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Molecular laboratory testing for dengue serotypes at Bamrasnaradura Infectious Diseases Institute

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Abstract Dengue virus (DENV) infection is a major serious health problem in many tropical countries including Thailand. DENV comprises of 4 serotypes (DENV-1 to DENV-4) and co-circulation of DENV and Chikungunya virus (CHIKV) has been reported in areas where dengue is common. This study aims to investigate genetic materials of DENV and CHIKV including serotype distribution of DENV in dengue-suspected patients using real-time polymerase chain reaction (real-time PCR). The study was conducted during 2016-2017 at Bamrasnaradura Infectious Diseases Institute and a total of 100 patients (29 children and 71 adults) were enrolled. The laboratory findings in dengue-suspected patients was screened by platelet count of ≤ 100,000 cells/μl, DENV NS1 antigen positive, or anti-DENV IgM positive. From 100 DENV-suspected cases, 37 cases were detected for DENV by real-time PCR. No CHIKV was detected. The results showed that 27 cases (73%) were admitted to the hospital, 18 cases (48.6%) had platelet count <100,000 cells/μl, 37 cases (100%) had NS1 antigen positive and 17 cases (45.9%) had anti-DENV IgM positive. The distribution of DENV-2, DENV-4, DENV-3 and DENV-1 serotypes was 43.2%, 32.4%, 18.9% and 5.4%, respectively. Rapid screening test results for NS1 antigen were concordant with those by real-time PCR. Serotype distribution using molecular testing could be useful for studies on correlation between viral diversity and host immune response.

Keywords: dengue, serotype, molecular testing

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Introduction

Dengue virus (DENV) infection has been becoming a serious public health problem in many tropical countries[1-3] including Thailand. Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are characterized by increased vascular permeability and are causes of death[4-6]. Fever caused by Chikungunya virus (CHIKV) shares some clinical signs with dengue and can be misdiagnosed in areas where dengue is common[7]. DENVs are positive stranded-RNA viruses belonging to genus *Flavivirus*, family *Flaviviridae*. All serotypes of DENV are transmitted by mosquitoes *Aedes aegypti and Aedes albopictus*. Since *Aedes* mosquitoes can be vectors of both DENV and CHIKV, and endemic areas of these two viruses often overlap, co-circulation of DENV and CHIKV has been reported in various geographic areas[7, 8]. DENVs are classified into 4 serotypes, DENV-1, DENV-2, DENV-3 and DENV-4[9] with a broad range of symptoms[5, 6]. Factors that are important for a spread of disease vary in each area depending on host immunity, type of virus and vector, population density, and mobility[5]. Awareness on the removal of mosquito breeding sites is essential to prevent and control dengue outbreaks[5].

The dengue virus has been a major health problem in Thailand since the first outbreak in 1958[10-11]. There was an epidemic of dengue diseases widespread among the country[12]. Co-circulation of 4 serotypes of DENV was also demonstrated in Bangkok[11]. A meta-analysis of dengue severity in Southeast Asia region showed that DENV-3 and DENV-4 were strongly associated with dengue hemorrhagic fever (DHF) whereas DENV-2 and DENV-4 were strongly associated with dengue shock syndrome (DSS)[13].

The objective of the present study was to investigate the distribution of DENV and CHIKV circulating among clinically suspected patients at Bamrasnaradura Infectious Diseases Institute and to identify the serotype of DENV using real-time PCR.

Materials and Methods

Ethical considerations

The study was approved by the Institutional Review Board of Bamrasnaradura Infectious Diseases Institute on August 5, 2016 with the project code S003h/59.

Study site and population

This study was conducted at Bamrasnaradura Infectious Diseases Institute, Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand. A prospective study with suspected dengue patients was conducted between 2016 and 2017. The study included 100 febrile cases of children aged between 7 and 15 years and adults aged between 15 and 60 years, whose parents or patients themselves gave written informed consent to be enrolled. Dengue suspected symptoms were identified by following laboratory findings; platelet count less than or equal to 100,000 cell/μl, dengue NS1 antigen-positive, or anti-dengue IgM-positive. The participants should have at least one DENV-confirmed laboratory finding as inclusion criteria.

