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Genomic analysis and growth characteristic of dengue viruses from Makassar, Indonesia

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ABSTRACT

Dengue fever is currently the most important mosquito-borne viral disease in Indonesia. In South Sulawesi province, most regions report dengue cases including the capital city, Makassar. Currently, no information is available on the serotypes and genotypes of the viruses circulating in the area. To understand the dynamic of dengue disease in Makassar, we carried out dengue fever surveillance study during 2007–2010. A total of 455 patients were recruited, in which antigen and serological detection revealed the confirmed dengue cases in 43.3% of patients. Molecular detection confirmed the dengue cases in 27.7% of patients, demonstrating that dengue places a significant disease burden on the community. Serotyping revealed that dengue virus serotype 1 (DENV-1) was the most predominant serotype, followed by DENV-2, -3, and -4. To determine the molecular evolution of the viruses, we conducted whole-genome sequencing of 80 isolates. Phylogenetic analysis grouped DENV-2, -3 and -4 to the Cosmopolitan genotype, Genotype I and Genotype II, respectively. Intriguingly, each serotype paints a different picture of evolution and transmission. DENV-1 appears to be undergoing a clade replacement with Genotype IV being supplanted by Genotype I. The Cosmopolitan DENV-2 isolates were found to be regionally endemic and is frequently being exchanged between countries in the region. By contrast, DENV-3 and DENV-4 isolates were related to strains with a long history in Indonesia although the DENV-3 strains appear to have been following a distinct evolutionary path since approximately 1998. To assess whether the various DENV serotypes/genotypes possess different growth characteristics, we performed growth kinetic assays on selected viruses. We observed the relatively higher rate of replication for DENV-1 and -2 compared to DENV-3 and -4. Within the DENV-1, viruses from Genotype I grow faster than that of Genotype IV. This higher replication rate may underlie their ability to replace the circulation of Genotype IV in the community.

1. Introduction

Dengue fever is an acute febrile disease caused by dengue virus (DENV) infection, transmitted through the mosquito vector *Aedes aegypti*. The symptoms of the DENV infection vary from classic Dengue Fever (DF) to severe forms such as Dengue Hemorrhagic Fever (DHF) and the potentially fatal Dengue Shock Syndrome (DSS). DENV is an enveloped flavivirus with a 10.7 kb single-stranded positive-sense RNA genome. There are four antigenically distinct serotypes of the virus (DENV-1, -2, -3, and -4), with around 65% genome similarity, circulating throughout the tropical and sub-tropical regions of the world (Gubler, 1998; Guzman et al.,

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2010). The disease caused by the four serotypes is symptomatically identical.

Indonesia is a dengue endemic country that experiences periodic major outbreaks of the disease, such as those in 1998 (Corwin et al., 2001) and 2004 (Suwandono et al., 2006). In addition, annual and sporadic occurrences regularly afflict all the 33 provinces of the vast Indonesian archipelago (Sumarmo, 1987). While there have been studies describing the molecular genetics of dengue viruses isolated from Western part of the Indonesian archipelago (Sumatra and Java Islands) (Ong et al., 2008; Raekiansyah et al., 2005), no data is available for other major islands in the Eastern part of Indonesia, such as Sulawesi island. Data from the South Sulawesi provincial health authority (www.dinkes-sulsel.go.id) described the increasing number of dengue incidents in South Sulawesi province in 2007, in which 5333 patients were admitted to hospitals. In the capital city, Makassar, the dengue incidence was recorded at 425 cases with 5 fatalities. Dengue diagnosis in Makassar is commonly performed based on clinical symptoms only as the lack of resources and relatively high cost of laboratory tests prohibit serological confirmation. Because of this, the reported incidence may not represent the true disease burden.

Comparative analysis of dengue genomes has been used widely to study the genetic diversity of the dengue viruses (Holmes and Burch, 2000; Holmes and Twiddy, 2003) and previous studies have also suggested a correlation between viral pathogenicity with its genetic structures in which mutations in viral genome enhances the viral pathogenesis (Leitmeyer et al., 1999; Pandey and Igarashi, 2000). To understand the dengue disease dynamic in Makassar city, Indonesia, we studied the molecular evolution of circulating dengue viruses by analyzing the genomes of the viruses isolated in the area. We observed the diversity of serotypes and genotypes of dengue viruses and hypothesized that spatial isolation may contribute to the diversity of dengue viruses circulating in Makassar, South Sulawesi, Indonesia. To complement the viral genetic data, we performed viral growth characterization of various serotypes/genotypes. This study provides information in the genetic and biological characteristics of the diverse dengue virus strains circulating in Makassar, Indonesia.

