Molecular Epidemiology of Dengue Viruses Co-circulating in Upper Myanmar in 2006

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Abstract: To understand the molecular epidemiology of circulating dengue viruses (DENV) in Upper Myanmar, DENV isolation was attempted by inoculating the sera of a panel of 110 serum samples onto a C6/36 mosquito cell line. The samples were collected from dengue (DEN) patients admitted at Mandalay Children’s Hospital in 2006. Infected culture fluids were subjected to a RT-PCR to detect the DENV genome. Three DENV strains were isolated. This was the first DENV isolation performed either in Mandalay or in Upper Myanmar. One strain belonged to DENV serotype-3 (DENV-3), and two other strains belonged to DENV serotype-4 (DEN-4). The sequence data for the envelope gene of these strains were used in a phylogenetic comparison of DENV-3 and DENV-4 from various countries. Phylogenetic analyses revealed that this DENV-3 strain was clustered within genotype II, and the two DENV-4 strains were clustered within genotype I in each serotype. The Myanmar strains were closely related to strains from the neighboring countries of Thailand and Bangladesh. These results are important for elucidating the trends of recent and future DEN outbreaks in Myanmar.

Key words: Dengue virus, Molecular epidemiology, Upper Myanmar

INTRODUCTION

The dengue virus (DENV) belongs to the genus Flavivirus of the family Flaviviridae, which exists as four serotypes (DENV-1,-2,-3, and -4) [1]. DENV infection is the most important of the mosquito-borne viral diseases, and it affects mainly tropical and subtropical countries [2]. It is well documented that all four serotypes of DENV co-circulate in Asian countries including Myanmar [3, 4]. The first major epidemic of dengue hemorrhagic fever (DHF) occurred in Myanmar in 1970 [5]. Currently, DHF occurs throughout the country, with the notable exception of the Chin State. Almost 80% of cases are reported from three divisions (Yangon, Bago and Mandalay) and one state (Mon), with more than 50% of cases recorded exclusively from the Yangon Division [5]. DEN outbreaks have been recorded in Upper Myanmar, especially in Mandalay, the largest city in the region. However, an extensive study has never been accomplished due to insufficient laboratory facilities. The present study focused on highlighting current DENV infections in Upper Myanmar with a special emphasis on molecular epidemiology.

METHODS

Patients

In total, 110 serum samples were obtained from 110 patients (≤ 12 years old) who were clinically suspected for DEN according to World Health Organization [6] criteria and who were admitted to the 550-bed Mandalay Children’s Hospital (MCH), Mandalay City, Upper Myanmar in 2006 with the informed consent of parents or legal guardians.

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guardians. The study protocol was reviewed and approved by the Ethical Committee on Medical Research Involving Human Subjects, Department of Medical Research (Upper Myanmar), Pyin Oo Lwin, Myanmar. The sera were stored at −70°C until further use.

Methods

Both IgM- and IgG-capture ELISAs were performed using Dengue Duo IgM-capture and IgG-capture ELISA Kits (PANBIO, Brisbane, Australia) to determine primary and secondary DENV infections. All the commercial kits in the present study were used following the manufacturer’s instructions.

The frozen sera were transferred to Japan, and the virus culture was conducted in the Department of Virology, Institute of Tropical Medicine, Nagasaki University, Japan. Each serum sample was inoculated onto *Aedes albopictus* clone C6/36 mosquito cells and incubated at 28°C for 7 days [7]. The presence of DENV in the infected culture fluid (ICF) was verified by in-house Flavivirus antigen detection ELISA (Ag-ELISA) [8] and RT-PCR. RNA extraction from ICF was performed using a viral RNA Mini Kit (QIAGEN, Hilden, Germany). DENV serotyping was done using 4 sets of serotype-specific primers [9–11] employing the PrimeScript™ One Step RT-PCR Kit (Takara Bio Inc., Shiga, Japan).

