Dengue virus surveillance: circulation of DENV-1, DENV-2, DENV-3 and DENV-4 in mosquitoes around Taman Connaught, Cheras

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Abstract. Dengue, a mosquito-borne viral infection that causes dengue fever, hemorrhagic fever and dengue shock syndrome, is on a drastic rise in recent years. In Malaysia, the distribution of dengue viruses is determined based on localized studies, where these data are essential to predict the epidemiology of dengue infections. Therefore, a study was conducted in Taman Connaught, Cheras, determining dengue virus serotypes in field caught *Aedes* mosquitoes. Out of 1,147 mosquitoes captured from September to December 2017, 156 *Aedes* spp. mosquitoes were identified based on their physical features using a digital microscope. Total RNA was then extracted from the head and thorax of the mosquitoes and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with DENV-1, DENV-2, DENV-3 and DENV-4 primer sets, respectively. The results show that at least one DENV serotype was detected in 91% (n=142) out of 156 *Aedes* spp. mosquitoes. The predominant serotype detected was DENV-2 [47.44% (n=74)], followed by DENV-4 [8.97% (n=14)], DENV-3 [7.05% (n=11)] and DENV-1 [3.85% (n=6)], respectively. Co-circulation of DENV-2 and -4 was the predominant co-infection detected in *Aedes* spp. mosquitoes. We also present the first report of DENV-1, -2 and -4 co-infection in field-caught *Aedes* spp. Localized surveillance mechanisms based on serotype circulation in the mosquito vector can be an effective monitoring system for the government and public health sector in order to reduce the incidence of dengue hotspots.

Keywords: *Aedes* spp., dengue virus, RT-PCR, serotypes, surveillance

INTRODUCTION

The *Aedes* mosquito is the vector for the dengue virus, which is the etiological agent for dengue fever, hemorrhagic fever and dengue shock syndrome. Dengue is now the most epidemiologically-important viral mosquito-borne disease in the world, with a steady increase in the number of dengue cases globally (World

Health Organization, 2019; Armstrong *et al.*, 2003).

Four dengue virus serotypes, DENV-1, DENV-2, DENV-3 and DENV-4, found at different frequencies in different geographic regions, are associated with the epidemic potential of DENV (Quintero-Gil *et al.*, 2014). DENV serotypes use the same putative receptors in the

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mosquito midgut, where the susceptibility of DENV infection in mosquitoes can alter the accuracy of predictions on DENV transmission (Carrington *et al.*, 2014). Some viral variants may have a strong fitness advantage, such as better ability to produce and or utilize genetically different polymerases or capsid proteins (Pepin *et al.*, 2008). This intra-host competition within the mosquito vector can occur between different serotypes of the same virus (Quintero-Gil *et al.*, 2014).

Virus surveillance is crucial in detecting and predicting infection outbreaks, where it is an important infection-control tool to understand spatial or temporal infection dynamics and evaluate control interventions (Pérez-Castro *et al.*, 2016). Virus surveillance can also serve as an important mechanism in the identification of viruses with higher virulence in studies of natural viral evolution (Cologna *et al.*, 2005).

In Malaysia, the Dengue Situation Update for July 2019 reported a half-year incidence of 62,421 cases as of 29th June 2019, which is nearly double the incidence reported for the same duration in 2018 (World Health Organization, 2019). The extraordinary rise of dengue cases from year to year has been reported in previous studies where records show a 160% increase in dengue cases reported within the Kuala Lumpur metropolitan area from 2015 to 2016. Of a total of 8664 dengue cases reported in 2016, the Cheras district contributed 20% of these dengue cases (Mahmud *et al.*, 2018) in comparison to other urban districts within Kuala Lumpur. Cheras was previously reported with the highest number of dengue cases in Kuala Lumpur, with the prevalence of all four DENV serotypes (Chew *et al.*, 2012).

Hence, data on vector carriage may be useful in developing effective intervention strategies in dengue hotspot areas. Thusly, we report the surveillance of dengue-infected mosquito populations around Taman Connaught in Cheras to identify dengue virus serotypes at risk for transmission from vectors to humans.