2. Materials and methods

2.1. Study site and patient recruitment

We conducted surveillance of febrile patients in Makassar, the capital city of South Sulawesi province, Indonesia. The city resides in an area of about 175.77 km^2, inhabited by about 1.3 million people (http://makassarkota.go.id). Makassar city is located about 1400 km east of the Indonesian capital of Jakarta. Dengue-suspected febrile patients admitted to Dr. Wahidin Sudirohusodo, Labuang Baji and Daya Hospitals and primary health care centers in Makassar were enrolled in the study upon obtaining written consent. For the minors/children participants, written consents were obtained from parents/legal guardians. We obtained ethical clearances for this study from the Hasanuddin University Medical Research Ethics Committee and the Eijkman Institute Research Ethics Committee. All patients aged between 5 and 100 years old with fever >38.0 °C for less than 72 h and without concurrent signs of upper respiratory infection or obvious alternate diagnoses to dengue were invited to participate in the study. In total, we obtained 455 serum samples during the patient recruitment period (May 2007–August 2010).

Initial screening on dengue-suspected patients was determined serologically using anti-dengue IgM and IgG (Panbio Dengue Duo IgM/IgG enzyme-linked immunosorbent assay (ELISA) kit (Panbio, Brisbane, Australia)). Dengue confirmation by detection of NS1 antigen was done using NS1 rapid test kit (Standard Diagnostics, Korea). Primary versus secondary dengue infection was determined using the ELISA results according to manufacturer's protocol. Briefly, the positive IgM (>11 Panbio Units) and negative IgG (<22 Panbio Units) indicated primary infection while positive IgG (>22 Panbio Units), which may be accompanied by elevated IgM levels, indicated secondary infection. The presence of viral RNA in NS1-positive samples was further confirmed by reverse transcription-polymerase chain reaction (RT-PCR) using method described by Lanciotti et al. (1992), with modification according to Harris et al. (1998). All dengue positive cases were categorized either dengue fever (DF) or Dengue Hemorrhagic Fever (DHF) according to criteria described by the WHO (WHO, 1997).

2.2. Virus propagation, cell lines, RNA extraction and serotyping

Viruses were isolated from RT-PCR positive samples, either directly from patients' sera or after one passage (or maximum of two passages for low titer isolates) of virus propagation in C6/36 *Aedes albopictus* gut cell lines. Baby hamster kidney (BHK-21) and C6/36 cell lines were maintained in RPMI 1640 medium (Gibco, Carlsbad, CA). African green monkey kidney (Vero76) cell line was maintained in Minimum Essential Medium (MEM) with Earle's salts (Gibco). Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) for cell maintenance. BHK-21 and Vero76 cell culture was maintained in 37 °C incubator (ESCO) supplemented with 5% CO_2. C6/36 cell culture was maintained in 28 °C incubator. Virus stock titer was measured using modified standard plaque assay method (Fink et al., 2007) and recorded as plaque forming unit (PFU) equivalent/ml.

QIAamp Viral RNA Mini Kits (Qiagen, Hilden, Germany) were used to extract viral genomic RNAs from patients' sera or cell culture supernatant according to the manufacturer's instructions. Dengue viral RNA was reverse-transcribed into cDNA using Superscript III reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) and DENV-specific primers (Lanciotti et al., 1992). Subsequently, cDNA was amplified using *Taq* DNA polymerase (Roche, Mannheim, Germany). The four dengue serotypes were distinguished by PCR product size.

2.3. Whole genome sequencing

Five overlapping PCR fragments covering the whole dengue genome were amplified using methods described previously (Ong et al., 2008; Schreiber et al., 2009) with slight modifications. Briefly, genomic RNAs were reverse-transcribed into cDNA using primers specific for each serotype. The primers used for DENV-4 sequencing were as listed in Supplementary Table 1. Fragment amplification was performed using high-fidelity *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). Typical PCR conditions were as follows: templates were denatured for 2 min at 95 °C, followed by 45 cycles of 30 s denaturation at 95 °C, 1 min annealing at 55 °C, and 4.5 min extension at 72 °C. Templates were allowed a 10 min final extension step at 72 °C followed by storage at 4 °C. PCR products were purified from 0.8% agarose gel using QIAquick gel extraction kit (Qiagen) and used as template for cycle sequencing. DNA sequencing was performed using BigDye Dideoxy Terminator sequencing kits v3.1 (Applied Biosystems, Foster City, CA) using the method described in the kit. In addition to the sequencing and amplification primers previously described by Ong et al. (2008), a new set of primers for DENV-4 were used. Primer sequences and predicted genome binding positions of RT-PCR primers for DENV-4 are listed in Supplementary Tables 2 and 3, respectively. Purified sequencing reactions were then run on