The desired DNA bands were excised from the agarose gel, and were extracted and purified using QIAEX® II Gel Extraction Kit (QIAGEN, Hilden, Germany). The primer extension dideoxy chain termination method was used for direct sequencing of the PCR product. DNA sequencing analysis was performed with BigDye® Terminator version 3.1 Cycle Sequencing Ready Reaction Mixture (Applied Biosystems, Foster City, USA) following the thermal cycle sequencing parameters described previously [12]. The reaction mixture was then purified using an AGENCOURT® CLEANSEQ® Sequencing Reaction Clean-up system (Agencourt Bioscience Corp., Massachusetts, USA). The final product was loaded on an ABI Prism™ Capillary Sequencer 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, USA). Nucleotide sequences were edited and homology searches and comparisons of the sequences done using DNASIS (Mac version 3.6 Software system; Hitachi, Tokyo, Japan). Nucleotide sequence alignments were carried out using CLUSTAL X, version 2.0 [13], and the phylogenetic analysis was performed using either the heuristic or the branch and bound algorithm of PAUP version 4.0b10 (Altivec) software [14]. The neighbor-joining method was used to construct the phylogenetic tree with a bootstrap analysis of 1,000 replicates [15].

The Genbank accession numbers, EU478408, EU478409 and EU478410, for the three Myanmar isolates used in the present study, and the accession numbers of all the other strains used for the phylogenetic analysis, are listed in Tables 1 and 2 with the geographic origin and the year of isolation.

**RESULTS**

Among 110 clinically diagnosed DEN patients, 70 (64%) were positive for both IgM and IgG by DEN IgM capture and DEN IgG capture ELISA and were confirmed as secondary DENV infections. Primary DENV infection was confirmed in 26 (24%) patients who were positive only for IgM. The remaining 14 (13%) patients were not confirmed to have DENV infections. Among the 96 dengue-confirmed patients, dengue virus strains were successfully

| Parameters          | Confirmed dengue cases* (%) | Non dengue cases* (%) |
|---------------------|----------------------------|----------------------|
| number of cases     | 96 (87)                    | 14 (13)              |
| Mean age in years (± SD) | 5.5 (± 3.2)                | 5.6 (± 4.0)          |
| Male/Female         | 49/47                      | 6/8                  |
| DHF I               | 26 (27)                    | 5 (36)               |
| DHF II              | 32 (33)                    | 5 (36)               |
| DHF III             | 21 (22)                    | 2 (14)               |
| DHF IV              | 1 (1)                      | 0 (0)                |
| DSS                 | 16 (17)                    | 2 (14)               |

Confirmed dengue cases were positive for dengue IgM capture ELISA. Non-dengue cases were negative for dengue IgM capture ELISA.

DHF I, dengue hemorrhagic fever grade I; DHF II, dengue hemorrhagic fever grade II; DHF III, dengue hemorrhagic fever grade III; DHF IV, dengue hemorrhagic fever grade IV; DSS, dengue shock syndrome. DHF grading were classified according to WHO criteria (WHO, 1997).
Table 2. DENV-3 strains used for phylogenetic analysis

