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Serotyping of dengue virus in 2016-17 outbreak in Kudat, Sabah, Malaysia

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Abstract. An outbreak of dengue in Kudat, northern Sabah in 2016-2017 provided an opportunity to investigate the circulating serotypes of dengue viruses of cases at Hospital Kudat. Between September 2016 and December 2017, a total of 156 dengue positive sera (tested positive by either NS1 antigen, or IgM & IgG antibody rapid test) were collected from dengue patients who had acute fever and showed signs and symptoms suggestive of dengue. RNA was extracted from the sera using QIAamp RNA Blood Mini Kit, and molecular amplification was performed using one-step RT-PCR kit, followed by nested PCR using HotStart Taq master mix kit with the primers of the dengue C-prM gene. There were 81 (52%) male and 75 (48%) female cases. The age group with the highest number of cases was the 10-19 years old, while the youngest infected was 8 months old and the oldest was 83 years old. RT-PCR results showed 88 sera dengue positive, 48 infected with a single serotype while another 40 with multiple serotypes. All four DENV serotypes were co-circulating during the outbreak period and DENV-1 was predominant. Molecular analysis also indicated 69.2%, 50.0%, 51.9% and 48.9% respectively of the NS1, IgM, IgG and IgM & IgG positive sera were RT-PCR positive for dengue. High number of cases were seen in December 2016, February and May 2017. The dengue outbreak might be related to switching of predominant serotype from DENV 4 to DENV 1.

INTRODUCTION

Dengue is a mosquito-borne viral infection caused by Dengue virus (DENV) which is a single-stranded RNA virus comprised of 4 serotypes DENV 1 to DENV 4 (WHO, 2009) The viral RNA genome encodes three structural proteins (C: capsid, M: membrane and E: envelope) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (WHO, 2009; Klungthong *et al.*, 2004). Dengue is transmitted by the bite of two *Aedes* species, *A. aegypti* and *A. albopictus*, which are found throughout the world. Subsequent infections with different dengue virus serotypes could lead to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (CDC, 2014). Dengue is prevalent in tropical and subtropical regions of the world particularly in urban and semi- urban areas, although there is also evidence to show it is currently spreading to the rural areas all over the world (WHO, 2011). The first dengue haemorrhagic fever (DHF) was reported in Philippines in 1953-1954. Since then major dengue outbreaks have been reported from countries of South-East Asia (SEA) and Western Pacific (WP) regions (WHO, 2011).

There are four dengue serotypes, and detection of which serotypes circulating in an outbreak is important from the perspective of public health intervention, as Vicente *et al.* (2016) have pointed out that early detection of serotypes, especially DENV 2, circulating in an area could help prevent increasing numbers of severe outcomes during dengue outbreaks. Serotyping in

Malaysia has been previously done mainly in West Malaysia by Nizal *et al.* (2012) in Negeri Sembilan State, Chew *et al.* (2012) and Suppiah *et al.* (2018) in Selangor-Kuala Lumpur area, and Gintarong (2018) in Sandakan, Sabah.

Dengue cases in Kudat had been reported yearly. In 2015, Kudat had only 11 dengue cases, but this increased to 95 in 2016 with more cases being reported during the outbreak between September to December 2016 and 107 in 2017 (Jabatan Kesihatan Negeri Sabah, 2016; 2017). During this outbreak, campaigns to eradicate mosquito breeding grounds and to watch out for symptoms of dengue were conducted by the Sabah State Department of Health.

The main objective of this study is to investigate the circulating serotypes of DENV during the dengue outbreak in Kudat, Sabah, although we also looked into the distribution of dengue cases reported in the hospital in relation to month, age and gender.

MATERIALS AND METHODS

Study site

Kudat in Sabah, Malaysia, is situated at the most northern part of Boneo island (Fig. 1). The temperature ranges from 28°C to 34°C with the higher temperature recorded between April to September. Rainfall is high with range 20-320 mm per month, and maximum rainfall occurs between November to January (https://www.world weatheronline.com/kudat-weather-averages/sabah/my.aspx).