Laboratory testing

SD Bioline Dengue NS1 Antigen immuno-chromatography assay (SD Bioline, South Korea) is designed to detect DENV NS1 antigen with one step assay. Panbio Dengue Duo cassette (Panbio, Australia) was used to detect Dengue IgM and IgG in serum.

DENV serotyping and CHIKV detection by real-time PCR were performed according to the manufacturer's instructions. Briefly, 400 µl of serum specimens were extracted for total nucleic acid [MagnaPure Compact, (Roche Diagnostics GmbH, Germany)] and detected by DENV-CHIKV multiplex real-time reverse transcription polymerase chain reaction (RT-PCR) assay (*ab*TES™ DENV/CHIKU5 qPCR I kit, AITbiotech, Singapore) using a CFX96™ real-time PCR cycler (Bio-Rad, Hercules, CA, USA). Five microliter (µl) of extracted nucleic acid was added to 20 µl of PCR mixture which was composed of reaction buffer, reverse transcriptase/*Taq* enzyme mix, primer/probe mix, PCR enhance template and Nuclease-free water. PCR cycling conditions were established and validated on Bio-Rad CFX96 as indicated in the package insert. It was composed of 3 phases in cDNA synthesis for 1 cycle at 53°C for 10 minutes, *Taq* activation for 1 cycle at 95°C for 2 minutes 30 seconds, amplification for 42 cycles at 95°C for 17 seconds, 59°C for 31 seconds, 68°C for 32 seconds. The signal detection of DENV-1 to 4 and CHIKV were determined at Cy5, FAM, Texas Red, Quasar 705 and HEX channels, respectively. Positive and negative controls were included in every run of PCR. Nuclease-free water was used as a negative control. The result was regarded true negative for DENV-1, DENV-2, DENV-3, DENV-4 and CHIKV when the CY5, FAM, Texas Red, Q705 and HEX channels were not detected. An amplification signal detected in the respective fluorescence channel was considered as a positive result.

Data analysis

For descriptive analyses, data were summarized for median and ranges for hematological parameters. The percentage was used for categorical variables and calculated for serotype distribution among 100 dengue suspected patients.

Results

Sample population and testing results

A total of 100 febrile cases of 7 to 58 years old with suspected dengue infection between 2016 and 2017 were enrolled in the present study. There were 29 children (14 boys and 15 girls) and 71 adults (30 males and 41 females) participated in this study. Study population was 65% of in-patient department (IPD) cases and 35% of out-patient department (OPD) cases. Laboratory testing of NS1 antigen-positive, anti-dengue IgM-positive, and platelet count less than 100,000 cells/µl were 59, 67 and 29 cases, respectively. The median of fever duration was 4 days (1-9 days). The median of white blood cell counts on the blood collection date was 4,750 cells/µl (1,600-17,900 cells/µl) and the median of percentage of hematocrit was 42% (27-53%).

For laboratory findings of at least 1 inclusion criteria, there were 37 cases (100%) with NS1 positive, 17 cases (45.9%) with IgM positive and 18 cases (48.6%) with platelet less than 100,000 cells/µl. Among negative DENV RNA cases, there were 37 IPD cases (58.7%) and 26 OPD cases (41.2%). There were 22 cases (34.9%) with NS1 positive, 50 cases (79.3%) with IgM positive, and 11 cases (17.5%) with platelet less than 100,000 cells/µl. All dengue suspected cases with inclusion criteria of laboratory findings were summarized in Figure 1.