MATERIALS AND METHODS

Specimen collection and identification

Convenience sampling of 1,147 field-caught mosquitoes was carried out from September 2017 to December 2017 (4 months) within a one km radius sampling area in Taman Connaught, Cheras which covered commercial, residential and recreational facilities. Five commercial ultraviolet light-emanating mosquito traps were placed at sheltered strategic locations around the sampling area, along building corridors with a high frequency of daily human activity. Mosquitoes were continuously collected in the mornings between 8-10am throughout the 4 months, stored in sterile containers kept on ice, then transported to the laboratory for species identification on the same day. Mosquitoes were categorized into *Aedes* and non-*Aedes* mosquitoes using a digital microscope (Dino-Lite Edge, AnMo Electronics Corporation, Taiwan) at 80X magnification, where the physical morphology of mosquitoes was determined based on the guidelines of Walter Reed Biosystematics Unit, by accessing information available at http://www.wrbu.org/mqID/mq_gnra/aedes.htm. Once identified, mosquitoes were stored at -80°C prior to RNA extraction.

RNA extraction and reverse transcription

Viral titers of dengue are high in the head, thorax (where the salivary glands are located) and midgut of the mosquito (Tsai *et al.*, 2017), which provide reliable dengue positivity screening via molecular methods (Pankhong *et al.*, 2002; Thavara *et al.*, 2006). Thus, in the present study, the head and thorax of individual *Aedes* mosquitoes were dissected using a forceps and scalpel under a digital microscope (Dino-Lite Edge, AnMo Electronics Corporation, Taiwan) and used for subsequent reverse-transcription Polymerase Chain Reaction (RT-PCR). RNA extraction was done for the head and thorax of individual mosquitoes using TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Reverse transcription was then carried out in 20 µL reactions. Briefly, 0.5 µg of total extracted RNA and 1 µL of 0.2 µg of 100 pmol random hexamers (Thermo Fisher Scientific, USA) were added to PCR tube and topped up

with 0.1% DEPC water. Subsequently, the mixture was added with 4 μ L of 5X reaction buffer, 0.5 μ L of 20 U/ μ L RNase inhibitor, 2 μ L of 10mM dNTP, and 1 μ L of 200 U MMLV-Reverse Transcriptase (all from Thermo Fisher Scientific, USA). The resulting reverse-transcribed cDNA was used for subsequent PCR confirmation of *Aedes* spp. and PCR screening of DENV serotypes 1 to 4.

PCR confirmation of *Aedes* spp.

PCR primers to amplify the consensus region of the small unit ribosomal RNA (18S rRNA) gene of *Aedes* spp (as in Table 1) were designed based on the GenBank IDs AY988440.1 (*A. aegypti*) and HQ010437 (*A. albopictus*), respectively. PCR was carried out in 25 μ L reactions with 2.5 μ L of 10X Standard *Taq* Reaction Buffer, 0.5 μ L of 10 mM dNTP, 0.125 μ L of 5000 U/mL *Taq* DNA polymerase (all from New England Biolabs, USA), 0.5 μ L of each 10 μ M forward and reverse primers (IDT, Singapore), and 1.0 μ L of reverse-transcribed cDNA, and then topped up with nuclease-free water. The PCR reactions were subjected to the following cycling conditions in a thermal cycler (Nexus Gradient, Eppendorf, Germany): initial denaturation at 95°C for 30 s; then 35 cycles of denaturation at 95°C for 15 s, annealing at 53.3°C for 30 s, and extension at 68°C

for 30 s; followed by a final extension at 68°C for 5 min. PCR products were analyzed using 1% agarose gel electrophoresed in standard Tris Borate EDTA buffer at 80 V for 30 min, and visualized under ultraviolet light (Quantum ST4 Transilluminator + Quantum Capt Softwares, Viber Lourmat, France). Representative PCR products were gel-purified (GF-1 Gel DNA Recovery Kit, Vivantis Technologies Sdn. Bhd, Malaysia) for DNA sequencing (MyTACG, Taiwan) with the respective forward and reverse primers for PCR amplicon confirmation.

PCR screening of DENV-1 to DENV-4

PCR primers previously designed by Tanaka (1993) were used to detect DENV-1, DENV-2, DENV-3 and DENV-4 in *Aedes* spp.-positive samples. PCR was carried out using the cDNA of individual mosquitoes as before, with the exception of the specific DENV primers and the respective annealing temperature (Table 1). Every PCR run for a specific DENV included the respective DENV-specific positive control (a kind gift from Dr. Chee Hui Yee of Universiti Putra Malaysia), and a no-template control. DENV positive controls and representative were confirmed for primer specificity via DNA sequencing with the respective DENV primers.

Table 1. All PCR primers used in this study.

