Double Dengue Serotypes in Asymptomatic Populations Living in An Area of Thailand Endemic for Dengue Hemorrhagic Fever

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Abstract

Dengue virus infection remains a major health problem worldwide. Understanding dengue infection and characterizing circulating viruses are essential for disease prevention and control as well as vaccine development. In this study, we aimed to identify dengue virus in healthy people living in an area endemic for dengue disease. Blood samples were collected from 52 healthy local subjects living in a dengue-endemic area of Thailand. Viral RNA was detected using a nested reverse transcription polymerase chain (RT-PCR) that amplified the E gene. Phylogenetic trees were constructed by the neighbor-joining method using MEGA6.06. Dengue virus was detected in 5 of 52 samples (9.62%). Double dengue virus infection (Den 1&4 and Den 3&4) was found in two samples. Sequence analysis of the viruses showed that dengue serotype 1 belonged to sylvatic genotype. A mix of genotype I and II was found in a dengue serotype 3 sample while all dengue virus serotype 4 in this study belonged to genotype II. These preliminary results may provide better understanding of dengue infection and viral transmission between populations and mosquitoes. Therefore, it could be used for epidemiological studies and control of dengue hemorrhagic fever in the future.

Keywords: Aedes aegypti, asymptomatic infection, double dengue virus infection, endemic, phylogenetic tree

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**Introduction**

Dengue viruses (DENV-1 to DENV-4) are transmitted to human hosts through bites of infected female *Aedes aegypti* and *Ae. albopictus* mosquitoes (Thavara et al., 2006). The infection can cause dengue fever (DF), dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS) (WHO, 2009). The disease is problematic worldwide, especially in tropical and subtropical areas. Of 390 million infected patients reported annually, 50-100 million dengue cases (10-15%) are symptomatic, approximately 500,000 develop DHF (Guzman et al., 2010), and up to 300 million are asymptomatic or mild cases (Bhatt et al., 2013). However, the relationship between asymptomatic and symptomatic infections and the role asymptomatic infection play in disease transmission remain unknown.

Thailand often experiences dengue outbreaks and epidemiological data report high annual levels of DHF and DSS. Based on historical dengue incidence data during 2007 and 2012 obtained from the Department of Disease Control in Thailand’s Ministry of Public Health, provinces in the central region such as Samut Sakhon are at high risk of dengue outbreaks (Saungtho et al., 2013). According to cumulative provincial surveillance data, the 2012 incidence rate of DHF in Samut Sakhon was 252.26/100,000, making it a severe epidemic year for dengue disease (90th percentile) (Thavara et al., 2006; and Chompoosri et al., 2012). Several factors such as environmental conditions and human migration, particularly of foreign workers, contribute to increased dengue transmission (WHO, 2009). A previous study identified dengue infection by detecting immunoglobulin M and G (IgM and IgG, respectively) antibodies and an IgG and IgM immune response in adult residents of endemic areas (Barde et al., 2012). Various sample types, including white blood cells (Wang et al., 2000), platelets (Noisakran et al., 2009), saliva and urine (Ma et al., 2014), can be used to test for infection. As mentioned previously, the role of asymptomatic dengue infection in disease transmission and severity have not been fully evaluated; therefore, this study tested for presence of the virus in local adult volunteers with no reported dengue signs, symptoms, or diagnoses. These findings could provide a better understanding of dengue pathogenesis and the relationship between the virus and its hosts in the mosquito-human-mosquito cycle within endemic areas. This information could be of interest to epidemiological studies and inform development of effective dengue control measures.

**Materials and Methods**

**Sample collection:** Adults between 20-60 years of age living in Ban Phao district in Samut Sakhon province with no history of dengue infection in the previous year were enrolled in the study. Blood samples were collected from individuals who had no clinical signs of dengue for at least 1 year before the study. Enrolled subjects signed consent forms for their participation in the study. The study was approved by the Ethic Committee of Research Affairs Unit, Faculty of Medicine, Chulalongkorn University (COA No. 328/2014, IRB No. 182/2014). Three milliliters of K2EDTA-blood samples were collected from each subject. The blood samples were kept at 4°C and transferred to the laboratory on the same day. Plasma was separated by centrifugation at 5,000 rpm for 10 min at 4°C.

**RNA extraction:** The plasma samples were extracted for total RNA using an Invisorb® Spin Virus RNA Mini Kit (STRATEC Molecular GmbH, Birkenfeld, Germany) according to the manufacturer’s instructions. RNA quality and concentration were determined using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA). The extracted RNA samples were stored at -80°C until further processing.

