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Research Note

Dengue serotype surveillance among patients admitted for dengue in two major hospitals in Selangor, Malaysia, 2010-2011

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Abstract. Dengue serotype surveillance is important as any changes in serotype distribution may result in an outbreak or increase in severe dengue cases. This study aimed to determine circulating dengue serotypes in two hospitals in Selangor. Serum samples were collected from patients admitted for dengue at these two major public hospitals i.e. Hospital Sungai Buloh (HSB) and Hospital Tunku Ampuan Rahimah (HTAR) between November 2010 and August 2011 and subjected to real-time RT-PCR using SYBR® Green. All four dengue serotypes were detected in samples from both hospitals. The predominating serotype was dengue 1 in samples from both hospitals (HSB, DENV-1; 25.53 % and HTAR, DENV-1; 32.1 %).

Dengue is an endemic disease affecting tropical and subtropical regions around the world, predominantly in urban and semi-urban areas (WHO, 1997). Dengue virus (DENV) belongs to the genus of *Flavivirus* and family *Flaviviridae* (Rivetz *et al*., 2009) and the this virus is transmitted to human through the bites of *Aedes* mosquitoes (WHO, 1997). There are four serotypes of DENV which are DENV-1, DENV-2, DENV-3 and DENV-4 (Rivetz *et al*., 2009). In Malaysia the first dengue case was reported in 1902 (Skae, 1902) in Penang and the first major outbreak of dengue hemorrhagic fever (DHF) was reported in 1973 (George, 1992). The number of dengue cases has been gradually increasing from the 1990s to the present times. For example, the reported dengue cases in Malaysia for 2011, 2012 and 2013 were 19884, 21900 and 43346 cases respectively (Ministry of Health Annual Reports). Among the possible reasons for the increase in dengue cases are changes in the circulating dengue serotypes or genotypes, better surveillance and reporting mechanism, increased in patient susceptibility of dengue infections or environmental factors.

Here we report the result of dengue serotype surveillance which was limited to two hospitals located in Selangor. Patients suspected with dengue from Hospital Sungai Buloh (HSB) and Hospital Tengku Ampuan Rahimah (HTAR), between November 2010 and December 2011 were included in the study. The study was approved by the Medical Review and Ethical Committee of the Ministry of Health, Malaysia (Registration number: NMRR-09-883-4768). Blood were collected from 290 patients who were

clinically diagnosed as dengue (n= 47 for HSB, n=243 for HTAR) and centrifuged at 3000 rpm for five minutes. Serum samples were then tested for dengue by using SD Bioline™ Dengue Duo NS1 Ag and IgG/IgM Rapid Test (SD Standard Diagnostics Inc, Korea) that detects dengue NS1 Ag and dengue IgM/IgG. Subsequently 140 µl of serum was used for RNA extraction using the QIAmp® viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's procedure, followed by real-time SYBR® Green RT-PCR amplification as described by Yong *et al.* (2007) in a Rotor-Gene-Q® (Qiagen, GmbH, Germany). The reaction mixture contained 5.0 µl of extracted RNA, 12.5 µl of 2XQuantiTect SYBR® Green PCR Master Mix (QIAGEN, Hilden, Germany), 10 µM Dengue Forward Primers, 10 µM Dengue Reverse Primers, 0.25 µl Quantitect RT® and nuclease-free water to a final volume of 25 µl.

After amplification, melting curve analysis was performed by increasing the temperature from 75ºC to 95ºC with 1.0ºC intervals for 5 seconds. The melting temperature (Tm) for each dengue serotype in the sample was determined by comparing to the Tm of positive controls used for each of the dengue serotype. In addition, any variations in the melting temperature of the unknown samples compared to the positive controls were confirmed by sequencing the PCR product (selected sequence data is given in Table 5). The four strains of dengue serotypes that were used as positive control were DENV type 1 Hawaii strain (Tm= 85.0ºC), DENV type 2 NGC strain (Tm= 82.5ºC), DENV type 3 H87 strain (Tm= 85.8ºC) and DENV type 4 H241 strain (Tm= 83.2ºC) (Yong *et al.*, 2007).

Tables 1 and 2 listed the results of NS1 Ag and IgG/IgM for the 290 patients selected for the study. Overall, a total of 129 (44.5%) of all dengue patients recruited tested positive for SD Duo NS1 Ag; while 223 (76.9%) of these patients also tested positive for SD Duo IgM/IgG for both hospitals. In addition, all four dengue serotypes were found circulating among the patients admitted to these two major hospitals. The most dominant serotype in HSB was DENV-1 (n=12 or 25.53%), followed by DENV-3 (n=11 or 23.40%), DENV-4 (n=6 or 12.77%) and DENV-2 (n=5 or 10.64%). The results are shown in Table 3. In HTAR, the most dominant serotype was also DENV-1 with 32.1% (n= 78), followed by DENV-2 (n= 47 or 19.34%), DENV-3 (n= 37 or 15.23%) and DENV-4 (n=29 or 11.93%) as shown Table 4. Figure 1 shows examples of the melting curves from patients and positive controls. All samples that had recorded the presence of melting curves but had slight variation in the melt curve temperature as compared to the four dengue prototype positive controls were sequenced to identify the dengue serotypes. This was done to confirm the identity of the dengue virus serotypes (Table 5). The correlations between Dengue NS1 and Real Time SYBR® Green RT-PCR are shown in Table 6. Overall the detection rate of Real Time SYBR® Green RT-PCR was better compared to the Dengue NS1 assay where the former assay detected the presence of dengue RNA in 78% of the samples as compared to only 44% by the later assay.