Primers	Sequences (5'-3')	Optimized annealing temperature (°C)	PCR product size (bp)
<i>Aedes</i> spp. 18S rRNA	F: 5'-CATCGTGGTTGACTTCTGCT-3' R: 5'-AAAGGGCAGGGACGTAATC-3'	53.3	262
DENV-1 ^[16]	F: 5'-GGACTGCGTATGGAGTTTGTG-3' R: 5'-ATGGGTGTGGCCTAATCAT-3'	49	490
DENV-2 ^[16]	F: 5'-GTTCTCTGCAAACACTCCA-3' R: 5'-GTGTTATTTTGATTTTCCTTG-3'	45	230
DENV-3 ^[16]	F: 5'-GTGCTTACACAGCCCTATTT-3' R: 5'-TCCATTCTCCCAAGCGCCTG-3'	51.5	320
DENV-4 ^[16]	F: 5'-CCATTATGGCTGTGTTGTTT-3' R: 5'-CITTCATCCTGCTTCACTTCT-3'	46	398

RESULTS

Out of a total of 1,147 mosquitoes caught in this study, 156 were morphologically identified as

Aedes spp., of which 74.7% were *A. aegypti* while 18.6% were *A. albopictus*.

The average concentration of the extracted RNA from the 156 *Aedes* spp. individuals was 90 ng/ μ L. Reverse transcription-PCR amplification

with 18S rRNA primers specific for *Aedes* spp. confirmed the morphological identification of all individuals as *Aedes* spp. (Figure 1).

Figure 1. Representative agarose gel electrophoresis image of *Aedes* spp. 18S rRNA PCR products. Lanes 2 to 9: PCR products of samples positive for the *Aedes* spp. 18S rRNA (~262bp), Lane 1: 100bp DNA ladder marker (New England Biolabs, USA).

Subsequent PCR with DENV-1 to DENV-4 primers specific to the respective dengue virus serotypes (Figures 2A and 2B) revealed that out of 156 *Aedes* spp., DENV-2 was the most prevalent single serotype detected [47.4%, n=74], followed by DENV-4 [9% (n=14)], DENV-3 [7.1% (n=11)] and DENV-1 [3.8% (n=6)], respectively (Table 2). Fourteen mosquitoes (9%)

did not carry any of the four dengue virus serotypes.

Our results also revealed multiple dengue virus serotypes in 37 mosquitoes (23.7%), where DENV-2 + DENV-4 was the predominant co-infection detected (Table 3), occurring in 12 individuals. Co-detection of up to three dengue virus serotypes (DENVs-1, -2 and -4) was observed in four mosquitoes.

Figure 2A. Agarose gel electrophoresis picture for DNA of positive controls, from P1 to P4, tested with four dengue primer pairs through PCR reaction. Lane 2 to 5: PCR products of positive controls of DENV-1 to DENV-4, for P1 (~490bp), P2 (~230bp), P3 (~320bp), and P4 (~398bp), Lane 1: 100bp DNA ladder marker (NEB, USA).

Table 2. *Aedes* spp. and DENV serotypes detected in this study. *Aedes aegypti* is the predominant *Aedes* spp. (74.4%), while DENV-2 is the predominant serotype (47.4%) in this study.

<i>Aedes</i> species	DENV serotypes detected in individual mosquitoes [%]						Total
	DENV-1	DENV-2	DENV-3	DENV-4	Co-detected DENV [^]	No DENV	
<i>A. aegypti</i>	5 [3.2]	48 [30.8]	10 [6.4]	14 [9.0]	27 [17.3]	12 [7.7]	116 [74.4].
<i>A. albopictus</i>	0	19 [12.2]	1 [0.6]	0	7 [4.5]	2 [1.3]	29 [18.6]
Other*	1 [0.6]	7 [4.5]	0	0	3 [1.9]	0	11 [7.1]
Total	6 [3.8]	74 [47.4]	11 [7.1]	14 [9.0]	37 [23.7]	14 [9.0]	156

*Other *Aedes* spp.: *A. togoi* and *A. pseudoniveus*

[^]Refer to Table 3

Table 3. DENV serotypes co-detected in this study. DENV-2 + DENV-4 are the predominant co-detected serotypes (32.4%) in this study.