**Amplification and sequencing of dengue virus envelope (E) gene fragment:** A region of the dengue virus E gene was targeted for amplification in a nested reverse transcription polymerase chain reaction (RT-PCR). The nested RT-PCR was performed using type-specific oligonucleotide primers described previously (Yenchitsomnanus et al., 1996). This technique was used to screen for dengue virus infection in the study samples. PCR was performed using One-Step RT-PCR Kit (Qiagen, Düsseldorf, Germany) and BIOTAQ™ PCR Kit (Qiagen, Düsseldorf, Germany), respectively, according to manufacturers’ directions. Amplification was carried out on a Mastercycler® pro thermalcycler (Eppendorf, New York, USA) for cDNA synthesis and nested PCR that produced 641 bp and 434 bp products, respectively. The nested PCR was performed with 1 µL of 100-fold dilution (with ddH2O) from the first reaction as template DNA. Samples positive for dengue in the nested reaction were then subjected to PCR with serotype-specific primers (Yenchitsomnanus et al., 1996) to determine virus serotypes. The template DNA for this PCR was obtained by diluting the first-round PCR products (641 bp) as described previously. The dengue-specific primer sets produced 504, 346, 198, and 143 bp amplicons for serotypes DENV 1-4, respectively. The following positive controls and template concentrations were used: Hawaii strain (DENV-1), 5 × 10^4 PFU/mL; 16681 strain (DENV-2), 4.75 × 10^5 PFU/mL; H87 strain (DENV-3), 2.75 × 10^6 PFU/mL; and 814609 strain (DENV-4), 2.5 × 10^6 PFU/mL.

The amplified PCR products were then ligated into pGEM®-T Easy Vector Systems (Promega, Wisconsin, USA) and plasmids transformed into *Escherichia coli* (DH5α). The plasmids were extracted using the Invisorb Spin Plasmid Mini Two kit (STRATEC Molecular GmbH, Birkenfeld, Germany) according to manufacturers’ directions. Plasmid concentration and purity were determined with a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA) before sequencing (ATI, Singapore) based on M13 primer.

Sequences were aligned using ClustalW (Bioedit v2.0.2). The aligned sequences matched, with the sequence histograms of the amplified region showing clear individual base peaks. The sequences were then compared with exiting reference nucleotides on the NCBI online webservice (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The FASTA
sequences were deposited in GenBank and assigned accession numbers. Descriptive statistics, including percentage match to reference dengue sequences, were then calculated.

**Phylogenetic tree construction:** A phylogenetic tree was constructed using partial dengue virus E gene sequences by the neighbor-joining (NJ) method using Kimura’s 2-parameter model and bootstrap with 1,000 replications in MEGA version 6.06. Sequences with GenBank accession numbers D00502 (DENV-1), JF967989 (DENV-2), JF968088 (DENV-3), and U18429 (DENV-4) were used as out-groups.

**Results**

A total of 52 subjects, including 15 males and 37 females between 26 and 60 years of age, enrolled in the study. Of the 52 samples, 5 (9.62%) were positive for dengue infection (Fig 1A). All dengue-positive samples were serotyped using techniques described previously. Samples S27 and S36 had double infection with DENV-1 (504 bp) and DENV-4 (143 bp), and DENV-3 (198 bp) and DENV-4 (143 bp), respectively (Fig 1B, Table 1). Single DENV-4 infection was found in samples S34, S43, and S51. The nucleotide sequences were deposited in GenBank with the following accession numbers: KM003963 (DENV-1) and KM003995-KM003998 and KM004003 (DENV-4). Since the DENV-3 nucleotide sequences were less than 200 bp in length, they were not accepted for deposit in the NCBI system.

For the analysis of DENV-1 and DENV-4 serotypes, evolutionary relationships based on the phylogenetic tree showed that the virus belonged to the sylvatic genotype (Fig 2) and genotype II (Fig 4), respectively. Interestingly, the DENV-3 tree showed that the virus belonged to genotypes I and II (Fig 3).