The World Health Organization (WHO) estimated that more than 70% of the population at risk of dengue worldwide lives in the Southeast Asia region and the Western Pacific region (WHO, 2009). Malaysia has been recording an increase in the number of dengue cases since the 1980s (Lam, 1993). Furthermore, the more developed regions in Malaysia including parts of Selangor and the Federal Territories accounted for a great percentage of the total cases. This could be due to improved detection ability at the testing laboratories located in the Klang Valley, higher population density, rapid increase in construction sites and potential haphazard dwellings lacking in basic infrastructures such as supply of piped clean water, perpetuating in the increase in mosquito breeding sites.

The number of dengue cases in Selangor has been a major concern. There were a total of 16, 367 cases with 45 fatalities in 2010 as compared to 2008 where the state recorded its highest dengue cases with 21 262 cases and 46 deaths (Nusyirwan, 2010). As such this

study had intended to determine the dengue serotypes among patients who were admitted in two hospitals in Selangor. This data may provide information on the pattern of circulating dengue serotype in certain parts of Selangor. The importance of dengue serotype surveillance for monitoring dengue outbreaks has been reported (Thayan *et al.*, 2001; Hilaire *et al.*, 2008). Among the reasons associated with an increase in dengue cases are the changes in the circulating dengue serotype and genotypes in the population.

All four dengue serotypes co-circulates in Malaysia (Mudin, 2009). Usually a particular dengue serotype pre dominates the other strains over a certain period of time, which can last up to 2 to 3 years. As the population is sensitized to the prevalent dengue serotype and the development of herd immunity in the population may limit the transmission of the virus, a different dengue serotype takes over the pre dominance. This usually causes an increase in dengue cases due to the presence of un-sensitized population (Thayan *et al.*, 2001). According to the report from the Malaysia's Ministry of Health, DENV-3 was the dominant serotype in the early 90s until it reached its peak in 1993 and then re-emerged in 2001. DENV-1 pre-dominated between 2003 until 2006 and was replaced by DENV-2 as the pre dominating serotype in the country in 2007 (Ministry of Health Annual Report, 2010). In the present study, we have found a predominance of DENV-1 among patients admitted for dengue in two hospitals in Selangor during 2010-2011. We also found the presence of other co-circulating strains such as DENV-2 and DENV-3 in both hospitals. A recent study by Nizal *et al.* (2012) also found a similar predominance of DENV-1 serotype among their study subjects in Negeri Sembilan.

The scenario among the countries surrounding Malaysia is quite similar. In a study carried out in Bangkok, Thailand, different types of DENV was seen circulating between 2004 and 2010. DENV-1 was the predominant serotype in 2004 (56.41%), DENV-4 in 2007 (50%), DENV-1 in 2008 (57.41%), and DENV-2 in 2010 (38.7%) (Pongsiri *et al.*, 2012). Also another study showed that both DENV-2 and DENV-3 have been associated with severe dengue disease, DHF, whereas DENV-4 is found more frequently among primary dengue infections (Nisalak *et al.*, 2003). All four dengue serotypes have been found in Singapore too. During a dengue outbreak in 2005, a total of 14 006 cases were reported with 27 deaths where DENV-2 was found to be the dominant serotype (Koh *et al.*, 2008). This was followed by DENV-1 which became the dominant serotype in 2006, and continued to pre dominate, causing another outbreak in 2007 (Lee *et al.*, 2010). However, in 2008, the predominant circulating serotype switched back from DENV-1 to DENV-2, and the number of dengue notifications in that year increased by almost three-folds to that of 2006.

Usually molecular methods are used to determine circulating dengue virus serotypes as the real time RT-PCR assay has the ability to detect genomic materials during the viraemic stage of the infection (Johnson *et al.*, 2005). The method also has the ability to serotype simultaneously in a single sample (Yong *et al.*, 2007). By comparison, the dengue NS1 assay has also been useful to detect viral proteins in the initial stage of the infections as the protein has been known to last up to 7-9 days after onset of symptoms. (Wang & Sekaran, 2010).

There are a few limitations in this study. We found that the Real Time SYBR® Green RT-PCR detected more dengue cases (patients) compared to the rapid NS1 assay. This is unusual as usually NS1 antigen assay is more sensitive than the PCR method as NS1 protein stays longer in serum compared to genomic materials from the virus. Among the possible reasons for this anomaly is interpretation of the rapid NS1 test which is very much dependent on the ability of the reader to visualize a band on the strip. Hence there are possibilities of under-reporting the NS1 band. Even though a rapid test seems to be simple test, training should be provided to the reader to correctly interpret the bands. Secondly the strip must be read within the specific time recommended (usually within 10 minutes) failing which affects the

accuracy of the readings. Another possible reason is that many of these samples have also demonstrated the presence of dengue IgM or IgG which can inhibit the assay (Wang & Sekaran, 2010). In addition, there could also be over-reporting by the real time SYBR Green RT-PCR as this method has less specificity due to the use of melt curve to determine the dengue serotypes.

The use of SYBR green chemistry is not ideal for performing a multiplex RT-PCR as the interpretation of the results is dependent on the melt curve analysis. As such any variation in the melting temperature of unknown samples compared to positive control makes interpretation difficult and may result in over-reporting. An alternative to SYBR green chemistry is the use of hydrolysis probes as each of the probe is specific to a particular dengue serotype. Also the study involved collection of samples from only 2 hospitals in Selangor and thus is inadequate to give representation on the circulating dengue serotypes in the State. Ideally more hospitals from the State should have been included to give an overall representation on the circulating dengue serotypes in Selangor. But this is beyond our control as only two hospitals were willing to participate in the study.

This study has shown that the most dominant serotype in two hospitals in Selangor was DENV-1. Even though the sample size was inadequate to represent the scenario for Selangor, the result seems to correspond with that for Malaysia in 2011 where dengue serotype 1 was found to be the pre dominating serotype (Ministry of Health Annual Report 2011).

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