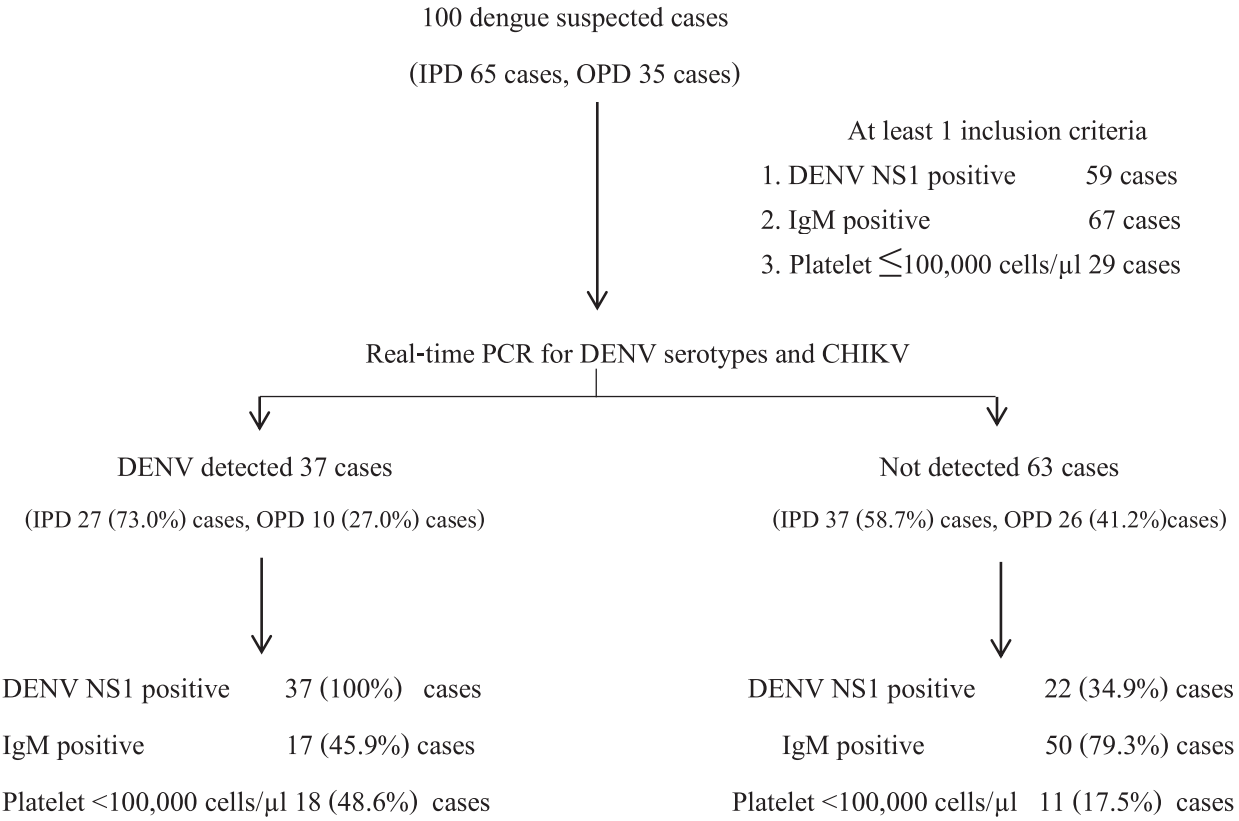

Figure 1. Results of laboratory findings of cases enrolled with at least one inclusion criteria.

The median age of 37 DENV RNA positive cases was 22 years (8-54 years). The median of fever duration was 3 with a range from 1-6 days. The median of white blood cell counts was 3,700 cells/µl (1,600-9,600 cells/µl) and for hematocrit was 43% (31-59%). The median of platelet count was 108,000 cells/µl (21,000-293,000 cells/µl). Laboratory testing for DENV NS1 antigen was positive in all 37 cases (100%). DENV IgM and IgG antibody was positive for 17 (45.9%) and 14 (37.8%) cases, respectively. The results were summarized in Table 1.