Sample collection

During the dengue outbreak in Kudat (Fig. 1), 156 dengue positive sera whether tested by NS1 antigen (Dengue Early rapid test, PanBio), or IgM & IgG antibody (Dengue Duo Cassette, PanBio) were collected between September 2016 to December 2017 from dengue patients attending Hospital Kudat, Sabah, Malaysia. These patients had acute fever and signs and symptoms suggestive of dengue. One ml of serum of each patient was available for the study. The serum samples were initially kept in the freezer compartment of the fridge in the hospital until collected a week later and transported by car to the University laboratory freezer at -20° for further analysis by RT-PCR.

Methodology

The patients' serum samples were serotyped using RT-PCR and nested PCR at the Department of Pathobiology and Medical Diagnostics, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah.

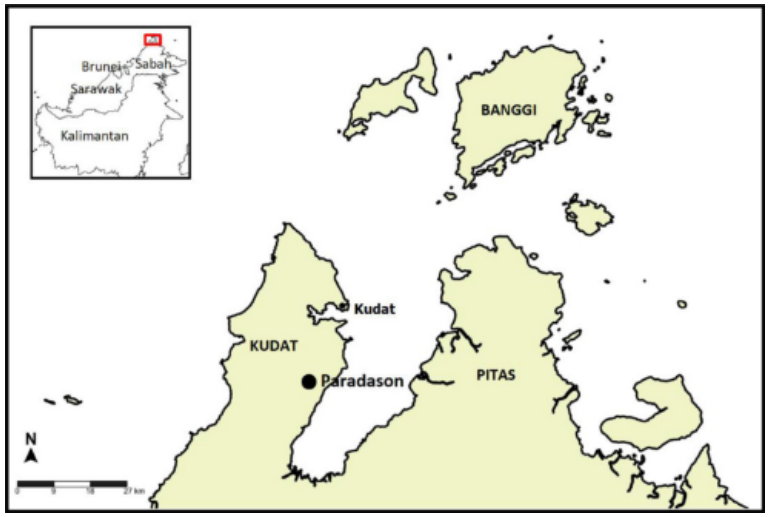

Figure 1. Location of Kudat town (Kudat district) at the northern tip of Sabah, where the dengue samples were collected.

Table 1. Primers used in the RT-PCR and nested PCR

Method		Primer pair	Size of product	Reference
One-step RT-PCR		mD1: TCAATATGCTGAAACGCGAGAGAAACCG D2: TTGCACCAACAGTCAATGTCTTCAGGTTC	511 bp	Lanciotti *et al.* (1992). Chien *et al.* (2006).
Nested PCR	DENV1	mD1 and rTS1: CCCGTAACACTTTGATCGCT	208 bp	Chew *et al.* (2012).
	DENV2	mD1 and mTS2: CGCCACAAGGGCCATGAACAGTTT	119 bp	
	DENV3	mD1 and TS3: TAACATCATCATGAGACAGAGC	288 bp	
	DENV4	mD1 and rTS4: TTCTCCCGTTCAGGA TGTTC	260 bp	

RNA was extracted from 140 µl of serum using QIAamp RNA Blood Mini Kit (QIAGEN, Inc., Germany). One-step Reverse transcriptase Polymerase Chain Reaction (one-step RT-PCR) was performed using one-step RT-PCR kit (QIAGEN). Nested PCR was done on the RT-PCR product using HotStart Taq master mix kit (QIAGEN). C-prM amplimers designed by Lanciotti *et al.* (1992) and redesigned by Chien *et al.* (2006) were used in this study. The sequences of the primers used are shown in Table 1.

In the RT-PCR amplification using one-step RT-PCR kit (QIAGEN), 5µl of the extracted RNA, 25 pmol each of mD1 and D2 primers with the reaction mixture made up to 25 µl. Amplification was done in a thermal cycler (Thermo Scientific) with the following PCR reactions: reverse transcription at 50°C for 30 minutes; followed by initial denaturation and activation of *Taq* DNA polymerase at 94°C for 15 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C 30 seconds, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes and hold at 4°C.

The RT-PCR product was further processed by nested PCR using HotStart Taq master mix kit (QIAGEN). Twenty pmol each of the mD1 primer coupled with one of the four serotype-specific primers (rTS1, mTS2, TS3, or rTS4) were added into a separate reaction mixture tubes containing the RT-PCR product and each made up to a total volume of 25 µl. The PCR conditions were polymerase activation at 95°C for 15 minutes followed by 25 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 30 seconds. Final extension was at 72°C for 10 minutes and hold at 4°C. For negative control distilled water was used. Agar gel electrophoresis was done with 2% agarose gel and stained with ethidium bromide solution for 15 minutes. Gel documentation was made after rinsing the gel with distilled water.

