Genotypes of the dengue virus in patients with dengue infection from Sabah, Malaysia.

N I Najri 1, Z Mazlan 1,2, J J Jaimin 3, R Mohammad 3, N H Md Yusuf 4, V S. Kumar 4, M Z Hoque 1*

1Faculty of Medicine and Health Sciences, University Malaysia Sabah, Kota Kinabalu, Malaysia
2Department of Pathology, Hospital Wanita dan Kanak-kanak Sabah, Kota Kinabalu, Malaysia
3Department of Infectious Disease, Public Health Laboratory, Kota Kinabalu, Sabah, Malaysia
4Biotechnology Institute Research, University Malaysia Sabah, Kota Kinabalu, Malaysia
E-mail: nin902@yahoo.com

Abstract. Dengue fever is an arthropod-borne viral disease caused by the Dengue virus (genus Falviviruses, family Flaviviridae). It has rapidly spread all over the world affecting approximately 400 million people annually. Human dengue infection is caused by four types of closely related viruses (also called serotypes) namely DENV-1, DENV-2, DENV-3, and DENV-4, all of which can be all found in Sabah, Malaysia. Each serotype can then be divided into unique groups based on its genotypes. In Malaysia, dengue has been reported as the most prevalent disease of the country with a ratio of 328.3 cases per 100,000 populations. Exacerbating this further, it was also recently reported in 2017 of the emergence of a newly identified Asian lineage dengue virus i.e. type 3 genotype II (D3GII) in Malaysia. We have aimed, through this study, to examine the serotypes and the genotypes of dengue virus circulating in Sabah. This study was conducted for a period of 8 months i.e. from January to August 2017. A total of 52 NS1 (50.9% were males and 49.1% were females) positive dengue patient serum samples were genotyped. Viral RNA was extracted from serum using QIAamp viral RNA mini kit and DNA sequencing was done on Applied Biosystems 3730xl DNA analyzer. The results showed that serotype DENV-3 was the most predominant dengue circulating virus in Sabah with 23 cases detected. These were further grouped under three genotypes namely D3GI (1 case), D3GII (14 cases) and D3GIII (8 cases). Serotype DENV-1 was the second most common circulating virus in Sabah with 23 cases detected. These were further grouped under three genotypes namely D3GI (1 case), D3GII (14 cases) and D3GIII (8 cases). Serotype DENV-1 was the second most common circulating virus in Sabah with 23 cases detected. These were further grouped under two genotypes, D1GI (15 cases) and D1Gic (2 cases), respectively. On the other hand, only one genotype (D4GII) was detected for DENV-4 (9 cases), and two genotypes (D2 Cosmopolitan Clade I and D2 Cosmopolitan Clade Ib) for DENV-2, each with one case per genotype, respectively. Understanding of genotype diversity will be useful in designing strategies for dengue management in epidemiological surveillance and vaccine design.
1. Introduction

In recent decades, dengue viral infection has grown dramatically around the globe. Dengue virus rapidly spread globally affecting approximately 400 million individuals yearly. About one third of the world’s populations are at risk of dengue infection which highly occurrence occurs mostly in developing countries [26]. Dengue virus is endemic in more than 128 countries, especially in the urban and suburban districts [4]. The surrounding regions of South East Asia are an endemic for dengue particularly in Thailand, Indonesia, Philippine and Malaysia which has a suitable climatic condition for the proliferation of the dengue virus (DENV).

Dengue is an arboviral disease that is caused by the dengue virus. The virus belongs in the genus Flavivirus and under the family Flaviviridae. There are four serotypes of dengue virus (DENV-1, DENV-2, DENV-3 and DENV-4) co-circulated globally. Dengue virus is transmitted to human through the day biting mosquito vectors, Aedes aegypti and Ae. albopictus [13]. The female Ae. aegypti bites multiple peoples daytime while, Ae. albopictus is considered as a highly adaptive second vector that can survive in cooler environments. Both species are sensitive to changes in environmental conditions such as temperature, precipitation and humidity [23].

The DENV is an enveloped virus with an icosahedral symmetry and with a genome size of about 11 Kb in length [11]. It has a positive single stranded RNA genome that encodes for a single open reading frame and can be translated into three structural proteins, that is, the core, (C), Pre-membrane / membrane (prM/M) and envelop (E) proteins, and seven non-structural (NS) proteins namely as NS1,NS2a, NS2b, NS3, NS4a, NS4b and NS5 [9]. Its structural glycoprotein E is responsible for cell recognition and for promoting entry host, which is mediated by a fusion process between the viral envelope and the cell membrane, while the NS protein aid viral genome replication.

