same country each year during 2003–2007 (Figures 2–5), strongly suggesting that majority of these epidemic strains remains stable in their geographic distribution.

Analysis of the countries of origin and the purposes for travel of the imported cases shows some interesting links. As
we previously reported, the distribution of the countries of origin accurately reflected the frequency of air travel between Taiwan and these nations, as well as the intensity of massive dengue outbreaks during the same period in the country of origin. During 2005–2007, the top five importing countries in Taiwan are also those countries having most of Taiwan's new immigrants (foreign spouses). Therefore, increasing family visit of new immigrants (visits by the Taiwanese family to the home of their new relatives in various countries) together with international trade and tourism between Taiwan and these endemic countries leads to increased imported dengue cases.

Ito and others recently reported the isolation and phylogenetic analysis of DENV strains from imported cases who were infected while traveling in endemic areas. When comparing the genomes of imported DENV strains with those already deposited in the GenBank database, accordant results were obtained with respect of the countries of origin. The study indicates that phylogenetic analysis of isolated DENV is a unique technique to determine the countries where infection occurred. Similar findings were observed in our study. Our results provide an updated view on the geographic distribution and dynamic transmission of epidemic DENV strains circulated in Southeast Asian countries during 2003–2007. Further analyses showed that the genotype distribution of epidemic DENV strains in these countries can be divided into two geographic regions. The northern region contains countries including Vietnam, Thailand, Cambodia, and Myanmar, and the southern region contains countries including Indonesia, the Philippines, Malaysia, and Singapore. The DENV strains circulated in each of these two regions usually locate in closely related clades in the same genotypes, suggesting close genetic relationship and frequent flow of viruses in these countries (Table 4; Figures 2–5). However, discordant genotype distributions were observed, such as DENV-1 genotype I strains in Malaysia and Singapore and DENV-4 genotype I strains in the Philippines, suggesting multiple introductions and expansions of epidemic strains resulting genotype co-circulation or shift in some of these countries (Table 4).
Previous studies on molecular evolution of DENVs provided supporting evidence on genotype shift and local evolution. Zhang and others reported molecular evolution of DENVs in Thailand showing clade replacement in DENV serotypes 1 and 3 are associated with changing serotype prevalence. Phylogenetic analyses showed that genotype replacement occurred in DENV-1 during the early 1980s, in which the genotype III viruses were replaced by those assigned to genotype I. Furthermore, the Thai strains in the 1980–1994 clade within genotype I of DENV-1 co-circulated with the 1990–2002 clade during 1990–1994 and then dropped dramatically and was largely replaced by the 1990–2002 clades. Our data agreed with their conclusion in that all of the 11 DENV-1 strains isolated in Taiwan imported from Thailand during 2003–2007 belong to genotype I clustered together with a 1995 Thailand strain (ThD1 0438 95) (Table 4; Figure 2). Similar finding was observed in DENV-3, in which an old clade in genotype I was replaced during 1990s by a new 1991–2002 clade in genotype II (Table 4; Figure 4). These findings showed the dynamic change of clade and genotype shifts caused by constant introduction and local evolution of epidemic DENV strains in new areas.

Overall, our results showed that geographic distribution of strains and genotypes of DENV-3 isolated from Southeast Asian countries remain unchanged during 2003–2007. However, the movement and new establishment of DENV-1, DENV-2, and DENV-4 strains were observed in certain areas of Asia. Therefore, although the majority of DENV-4 strains isolated from the Philippines belonged to genotype I, a strain (0409aTw) clustered in genotype II with Indonesia and Malaysia strains were observed (Figure 5). In addition, DENV-1 isolates from Indonesia and Malaysia were found to be shifting from genotype II to genotype I (Figure 2), whereas in the Philippines, DENV-2 isolates were found to be shifted from the Asian genotype 2 to the Cosmopolitan genotype during the study period agreed with a recent report (Figure 3). Our data also showed interesting findings in that DENV-1 and DENV-2 strains imported from Vietnam were dispersed at a variety of phylogenetic locations clustered with strains from Cambodia, Indonesia, and Thailand. The results suggest extensive introduction and expansion of these DENV strains from these countries to Vietnam. These findings showed that a comprehensive database containing sequences of currently epidemic strains is needed to correctly map the geographic distribution of DENV strains worldwide.

Our results witness the growing global threat of dengue in a new era with unparalleled human movement. The situation is expected to worsen in the future before an effective dengue vaccine is developed. This worrisome trend demands us to develop more effective surveillance system and control measures. The DENV strains isolated from these imported dengue cases and the establishment of a DENV genomic database may provide essential information of global expansion and genetic evolution useful for disease surveillance, laboratory diagnosis, pathogenesis investigation, and vaccine development.
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Figure 4. A phylogenetic trees of dengue virus type 3 (DENV-3). The phylogenetic trees is based on the complete E gene sequences of 31 strains of DENV-3 including 27 Taiwan isolates from imported cases. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation. GenBank accession numbers are shown in the parentheses.

Figure 5. A phylogenetic trees of dengue virus type 4 (DENV-4). The phylogenetic trees is based on the complete E gene sequences of 21 strains of DENV-4 including 17 Taiwan isolates from imported cases. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation. GenBank accession numbers are shown in the parentheses.
REFERENCES