**Discussion**

Dengue viruses were detected in plasma of individuals based on the nested RT-PCR, which amplified part of the E gene, with reported high detection sensitivity of 0.1-1.0 PFU/mL (Yenchitsomanus et al., 1996). DENV-1, DENV-3, and DENV-4 infections with both single and double infections were identified from the plasma samples collected in the present study. To our knowledge, this is the first report of DENV-1 & 4 and DENV-3 & 4 co-infections in asymptomatic individuals in Thailand (Figure 1B, Table 1). Recently, a DENV-1 and 2 co-infection has been described in a Belgian resident returning from Thailand (Cnops et al., 2014). It is possible for humans to acquire dengue infections via multiple bites from vector mosquitoes carrying single or multiple viruses (Thavara et al., 2006). Additionally, co-infection probably depends on host immune status which affects viral replication (Steinhauer and
However, the subjects with co-infections in this study did not show clinical evidence of dengue infection. Some survey studies reported similar infection incidence rates between asymptomatic and symptomatic children in primary schools (Endy et al., 2002; Yoon et al., 2012). The reports also suggested that asymptomatic dengue infection could play an important role in dengue virus transmission. Longitudinal studies found symptomatic and asymptomatic dengue infection prevalence of 18 and 56 cases per 1,000 person-years, respectively, in adult residents of the endemic area of Bandung, in west Java, Indonesia (Porter et al., 2005). However, co-infection in patients with acute-phase illness during outbreaks was reported (Figueiredo et al., 2011; Colombo et al., 2013). Previous studies reported that the virus could be detected in white blood cell (Wang et al., 2000), platelets (Noisakran et al., 2009), saliva, and urine (Ma et al., 2014) samples collected from patients during both acute and convalescent phases. However, no studies have suggested that these viruses are attenuated or virulent strains. This question should be addressed in further research. Moreover, patients in the convalescence phase of the disease could test positive for dengue by RT-PCR due to detection of lingering antibodies to the virus (Figueiredo et al., 2011). We ensured that the results of nested RT-PCR were not false positive findings through the pre- and post-analytical testing, which was performed based on standard PCR criteria to minimize false negative findings (Kwok and Higuchi, 1989). The samples with multiple infections were tested in triplicate with re-extracted template material. Long-term storage could degrade RNA or the sample itself might contain virus concentrations lower than the threshold for detection. High sensitivity methods such as the nested RT-PCR could be useful for detecting the virus in these cases. Therefore, long-term asymptomatic infection may be a viral adaption or its way of escaping the host’s immune response.

Figure 2 A phylogenetic tree of DENV-1 based on partial E gene sequences. The tree was constructed using 26 DENV-1 sequences, including 25 references and one sequence from studied participants (black solid diamond).

It is possible that some local residents were infected with homologous or heterologous viruses via single or multiple bites from vector mosquitoes (Thavara et al., 2006). Evidence of more than one dengue virus strain was reported in mosquito vectors collected from Thailand (Thavara et al., 2006). These situations might lead to persistent infections that may develop into secondary infections when exposed to heterologous dengue infection (Bhatt et al., 2013).

The phylogenetic tree analysis revealed sylvatic genotypes. These genotypes have been circulating in the local populations and represent an attenuated strain (Rico-Hesse, 2003). The strain is part of an enzootic cycle and might be transmitted to local humans through a bite from an infected non-local mosquito (Wang et al., 2000). This genotype might also be introduced to the area via a local mosquito transmitting the virus from an infected ‘working monkey’ (used to pick coconuts) or foreign workers such as Burmese laborers who commonly work in the local industry. Although there is no evidence for sylvatic DENV emergence into human transmission, a newly arrived sylvatic DENV strain could be carried by non-human primates (NHP) and local competent
vectors and spread a new cycle of transmission (Hanley et al, 2013). However, Vasilakis and Weaver (2008) stated that the sylvatic DENV emergence was found in Asia and West Africa and might remain a potential source of re-emergence since the mosquito vector and NHP were not responsive to interventions. Two DENV-3 genotypes might represent viral diversification and contribute to virus survival (Wang et al, 2000; Lin et al, 2004).

**Figure 3** A phylogenetic tree of DENV-3 based on representative partial sequences of the dengue virus E gene. The tree was constructed with 36 DENV-3 sequences including 24 reference sequences and two DENV-3 sample sequences (black solid diamond and black solid circle), which belonged to genotype I and genotype II, respectively.

**Figure 4** A phylogenetic tree of DENV-4 based on partial E gene sequences. The tree was constructed using 24 DENV-4 sequences, including 19 reference sequences and five DENV-4 sample sequences (black solid diamonds).
In summary, we identified asymptomatic dengue virus infection with both single and co-infections in a highly endemic area of Thailand. The study of subclinical infections could provide better understanding of disease pathogenesis in the human host. Moreover, basic knowledge of co-infection and concurrent transmission in endemic areas are key factors for epidemiological design of effective control programs. Additionally, data on variations in viral sequences could be used to support future development of diagnostic and treatment approaches as well as vaccine design.