| Strain          | Geographic origin | Year of isolation | Accession number |
|-----------------|-------------------|-------------------|------------------|
| BDH 02-01       | Bangladesh        | 2002              | AY 496871        |
| BDH 02-07       | Bangladesh        | 2002              | AY 496871        |
| 114             | Bangladesh        | 2000              | AY656669         |
| 165             | Bangladesh        | 2000              | AY656671         |
| 058             | Bangladesh        | 2000              | AY656674         |
| Jacob           | Bangladesh        | 2001              | AY656673         |
| 68784           | Brazil            | 2000              | AY038805         |
| 80-2            | China             | 1980              | AF317645         |
| Cuba-21/02      | Cuba              | 2002              | AY702031         |
| 29472           | Fiji              | 1992              | L11422           |
| 1416            | India             | 1984              | L11424           |
| 228761          | Indonesia         | 1973              | L11425           |
| 1280            | Indonesia         | 1978              | L11426           |
| 85-159          | Indonesia         | 1985              | L11428           |
| 1306            | Malaysia          | 1974              | L11429           |
| 29586           | Malaysia          | 1981              | L11427           |
| LN 5547         | Malaysia          | 1992              | AF147457         |
| LN 1746         | Malaysia          | 1993              | AF147458         |
| LN 6083         | Malaysia          | 1994              | AF147460         |
| D3/H/HTMSSA-MART/2001/2012 | Martinique     | 2001              | AY099930         |
| MEX0697         | Mexico            | 1995              | AY146763         |
| 1559            | Mozambique        | 1985              | L11430           |
| 31985 KLA       | Myanmar           | 1998              | AY145712         |
| DV3/Myanmar/0508aTw/2005 | Myanmar      | 2005              | DQ518666         |
| DV3/Mandalay.MYA/H58/2006* | Malaysia    | 2006              | EU478409         |
| 24/94           | Nicaragua         | 1994              | AY702033         |
| D3 PY/A59/03    | Paraguay          | 2003              | DQ 118885        |
| H 87            | Philippines       | 1956              | L 11423          |
| 168/AP-2        | Philippines       | 1983              | L11432           |
| PMH-I1-97       | Philippines       | 1997              | AY496879         |
| PR6             | Puerto Rico       | 1963              | L11433           |
| 1340            | Puerto Rico       | 1977              | L11434           |
| 1696            | Samoa             | 1986              | L11435           |
| 1326            | Sri Lanka         | 1981              | L11431           |
| 1594            | Sri Lanka         | 1985              | L11436           |
| 260698          | Sri Lanka         | 1989              | L11437           |
| 27833           | Sri Lanka         | 1991              | L11438           |
| D3/Sri Lanka 9912aTw/1999 | Sri Lanka    | 1999              | DQ 518679        |
| 2167            | Tahiti            | 1989              | L11619           |
| D3/Taiwan/813KH9408a/1994 | Taiwan     | 1994              | DQ 518667        |
| D3/Taiwan/701TN9811a/1998 | Taiwan       | 1998              | DQ 518662        |
| D3/Taiwan/807KH0509a/2005 | Taiwan   | 2005              | DQ 518659        |
| 5987            | Thailand          | 1962              | L11440           |
| CH3489D73-1     | Thailand          | 1973              | L11620           |
| D86-007         | Thailand          | 1986              | L11441           |
| MK315           | Thailand          | 1987              | L11442           |
| D88-303         | Thailand          | 1988              | AY145714         |
| D89-273         | Thailand          | 1989              | AY145715         |
| D91-393         | Thailand          | 1991              | AY145716         |
| D92-431         | Thailand          | 1992              | AY145719         |
| D93-044         | Thailand          | 1992              | AY145718         |
| D94-283         | Thailand          | 1993              | AY145720         |
| D95-0400        | Thailand          | 1994              | AY145723         |
| D 96-313        | Thailand          | 1995              | AY145725         |
| D 97-0291       | Thailand          | 1997              | AY145730         |
| 00-27-1 Hu NIID | NIID              | 2000              | AB111080         |
| LARD 5990       | Venezuela         | 2000              | AY46764          |
| LARD 6668       | Venezuela         | 2001              | AY46774          |
| D3/Vietnam/9609aTw/1996 | Vietnam      | 1996              | DQ518655         |
| D3/Vietnam/0409aTw/2004 | Vietnam    | 2004              | DQ518656         |
| D3/Vietnam/0507aTw/2005 | Vietnam    | 2005              | DQ518658         |

* New strain from Myanmar presented in this study
isolated from three patients whose sera were collected within 7 days from the onset of fever. One isolate was DENV-3 from a patient having a primary DENV infection with DHF grade (I), and two isolates were DENV-4 from a patient having primary DENV infection with DSS. The other patient had secondary DENV infection with DHF grade (I). The clinical information of the 110 patients is shown in Table 1.

The nucleotide sequence of the E gene of the newly isolated DENV-3 strain from Upper Myanmar, designated as the DV3/Mandalay.MYA/H58/2006 strain (Myan 06, code in tree), was compared with other published sequences of 61 DENV-3 strains originating from various geographic regions (Table 2). The phylogenetic tree constructed for the 62 DENV-3 strains, employing the DENV-2 New Guinea C strain as an out-group strain, is shown in Fig. 1. The tree reveals that the newly isolated DENV-3 strain from Upper Myanmar was grouped together with previously published strains from Lower Myanmar, as well as the strains from Thailand, Bangladesh, Malaysia, Vietnam and Taiwan in a well-defined genotype II.

Similarly, the nucleotide sequences of the E gene of two newly isolated DENV-4 strains from Upper Myanmar, designated as the DV4/Sagaing.MYA/H27/2006 strain (Myan 06 Sgg, code in tree) and the DV4/Mandalay.MYA/H64/2006 strain (Myan 06 Mdy, code in tree), were compared with other published sequences of 59 DENV-4 strains originating from various geographic regions (Table 3). The phylogenetic tree constructed for a total of 61 DENV-4 strains is shown in Fig. 2. The tree reveals that the two new strains from Upper Myanmar were grouped together with those from Thailand, Cambodia, Malaysia, India, Sri Lanka, China and the Philippines in the Asian genotype I.