Clinical manifestations which (ranges from asymptomatic, undifferentiated fever to classical dengue fever and with a life threatening severe infection) are presents in any of the four DENV serotype infection. More specifically, infections differential symptom from severe flu-like illness (systemic and dynamic infection with broad clinical spectrum) to lethal complications like dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Infection with one serotype provides lifelong serotype-specific immunity (homotypic immunity). Sequential infection of two serotypes leads to more severe type of disease, which leads to an advanced risk for DHF/DSS during secondary infections with heterogenous serotype [18].

The four serotypes, further divided into phylogenetically distinct clusters each with its own distinct genotypes [10]. Taxonomically, the differentiating component is the icosahedral viral genome which manifests itself as the DENV1-4 serotypes with 65–70% sequence homology [24]. Genome sequence characterizes the serotypes into multiple lineages with extensive genetic diversity [11]. The regional assimilation of virus serotypes and genotypes from nearby geographical proximity and its widespread distribution can result in regional population mobility and trans-border economic activities [17]. In addition, the viral genotypes may vary based on their geographical distributions, epidemic potential, fitness and virulence [11].

Malaysia reported its first case of dengue in 1902 [25]. Since then, dengue cases has gradually increased with major outbreaks in 1974, 1978, 1982 and 1990 [1]. From 2014 to 2016, Malaysia experienced an unprecedented outbreak that lead to a huge increased in the number of cases and mortality throughout the country [14]. More specifically, in 2014, a total of 108, 698 cases were reported in Malaysia, which was is equivalent to an incident rate (IR) 0f of 361.1 cases in per 10,000 populations individuals with 215 mortalities. Meanwhile, in 2016, the total number of dengue cases, while still considered high, decline to 101, 357 although the mortality rate was higher by 10% when compared to 2014 [19][21]. All the four serotypes were found co-circulating in Malaysia with the
presence of serotype specific geographical association. For example, DENV-1, DENV-2 and DENV-3 were found in Negeri Sembilan [2], while multiple cases of DENV-2 and DENV-4 were identified in Sarawak[10]. In addition, DENV-4 was found to dominate the populated regions of Kuala Lumpur and Selangor [5]. More recently, in 2017 a newly emerged Asian lineage dengue virus type 3 genotype II has been reported in Malaysia [15]. Most of studies in Malaysia were conducted in the Peninsular While reports from the Borneo Island, particularly Sabah remains scarce. As such, the aim of this study is to determine the main dengue serotype circulating in Sabah, and to examine its genotype and identify possible association with geographical distribution within Sabah.

2. Method

2.1 Medical Ethics Clearance

This research was approved by the Medical Research Ethics Committee (MREC), Ministry Of Health, Malaysia (No.NMRR-16-1163-30925) and Research and Ethical committee University Malaysia Sabah No. (UMS) JKEtika2/16(8). Samples were obtained after receiving informed consent from participants.

2.2 Dengue virus NS1 antigen detection

A total of 752 NS1 positive dengue patient serum samples within January to December 2017 were obtained from the Public Health Laboratory, Sabah. All the samples had previously been were tested for NS1 dengue using the SD Bioline NS1 rapid test (Alere, Australia) according to the manufacturer’s recommendations.

2.3 RNA Extraction

Dengue viral RNA was extracted from serum samples which were positive for the NS1 test. Viral RNA was extracted from 140μL supernatant of infected cells or from patients sera using the QIAamp® viral RNA extraction kit (Qiagen), following the protocol from the manufacturer.

2.4 Real-Time- quantitative Polymerase Chain Reaction (RT-qPCR) assays

Dengue virus serotyping were carried out by multiplex real-time qPCR abTEST™ DEN4 qPCR I Kit assay to simultaneously detect the presence of DENV-1, DENV-2, DENV-3, and DENV-4 in one reaction tube. All RNA samples were tested according to the manufacturer protocol (AITbiotech, Singapore). Real-Time PCR was performed using CFX96 real-time thermocycler Bio-Rad Laboratories, Hercules, USA. Samples were considered positive if the target amplification was recorded within 37 cycles.

2.5 PCR and sequencing of the Envelope gene

The synthesis of complimentary DNA (cDNA) from extracted RNA was carried out using Superscript™ III First-strand Synthesis System (Invitrogen, USA). The complete E gene (~1.5kb) was amplified using 0.5μM of DENV serotype-specific primer and 1X Phusion™ Flash High –Fidelity PCR Master Mix (Finnzymes, USA). The amplification protocol was as follows: initial denaturation at 98°C for 5 sec, annealing at 60°C for 8 sec, extension at 72°C for 25 sec and a final extension at 72°C for 1 minute. Amplified products were visualized in a 2% agarose gels stained with gelred (Biotum, USA). The amplicons were then sequenced using to determine its genotype.
3. Results

This cross sectional study was conducted from January to December 2017. A total of 752 NS1 positive dengue patients’ serum samples were tested for serotyped. The study shows that all age groups and gender were affected with dengue. Out of 752 examined patients, 404, (54%) were males and 348, (46%) were females, respectively (Table 1).