DENV serotype	Mixed dengue serotypes detected in individual mosquitoes [%]			
	DENV-1	DENV-2	DENV-3	DENV-4
DENV-1	/	2 [5.4]	1 [2.7]	-
DENV-2	6 [16.2]	/	-	1 [2.7]
DENV-3	-	2 [5.4]	/	4 [10.8]
DENV-4	1 [2.7]	12 [32.4]	4 [10.8]	/
DENV-1 + DENV-2	-	-	-	4 [10.8]

Figure 2B. Agarose gel electrophoresis image for DENV-1 to DENV-4 primer sets testing on representative mosquito. Lane 3 to 7, Lane 13 to 17, Lane 20 to 24, and Lane 30-34: PCR products of Mos 7, 9, 10, 11, and 12 tested by DENV-1 to DENV-4 primer sets, respectively. Lane 1, Lane 11, Lane 18 and Lane 28: negative controls. Lane 2, Lane 12, Lane 19, and Lane 29: positive controls, P1 to P4. Lane 9 and 26: 100bp DNA ladder marker (NEB, USA).

DISCUSSION

Of all 156 *Aedes* spp. mosquitoes caught, 142 tested positive for single or multiple dengue virus serotypes. The number of *A. aegypti* captured as compared to *A. albopictus* in this study is consistent with the general consensus that *A. aegypti* is gradually overtaking the native species of *A. albopictus* within the region (Rudnick, 1965). While *A. aegypti* is considered the primary vector for dengue, both species are significant vectors for arboviruses and are capable of carrying multiple arbovirus infections such as Chikungunya and Zika (Cogan, 2019).

A previous study in a hospital setting in Cheras found that although co-circulation of all four DENV serotypes were present, DENV-4

was the predominant serotype within 46 dengue positive cases with 97% being single or dual infection with DENV-4 (Chew *et al.*, 2012). The present study, which is the first report of dengue virus serotypes in *Aedes* spp. in Cheras, found that DENV-2 (47.4%) was the predominant serotype in the vector instead, followed by DENV-4 (9%).

From general clinical data, there is observed seasonal drift in dominant dengue serotypes over the years with DENV-3 dominating in 2014 and DENV-4 gaining prominence in recent years, whilst still constituting less than 20% of all serotypes between 2000-2012 (Chew *et al.*, 2012; Mohd-Zaki *et al.*, 2014; Cogan, 2019). However, there has yet to be a clear correlation between the dominant DENV serotype found in infected humans and the dominant DENV serotype within mosquitoes, as infection dynamics depend

on the number of infected mosquitoes, proximity to humans, weather patterns, virological factors and other elements (Bar-Zeev *et al.*, 1977; Cogan, 2019)

Co-infections are associated with more severe clinical manifestations of the disease, most notably with increased likelihood of developing Dengue Hemorrhagic Fever (DHF) (Gubler, 1998). The circulation of multiple DENV serotypes in mosquito vectors may increase both the occurrence of co-infection within humans and also the risk of subsequent secondary infection with a different dengue serotypes, leading to poorer prognoses (Dhanoa *et al.*, 2016). DENV-2 + DENV-3 co-infections in individual mosquitoes (10 mosquitoes with DENV-2 + DENV-3 of 15 total co-infections) were previously reported in Selangor (Lau *et al.*, 2015).

In a 2016 study conducted in Johor Bahru (Malaysia) using 262 Dengue positive patient sera samples, 40 (15%) of samples were identified as co-infections (with DENV-1/DENV-2 contributing 85% of co-infections) (Dhanoa *et al.*, 2016). The prevalence of several serotypes within a community or region collectively increases the impact of seasonal dengue outbreaks and co-infections and monitoring of dengue virus within the mosquito population and subsequent control measures may prove to be a vital precautionary step and serve as an early warning system to thwart the increasing burden of dengue outbreaks.

The present study found serotype co-infections in 37 individual *Aedes* spp. mosquitoes, with DENV-2 + DENV-4 as the prevalent co-infection. Interestingly, our study reports four individual *Aedes* spp. mosquitoes co-infected with three DENV serotypes (DENV-1, -2 and -4) which, to our knowledge, is the first such report of tri-DENV co-infections in field-caught *Aedes* spp.

CONCLUSION

In conclusion, this study suggests that DENV-2 is the predominant DENV serotype in the Taman Connaught area, with a large number of mosquitoes carrying co-infections of dengue serotypes. Data on localized DENV serotype circulation in mosquito vectors has the potential

provide an effective monitoring mechanism to the government, public health officials and the general public in order to develop better containment and dengue eradication strategies targeting the dengue mosquito vector.

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