Ethical approval

This study was approved by Medical Research & Ethics Committee, Malaysia. (NMRR-15- 2378-26766 (IIR)).

RESULTS

The youngest patient was an 8 months old infant, the oldest 83 years old, while those above 70 years of age had eight cases (Fig. 2). The highest number of cases was recorded in the 10-19-year-old group. There were more males: 81 versus 75 females, with the sex ratio of male: female of 1.08:1.0.

Out of 156 dengue rapid test positive cases from the hospital, 52 (33%) were NS1 antigen positive, 30 (19%) IgM positive, 27 (17%) IgG positive, 47 (30%) IgM & IgG positive (Table 2). However, RT-PCR was positive 88 samples (56%, range 50%–69%) with the highest percentage (69%) recorded for NS1 antigen positive sera. On the other hand, IgM, IgG and IgM & IgG) had about 50% positive RT-PCR.

Of the RT-PCR positive samples, 48 (55%) had a single serotype (either DENV 1, 3 or 4), 33 had two serotypes, while 7 had three (Table 3). Basing on the presence of serotypes

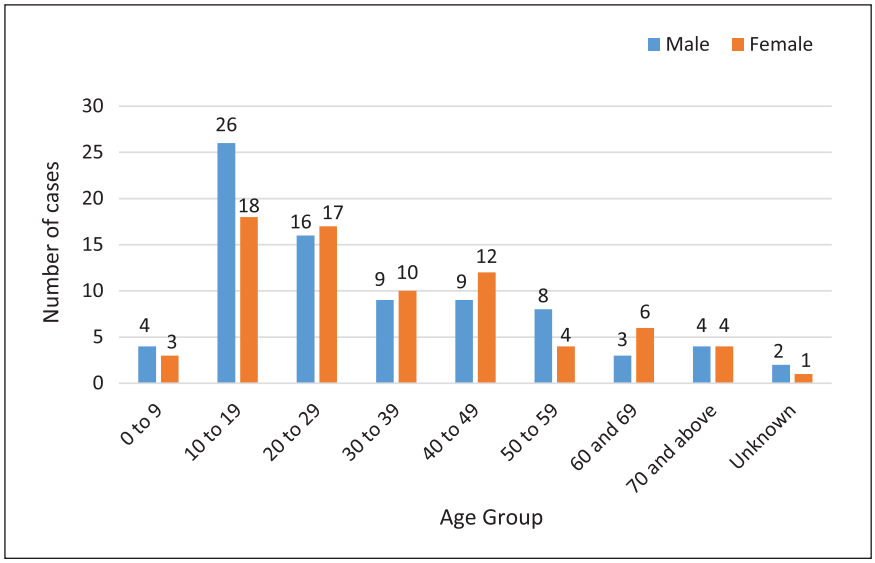

Figure 2. Age and sex distribution of dengue cases as detected positive by serology rapid test (N=156).

Table 2. Comparison of results of serology test and RT-PCR analysis

Serology test	No. Positive	No. (%) positive in RT-PCR
NS1	52 (33%)	36 (69.23%)
IgM	30 (19%)	15 (50%)
IgG	27 (17%)	14 (51.85%)
IgM & IgG	47 (30%)	23 (48.93%)
Total	156	88 (56.41%)

Table 3. DENV serotypes detected by RT- PCR in sera of dengue patients collected in Kudat

Number of serotypes in a sample	DENV serotypes	Number Positive
Single	DENV 1	31
	DENV 3	12
	DENV 4	5
Two	DENV 1+2	5
	DENV 1+3	25
	DENV 1+4	1
	DENV 2+3	2
Three	DENV 1+2+4	1
	DENV 1+3+4	6
	Total	88

whether in single or multiple serotype infections, DENV 1 was predominant (51%) and the DENV 2 was the least common (10%) (Table 4).

The distribution of dengue cases as detected by rapid test fluctuated with the month although a decreasing trend was discernible, presumably the result of intervention implemented by the Department of Health. More dengue cases were recorded in December 2016, February and May 2017 (Fig. 3).