There were more infected adults (67.8%) compared to children and adolescents (32.2%).

**Table 1**: Gender and age records of the dengue infected patients obtained from Jan-Dec 2017 (n=752).

| Gender | Number of individuals (%) |
|--------|---------------------------|
| Male   | 404 (54%)                 |
| Female | 348 (46%)                 |

| Age               | Number of individuals (%) |
|-------------------|---------------------------|
| Children (0-9)    | 88 (11.7%)                |
| Adolescent (10-19)| 154 (20.4%)               |
| Adults (20-79)    | 510 (67.8%)               |

All four serotypes were found to circulating in Sabah during the study period. DENV-3 was the predominant serotypes with 413 infected individuals (55%). This was, followed by DENV-4 and DENV-1 with 130 (17%) and 126 (17%) individuals, respectively. The DEN-2 serotypes infected the least number of people with 83 individuals (11%) (Figure 1).

**Figure 1**: Distribution of the four dengue serotypes circulated in Sabah from between January to December 2017 (n=752).
In terms of its geographical location, most of dengue cases were from the western division of Sabah from Kota Kinabalu, Papar, Putatan, Penampang, Ranau and Kota Belud a total of 489 cases (65%). This was followed by the eastern Tawau division, with its less denser population, comprising of Tawau, Lahad Datu, Kunak and, Semporna, accounting for 164 cases (21.8%). Meanwhile, the northern Kudat division comprising of Kudat, Pitas, Kota Marudu had 50 cases (6.6%). The Sandakan division comprising of Sandakan, Beluran, Kinabatangan) had 18 cases (2.89%). The Keningau division comprising of Keningau, Beaufort, Sipitang, Tambunan, Kuala Penyu had surprisingly only 2 cases (0.27%). Finally across the straits, the island of Labuan had 29 NS1 positive cases (3.86%).

DNA sequencing of the dengue Envelope (E) gene was performed for analysis 52 samples to obtain viral genotypes. Eight different genotypes were identified from the four dengue serotypes. The most common dengue genotypes were D1G1a (DENV-1) and D3G1 (DENV-3), each with 14 cases (Table 2). We identified three genotypes under DENV-3 (D3G1a, D3G1 and D3G1I11) with a total of 23 cases.

Table 2: Genotypes of the dengue virus detected from 52 samples that was sequenced.

| Serotype | Genotype | Number of Individuals |
|----------|----------|-----------------------|
| DENV-1   | D1G1a    | 15                    |
|          | D1G1c    | 2                     |
| DENV-2   | D2-Cosmopolitan Clade I | 1                  |
|          | D2-Cosmopolitan Clade Ib | 2                  |
| DENV-3   | D3G1     | 14                    |
|          | D3G1a    | 1                     |
|          | D3GIII   | 8                     |
| DENV-4   | D4GII    | 9                     |

When the genotypes were grouped according to geographical locations (based on patients’ record), we found most were found [48] circulating in the western division Sabah. Genotype D1G1a was the most dominant and found in almost throughout Sabah (Figure 2). Interestingly, D2-Cosmopolitan Clade I and Clade II was found exclusively in the Tawau division. Also, the D1G1a genotype was found in one sample from the Sandakan division. This genotyping shows specific distribution to geographical location for each genotype (Figure 2).

Figure 2: Distribution of the dengue genotypes based on the geographical divisions of Sabah
4. Discussion

Dengue is not a new disease and it has been a public health risk since the 1950’s. In recent decades, the prevalence of dengue has increased dramatically worldwide. The disease is now endemic in more than 128 countries worldwide with South East Asia and the Western Pacific regions being the most seriously affected. Dengue infection is a growing trend in the South East Asia including Malaysia over the past few years [20]. Most of the published data on dengue is based on patients from the Peninsular Malaysia. As such, there is a need to present data obtained from endemic regions of Borneo, and particularly from Sabah. Such information on the distribution and prevalence of dengue serotype and genotype is currently lacking. Thus, this study aims to address this matter.