Table 1  Results of RT- and nested RT-PCR amplification of the dengue virus E gene amplification from plasma samples. The samples were collected from local asymptomatic populations without history of dengue infection during the study period.

<table>
<thead>
<tr>
<th>No. (Samples)</th>
<th>Sex</th>
<th>Age</th>
<th>E gene (Typing)</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (S27)</td>
<td>Female</td>
<td>59</td>
<td>DENV-1</td>
<td>KM003963</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DENV-4</td>
<td>KM003995</td>
</tr>
<tr>
<td>2 (S34)</td>
<td>Female</td>
<td>59</td>
<td>DENV-4</td>
<td>KM003996</td>
</tr>
<tr>
<td>3 (S36)</td>
<td>Female</td>
<td>60</td>
<td>DENV-3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DENV-4</td>
<td>KM003997</td>
</tr>
<tr>
<td>4 (S43)</td>
<td>Female</td>
<td>45</td>
<td>DENV-4</td>
<td>KM003998</td>
</tr>
<tr>
<td>5 (S51)</td>
<td>Female</td>
<td>27</td>
<td>DENV-4</td>
<td>KM004003</td>
</tr>
</tbody>
</table>

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References


บทคัดย่อ

การติดเชื้อไวรัสเดงกีร่วมในผู้ที่ไม่แสดงอาการของโรคที่อาศัยอยู่ในพื้นที่ระบาดของโรคไข้เลือดออกของประเทศไทย

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การติดเชื้อไวรัสเดงกียังถือเป็นปัญหาสาธารณสุขหลักที่สำคัญของโลก ความเข้าใจเกี่ยวกับการติดเชื้อและการระบุไวรัสที่ยังคงอยู่ในอนุสิ่งแวดล้อมก่อนที่จะเนื่องนั้นถือว่าเป็นความจำเป็นสำหรับการวางแผนควบคุมและป้องกันการติดเชื้อตลอดจนการพัฒนาวัคซีน สำหรับการศึกษาในครั้งนี้คณะผู้วิจัยมีจุดประสงค์ที่จะศึกษาการติดเชื้อไวรัสเดงกีในผู้ที่ไม่แสดงอาการที่อาศัยอยู่ในพื้นที่ระบาดของโรคจากอาสาสมัครทั้งหมด 52 คน โดยการตรวจการติดเชื้อมีางานเชื้อไวรัสด้วยการตรวจด้วยปฏิกิริยาลูกโซ่โพลีเมอร์เรสแบบ nested reverse transcription และศึกษาวิวัฒนาการของเชื้อไวรัสด้วยวิธี neighbor-joining โดยโปรแกรม MEGA6.06 จากการศึกษาพบว่าไวรัสจะมีการติดเชื้อ 5 คน คิดเป็นร้อยละ 9.62 และสามารถตรวจพบการติดเชื้อของสายพันธุ์ของเชื้อไวรัสซีโรทัยปี 1-4 และซีโรทัยปี 3-4 ในตัวอย่างเหล่านี้อาสาสมัครได้จากการวิเคราะห์ข้อมูลด้านการพัฒนาหรือการย้ายเชื้อไวรัสแสนสี่ศักย์เร่งรัดเชื้อไวรัสซีโรทัยปีหนึ่งเป็น sylvatic genotype มีการตรวจพบ genotype I และ genotype II ในตัวอย่างของไวรัสซีโรทัยปีที่สามจากการวิเคราะห์เชื้อไวรัสซีโรทัยปีสี่ของการศึกษาซึ่งอยู่ในกลุ่ม genotype II จากผลการศึกษาข้างต้นนี้อาจเป็นข้อสรุปว่าการติดเชื้อไวรัสเดงกีในผู้ที่ไม่แสดงอาการไวรัสระบาดทั่วไปในประเทศไทย และจากการศึกษาเชื้อไวรัสในกลุ่ม genotype II อาจนำไปสู่การตรวจพบการย้ายเชื้อไวรัสเดงกีที่มีการติดเชื้อไวรัสเดงกีในกลุ่ม genotype II นอกจากนี้กระบวนการควบคุมและป้องกันการระบาดของไวรัสเดงกีในประเทศไทยยังมีความจำเป็นที่จะต้องมีการศึกษาเพื่อให้มีการติดตามและควบคุมไวรัสที่มีการติดเชื้อในอนาคต

คำสำคัญ: ยุงลายบ้าน การติดเชื้อแบบไม่มีอาการแสดงของโรค การติดเชื้อไวรัสเดงกีร่วม โรคประจำาท้องถิ่น แผนภูมิวงศ์วานวิวัฒนาการ

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