Table 1. Laboratory findings and real-time PCR of DENV serotypes of 37 DENV detected cases.
[+ : positive result, - : negative result, NA : No Assay]

ID	Gender	Age (Years)	Duration of fever (Days)	Platelet (cells/ μL)	DENV NS1 Ag	Anti-DENV IgM	Anti-DENV IgG	Serotype
DENV001	M	40	3	83,000	+	NA	NA	DENV-4
DENV005	F	40	3	129,000	+	-	-	DENV-2
DENV006	M	22	4	59,000	+	+	+	DENV-4
DENV008	M	32	6	21,000	+	NA	NA	DENV-4
DENV009	F	29	5	56,000	+	NA	NA	DENV-2
DENV010	F	37	4	142,000	+	+	+	DENV-4
DENV011	F	24	3	167,000	+	-	-	DENV-2
DENV012	M	41	3	92,000	+	+	+	DENV-4
DENV014	F	17	5	79,000	+	NA	NA	DENV-2
DENV016	F	54	1	184,000	+	-	-	DENV-3
DENV019	M	22	4	79,000	+	+	+	DENV-3
DENV020	M	18	4	72,000	+	-	-	DENV-4
DENV023	M	22	3	51,000	+	+	+	DENV-2
DENV025	F	8	3	81,000	+	+	+	DENV-3
DENV026	F	22	5	29,000	+	-	-	DENV-2
DENV031	F	33	4	68,000	+	-	-	DENV-1
DENV035	M	37	4	293,000	+	-	-	DENV-2
DENV037	M	22	2	139,000	+	NA	NA	DENV-4
DENV038	F	23	3	119,000	+	NA	NA	DENV-2
DENV044	F	33	5	31,000	+	+	+	DENV-2
DENV046	M	10	5	174,000	+	NA	NA	DENV-4
DENV049	M	17	2	156,000	+	NA	NA	DENV-3
DENV052	F	15	5	29,000	+	NA	NA	DENV-2
DENV055	F	29	3	192,000	+	+	-	DENV-3
DENV056	M	21	3	163,000	+	NA	NA	DENV-2
DENV059	M	13	2	149,000	+	-	-	DENV-3
DENV061	M	20	3	196,000	+	+	+	DENV-2
DENV066	F	26	5	148,000	+	NA	NA	DENV-3
DENV074	M	48	2	162,000	+	+	-	DENV-2
DENV077	M	10	2	210,000	+	+	+	DENV-2
DENV086	F	9	5	131,000	+	+	+	DENV-2
DENV087	F	16	3	99,000	+	-	-	DENV-1
DENV088	M	9	5	23,000	+	+	+	DENV-4
DENV091	M	15	2	91,000	+	+	-	DENV-4
DENV092	M	8	2	50,000	+	+	+	DENV-4
DENV093	F	39	4	150,000	+	+	+	DENV-2
DENV098	F	9	2	108,000	+	+	+	DENV-4

Real-time PCR results

The nucleic acid of dengue virus was detected in 37 serum specimens by real-time PCR while chikungunya virus was not detected. The number of cases of DENV-1, DENV-2, DENV-3 and DENV-4 were 2, 16, 7 and 12, respectively, as shown in Figure 2.