The relationship between monthly number of cases and the total monthly rainfall can be described by a decreasing exponential function with equation as

Table 4. Occurrence of DENV serotypes in the sera of dengue patients collected in Kudat. Data is based on presence of a serotype in both single and multiple serotype infections

DENV serotypes	Number (%)
DENV 1	69 (51.11%)
DENV 2	8 (5.93%)
DENV 3	45 (33.33%)
DENV 4	13 (9.63%)
Total	135 (100%)

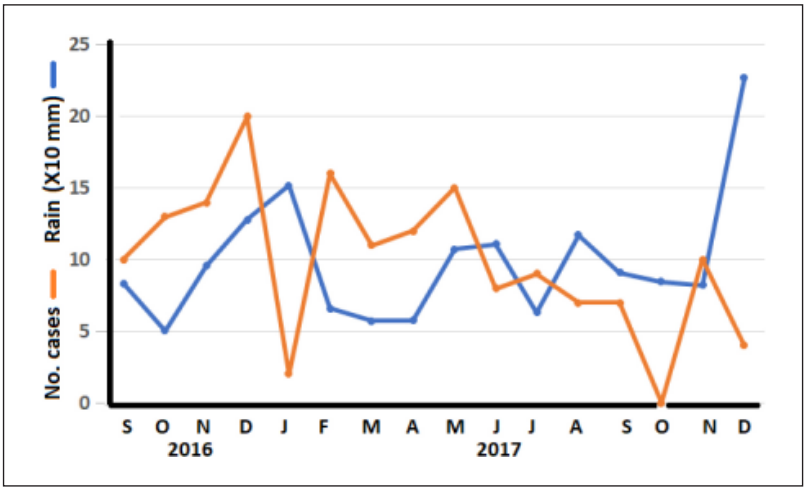

Figure 3. Monthly distribution of dengue positive cases (tested by serology rapid tests) from September 2106 – December 2017 in Kudat (N=156).

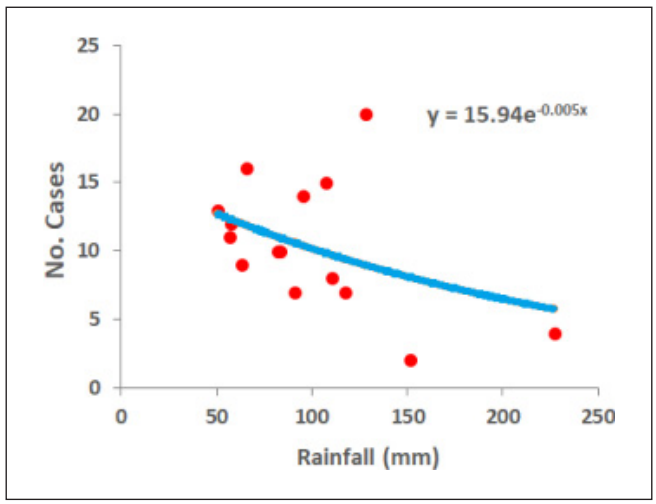

Figure 4. Regression of monthly number of cases against monthly rainfall.

y=15exp(-0.0044x) and residual standard error of 5.12 on 14 degrees of freedom (Fig. 4). The number of cases appeared to decrease with increasing rainfall.

DISCUSSION

Most of the dengue cases in Kudat were recorded in the 10-19-year-old age group (28.20%) followed by 20-29-year-old group (22%). The youngest dengue case was 8 months and the oldest was an 83-year-old male. This is similar to a previous study in Malaysia which also showed that although dengue affected all age groups, age 15 and above were more affected, with the youngest case of 8 months old and the oldest 89 years old (Nizal *et al.*, 2012). However, a study in Pakistan indicated that although all age groups were equally infected by dengue infection, the age group 20-30 years (Humayoun *et al.*, 2010) was slightly higher. In Bangladesh the age group most affected

by dengue and dengue haemorrhagic fever was the 18-25-year age group followed by 26-33 age group (Rahman *et al.*, 2001). The dengue outbreak in New Delhi, India in 2006 recorded the highest number of dengue RT-PCR positive cases (35.5%) in the 20-30 years old age group (Bharaj *et al.*, 2008). A communicable disease surveillance in Singapore done in 2016 showed the highest number of dengue cases (26%) was in the 25-34-year-old age group (Ministry of Health Singapore, 2017). In comparison, the most affected age group in Kudat was slightly younger than the other countries. This could be due to differences in socio-demographic factors, housing type, educational status and the nature of jobs all leading to different exposure risk to the disease vector.