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**Fig. 1.** Phylogenetic tree of DENV-3 strains (n = 62). The tree is rooted by the DENV-2-NGC (New Guinea C, Accession No. M29095) strain (Table 2). All horizontal branch lengths are drawn to a scale of nucleotide substitutions per site. Bootstrap support values are shown and the genotypes of DENV-3 are indicated. For simplicity, each strain name was replaced by a code that consists of the country and the year of isolation. The DENV-3 isolate presented in the present study is indicated by an asterisk (*).
| Strain            | Code in tree | Geographic origin | Year of isolation | Accession number |
|------------------|--------------|-------------------|-------------------|------------------|
| Bahamas A/98     | Bahamas A    | Bahamas           | 1998              | AY 152364        |
| Barbados B/93    | Barbados B/93| Barbados          | 1993              | AY 152376        |
| Barbados/99      | Barbados     | Barbados          | 1999              | AY 152368        |
| 1385/82          | Brazil       | Brazil            | 1982              | U 18425          |
| 02-21-1 Hu NIID  | Cambodia 02  | Cambodia          | 2002              | AB 111089        |
| China.GuangzhBoB5| China B      | China             | NA                | AF 289029        |
| 814669/81        | Dominica     | Dominica          | 1981              | AF 326573        |
| M 44/81          | Dominica M44 | Dominica          | 1981              | AY 152360        |
| 1411/83          | El Salva     | El Salvador       | 1983              | U 18426          |
| BC 6494/94       | El Salvador  | El Salvador       | 1994              | U 18427          |
| Hondurars/91     | Honduras     | Honduras          | 1991              | AY 152379        |
| 96-33-1 Hu NIID  | India        | India             | 1996              | AB 111086        |
| 30153/73         | Indonesia    | Indonesia         | 1973              | U 18428          |
| 1036/76          | Indonesia    | Indonesia         | 1976              | U 18429          |
| 1122/77          | Indonesia    | Indonesia         | 1977              | U 18430          |
| 02-12-1 Hu NIID  | Indonesia    | Indonesia         | 2002              | AB 111088        |
| Jamaica/81       | Jamaica      | Jamaica           | 1981              | AY 152389        |
| Jamaica/83       | Jamaica      | Jamaica           | 1983              | AY 152384        |
| P7-1006          | Malaysia     | Malaysia          | 1969              | AF 231722        |
| P73-1120         | Malaysia     | Malaysia          | 1973              | AF 231724        |
| P75-514          | Malaysia     | Malaysia          | 1975              | AF 231723        |
| MY 01-23096      | Malaya       | Malaya            | 2001              | AJ 428557        |
| 1492/84          | Mexico       | Mexico            | 1984              | U 18431          |
| Mexico/91        | Mexico       | Mexico            | 1991              | AY 152378        |
| Montserrat-A/94  | Montserrat   | Montserrat        | 1994              | AY 152369        |
| DV4/Sagai/MYA/H27/2006* | Myan 06 Sgg    | Myanmar          | 2006              | EU 478410        |
| DV4/Mandalay/MYA/H64/2006* | Myan 06 Mdy    | Myanmar          | 2006              | EU 478408        |
| 5489/84          | New Caledonia| New Caledonia     | 1984              | U 18432          |
| H241/56          | Philip       | Philipines        | 1956              | U 18433          |
| 12123/84         | Philip       | Philipines        | 1964              | U 18434          |
| M5/82            | Puerto Rico  | Puerto Rico       | 1982              | AY 152336        |
| M32/85           | Puerto Rico  | Puerto Rico       | 1985              | AY 152856        |
| M33/85           | Puerto Rico  | Puerto Rico       | 1985              | AY 152857        |
| 1650/86          | Puerto Rico  | Puerto Rico       | 1986              | U 18436          |
| 69/87            | Puerto Rico  | Puerto Rico       | 1987              | AY 152252        |
| 96/90            | Puerto Rico  | Puerto Rico       | 1990              | AY 152855        |
| 28/92            | Puerto Rico  | Puerto Rico       | 1992              | AY 152196        |
| 84/94            | Puerto Rico  | Puerto Rico       | 1994              | AY 152084        |
| 17/98            | Puerto Rico  | Puerto Rico       | 1998              | AY 152056        |
| S-44750/78       | Sri Lanka    | Sri Lanka         | 1978              | U 18437          |
| B/82             | Surinam      | Surinam           | 1982              | AY 152386        |
| A-94             | Surinam      | Surinam           | 1994              | AY 152372        |
| S-44754/79       | Tahiti       | Tahiti            | 1979              | U 18438          |
| 114-094-85/85    | Tahiti       | Tahiti            | 1985              | U 18439          |
| TC 2443/63       | Thai         | Thailand          | 1963              | U 18440          |
| Thai D4-0087/77  | Thailand     | Thailand          | 1977              | AY 618999        |
| Thai D4-0348/91  | Thailand     | Thailand          | 1991              | AY 618990        |
| Thai D4-0439/94  | Thailand     | Thailand          | 1994              | AF 231726        |
| Thai D4-0476/97  | Thailand     | Thailand          | 1997              | AY 618978        |
| Thai D4-0734/00  | Thailand     | Thailand          | 1999              | AY 618979        |
| Thai D4-0759/00  | Thailand     | Thailand          | 2000              | AY 618993        |
| Thai D4-0459/01  | Thailand     | Thailand          | 2001              | AY 618938        |
| Thai D4-0485/01  | Thailand     | Thailand          | 2001              | AY 618940        |
| Thai D4-0352/02  | Thailand     | Thailand          | 2002              | AY 618985        |
| Trinidad A/82    | Trinidad     | Trinidad          | 1982              | AY 152382        |
| Trinidad A/84    | Trinidad     | Trinidad          | 1984              | AY 152380        |
| Trinidad/94      | Trinidad     | Trinidad          | 1994              | AY 152377        |