From this study, we found that adults were mostly affected by dengue infections (Table 1). Mainly, working adults and students were the most infected. It is a similar trend in other studies done in Singapore, Bangladesh and Indonesia [8][16]. Dengue infection has conventionally affected children’s; but recently there has been a shift in the mean age of dengue cases towards older age groups dengue in hyperendemic countries in Southeast Asia, including Malaysia. Most dengue cases now occur in individuals aged 5–24 years. The higher prevalence of dengue in a higher age group, 20 to 45 years old may be due to the higher outdoor exposed exposure to the dengue mosquito. The elderly group (55 years to 79 years old) showed low dengue infection rate which could be due to the development of immunity from previous infections [5]. We also found, a higher exposure to dengue infection in males rather compared than to female was 54% and 46%, respectively (Table 1). It may due to those males have the tendency to do more outdoor work and travel compared to females making them favorable by targets of the Aedes mosquitoes.

Most of the dengue cases were from the western division of Sabah which encompassed the township of Kota Kinabalu, Papar, Putatan, Penampang, Ranau and Kota Belud. The capital of Sabah, Kota Kinabalu, had high number of cases especially since it is an urban areas where there are high density populations and rapid developmental activities, which are favorable for dengue transmission. Gubler [7] noted that the transmissions of dengue infection are influenced by several factors; among them is the increase of in population growth, rapid urbanization, rural-urban migration inadequacies in urban infrastructure, including solid waste disposal and arise in domestic and international air travelling. In addition, transmission of dengue viruses is also influenced by climate, such as high rainfall, high temperature or global warming. Changes in climate such as global warming high rainfall can also lead to an increase in transmission of mosquitoes and create new opportunities for the survival of pathogens and vectors [3].

In Malaysia, the serotype distribution of dengue virus has been inconsistent and varies from year to year. We found that in 2017, the most common serotype in Sabah was DENV-3 (Figure 1). It was interesting to note that all found dengue serotypes were present in Sabah. A previous report on the Sabah serotypes, found that DENV-4 was dominant in 2013, followed by DENV-1 in 2014 and DENV-2 in 2015 [22]. According to Abubakar and Shafee [1], the pattern of predominant circulation dengue virus serotype during outbreak period tends to switching yearly. How this affects the severity of the disease is still speculative. Concurrent infection with more than one serotype is also on the rise. It could be possible that viral genotypic pressures could be the key to the varying cyclic dominant serotypes.

We then sequenced the Envelope gene of 52 samples to determine its viral genotype. We identified eight genotypes from all four serotypes (Table 2). DENV-3 had three different genotypes. A similar finding by Jeyanthi et al. [14], reported that a DENV-3 strain was divided into genotype I and genotype III in Malaysia from 2007 to 2013. Interestingly, genotype I of DENV-3 was last documented in 2011 and found circulated in Malaysia during the epidemics of 1974 and 2007 [27]. Hence, the
appearance of genotype I that was equal in numbers to genotype III among DENV-3 in this study raises the possibility of a re-emergence of genotype I. Emergence of new genotypes or genotypic clade replacements have been reported during outbreak in apart of serotype replacement due to positive selection pressures and viral fitness for survival. In this study, DENV-1 strains were classified into genotype I, which subsequently formed branches within same genotype. Usually it is acknowledged that the monophyletic form specifies high genetic similarity among DENV-1 Malaysian strains. As for DENV-2, only the Cosmopolitan genotype was reported in Malaysia [6] and this was similar with our findings. It has been reported that DENV-4 is rare in Malaysia [14]. However, we found nine cases in this study with one genotype i.e. D4GII (Table 2). We also saw clear association between the genotypes and its distribution across the various geographical regions (Figure 2). We note that some genotypes were widely distributed (D1GIa) while others such as the D2-Cosmopolitan Clade I and Clade II were geographically restricted. The finding does seem to agree with previous findings of a selection pressure on the viral genome. We suggest further work to confirm this hypothesis.

5. Conclusion

From this study, we identified the serotypes of 752 dengue viruses in Sabah from January to December 2017. We also identified the genotypes of a selected number of samples. Surveillance work such as this is important as the introduction of novel dengue viral genotypes may result in epidemics, as not every new virus or clades results in dengue epidemics. To the best of our knowledge, this is the first observational study to investigate the relationship between dengue serotype and genotype in Sabah. The exact cause of high prevalence of multiple serotypes in Sabah may determine thorough epidemiological investigation and phylogenetic genome analysis of dengue virus serotypes. In this regard a follow up study is being conducted to find out the origin of the causal agent and cause for the high incidence of all serotypes in single locality. Employing molecular epidemiological studies with dengue virus sequencing would be a better approach to determine the origin of a newly imported virus.

6. References

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