Our study found that there were more male than female cases, concurring with the Malaysia National data published by WHO for 2000–2008 which states that males constituted 55%–62% of the dengue cases (Mohd-Zaki *et al.*, 2014). Similarly, in Singapore male cases in 2016 were 7,662 compared to 5168 females (Ministry of Health Singapore, 2017). This gender difference is likely be due to the differences in nature of their work and the work place between the males and females leading to unequal exposure to the vectors.

The initial increase in dengue cases was recorded in November-December, when rainfall was increasing, and with slightly higher temperature. These environmental factors of heavy rainfall, humidity and temperature are known risk factors associated with dengue disease outbreak (Mudin , 2015). However, that the relationship between the amount of precipitation and mosquito abundance or the number of dengue cases is complex and nonlinear (Cheng *et al.* 2016). Moderate precipitation can increase potential breeding grounds, but heavy rain may wash out immature mosquito populations, thus affecting the vector population and hence the dengue incidence. In Kudat, the decreasing number of cases in the later part of the outbreak was more likely due to intervention carried out by the authorities rather than rainfall.

The NS1 antigen could be detected in the blood of dengue patients starting from day 0 up to day 9 of fever and may persist longer even when RT-PCR was negative (Alcon *et al.*, 2002). Nevertheless, another study stated that RT- PCR positivity in dengue RNA detection decreased after day 6 to 10 of dengue infection (Moi *et al.*, 2013). Our results show that only 69.23% of NS1 antigen positive was positive in RT-PCR. The remaining 31% which turned out to be negative could be due to the patients coming to hospital when the dengue virus was no longer present in the blood or due to storing the samples in suboptimal temperature before they were collected for RT-PCR testing. The third possibility is that some of the cases were chikungunya infections which have similar symptoms as dengue, give positive result in tests for NS1 antigen because of cross-reactivity between flaviviruses (Matheus *et al.*, 2016), but negative in RT-PCR assay.

This study also showed that only about 50% of dengue IgG or IgM or IgM & IgG positive dengue cases were RT-PCR positive, which is quite consistent with other studies. For example, WHO (2009) stated that IgG is the dominant antibody in secondary dengue infection which is detectable at high levels, even in the acute phase of dengue infection (WHO 2009). Similarly, Schilling *et al.* (2004) also found that IgM antibody was detected in 55% of the primary dengue cases between day 4-7, and during that period either IgM antibody or DENV or both IgM and DENV are circulating in the blood. Between days 3-5 of dengue fever, IgM antibodies can be detected in 50% of patients and the percentage can increase to 80% by the 5th day of fever (WHO, 2009).

All the four DENV serotypes were found circulating in Kudat during the study period. Patients infected with single serotype had either DENV 1, 3 and 4, but not 2. DENV 2 was only detected from July to December 2017 in multiple serotype infections. More study is required to determine the reasons why this is so. A study in Kuala Lumpur (Chew *et al.*, 2012) also showed presence of four DENV serotypes, with the predominant DENV4 usually coinfecting with the other three

serotypes. They also reported only six cases with DENV4 and one case with DENV3 in single infection.

In 2013, DENV 4 was the most common serotype found in Sabah state (Ng *et al.*, 2015). However, in this study, DENV 1 was the predominant serotype while DENV 4 ranked only the third prevalent serotype (9.63%). In the outbreak in Sandakan (182 km away from Kudat) in January to February, and July to September in 2016, DENV 1 was also the most common serotype detected, followed by DENV 4, DENV 2 and DENV 3 (Gintarong *et al.*, 2018). This shows there was a shift of the predominant serotype from DENV 4 in 2013 to DENV 1 in 2016/ 2017 or earlier in Sabah which could have led to dengue outbreak.

It is known that dengue outbreak could arise because of changes in predominant circulating DENV serotypes (Mohd-Zaki *et al.*, 2014). According to the Malaysian Ministry of Health epidemiological surveillance monitoring system, dengue cases increased 4 to 6 months after the switching of predominant dengue serotype, because of the low population herd immunity against the new DENV serotype (Mudin, 2015). There is no doubt that increased international travel can certainly lead to the exchange of DENV among endemic countries (WHO, 2009). Introducing different serotypes by humans could result in predominant serotype shift and dengue outbreak.

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