* New strains from Myanmar presented in this study
The DENV-3 isolate from Upper Myanmar in the present study belonged to genotype II, like two previously published Lower Myanmar strains: the 31985 KLA strain (Myan 98, code in tree) and the DV3/Myanmar/0508aTw/2005 strain (Myan 05, code in tree). It clustered together with strains from Bangladesh in a well-defined sub-cluster. Further support came from the fact that three unique aminoacid (aa) changes, I140T, S447G and A489T, were found in this strain and were shared by Myan 05 and the Bangladesh strains. To examine the introduction of the DENV-3 genotype II to the country (although DENV-3 isolates from Myanmar are very few), we compared an older strain, Myan 98, to the two most recent ones: Myan 05 and Myan 06 [16]. In the phylogenetic tree, Myan 98 was clustered in a separate sub-cluster of genotype II together with earlier Thai strains. This clustering is supported by four aa changes, I140T, S447G, A489T and A479V, which are present in the two most recent Myanmar isolates, Myan 05 and Myan 06, but are not present in either the Myan 98 strain or in the Thai isolates that were clustered together with the latter strain. These results indicate that the genotype II of DENV-3 reached Myanmar most likely through independent entries from Thailand, a supposition supported by the appearance of the more recent lineage including the isolates from 2005 and 2006 (Fig. 1). The fact that the Bangladesh strains isolated from 2000 to 2002 showed little evidence of independent evolution suggests that genotype II was also introduced recently from neighboring countries. Our results support Podder et al.’s (2006) suggestion that recent DEN outbreaks in Indonesia in 2002 may have entered from neighboring countries [12]. Recently, it was reported that seven DENV-3 strains isolated from Yangon (Lower Myanmar) in 2007 belonged to genotype III [18]. Therefore, it ap-
pears that more than one DENV-3 genotype is circulating in the country. It would be interesting to analyze the time and route of introduction.

The two newly isolated DENV-4 strains from Upper Myanmar in the present study were clustered together with other Asian strains in genotype I being the closest related strains from Thailand and Cambodia [9], but V238M and L489P aa changes were unique to Myan 06 Mdy and Myan 06 Sgg strains, respectively. Although the existence of DENV-1 and DENV-2 among the circulating viruses in Upper Myanmar could not be ruled out, the present study demonstrated that DENV-3 and DENV-4 were co-circulating in the area in 2006. This is the first report on the molecular analysis of DENV-4 strains in Myanmar, particularly those circulating in the upper part of the country. Therefore, if DENV-3 is currently regarded as the prevailing serotype for recent outbreaks, then DENV-4 might be in the pipeline to take the lead in future outbreaks in Myanmar.

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POTENTIAL CONFLICT OF INTEREST

We declare no conflicts of interest.

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