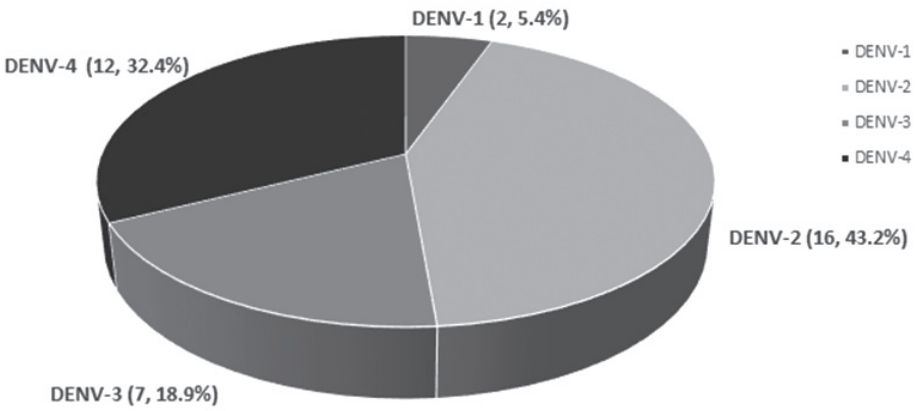

Figure 2. Dengue serotypes found in 37 serum specimens

Discussion

The co-circulation of multiple DENV serotypes occurred in Nonthaburi region, central part of Thailand, during 2016 to 2017, was shown in this study. All serotypes of DENV were found while CHIKV was not detected. DENV-2 was the most prevalence serotype (43.2%) found in our study, which was similar to another study conducted during May – October 2016. DENV-2 was predominantly found in 3 hospitals in the southern part of Thailand[14]. In contrast, a previous study showed that all 4 DENV serotypes co-circulated among 5 hospitals in Bangkok during 2015-2016 with DENV-4 as the predominant serotype[15]. The majority of serotype distribution was 45% of DENV-4, followed by 29% of DENV-3, 17% of DENV-2 and 8% of DENV-1[15], while DENV-2 was most frequently detected in our study. It indicated that the predominant DENV serotype varied by year with seasonal fluctuation concordance. Therefore, to determine the serotypes that spread in each endemic area where DENV can be repeatedly infected is essential[16, 17].

Dengue infection can be diagnosed by both serological test and molecular method. The dengue RNA can be detected in the acute phase of serum samples when patients developed clinical symptoms such as fever. DENV isolation, dengue antigen detection, and/or dengue antibody detection can be found in dengue-confirmed cases with clinical manifestation[5]. Our present study could demonstrate that all 37 DENV NS1 antigen positive samples, collected within 6 days after the onset of fever were DENV-positive by qPCR kit, suggesting that real-time PCR can be used to detect dengue infection within the period of dengue viremia.

This study indicated that the molecular testing provided more accurate diagnosis of dengue infection. It is also implied that serotype distribution and further genotype analysis could possibly provide information on a correlation of viral diversity and host immune response especially antibody dependent enhancement.

Conclusion

Our study demonstrated all 4 serotypes were circulated throughout the year. Information on serotype distribution would be beneficial for epidemiological studies for disease control and prevention.

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การตรวจสายพันธุ์ของเชื้อเดงกี ด้วยวิธีอณูชีวโมเลกุลที่สถาบันบำราศนราดูร

สุมนมาลย์ อุทยมกุล รวี นิธิยานนทกิจ สุภัทรษา เดชสถิตย์ ศาริณี เรี่ยวแรง ปฐมา สุทธา และวิศัลย์ มูลศาสตร์

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บทคัดย่อ โรคไข้เลือดออกที่เกิดจากเชื้อไวรัสเดงกี (Dengue virus: DENV) เป็นปัญหาสำคัญในประเทศแถบเขตร้อน รวมถึงประเทศไทย สายพันธุ์ไวรัสเดงกีประกอบด้วย 4 serotype (DENV-1 ถึง DENV-4) และมีรายงานพบการแพร่กระจายของ DENV และ Chikungunya virus (CHIKV) ในพื้นที่ที่มีการระบาดของไข้เลือดออก การศึกษานี้มีวัตถุประสงค์เพื่อตรวจหาสารพันธุกรรมของ DENV และ CHIKV ด้วยวิธี real-time polymerase chain reaction (real-time PCR) ในผู้มารับการรักษาที่สถาบันบำราศนราดูรที่สงสัยไข้เลือดออก ระหว่างปี พ.ศ. 2559-2560 โดยคัดเลือกผู้ป่วยที่ตรวจพบเกล็ดเลือด ≤100,000 เซลล์/ไมโครลิตร หรือพบผลบวกของ DENV NS1 antigen หรือ anti-DENV IgM จำนวน 100 ราย แบ่งเป็นผู้ใหญ่ 71 ราย และเด็ก 29 ราย ผลตรวจวินิจฉัยการติดเชื้อ DENV และ CHIKV ด้วยวิธี real-time PCR ไม่พบการติดเชื้อ CHIKV แต่พบ DENV 37 ราย ซึ่งเป็นผู้ป่วยที่พักรักษาในโรงพยาบาล 27 ราย (ร้อยละ 73) ผู้ป่วย 18 ราย (ร้อยละ 48.6) มีเกล็ดเลือด <100,000 เซลล์/ไมโครลิตร DENV NS1 antigen ให้ผลบวก 37 ราย (ร้อยละ 100) และพบผลบวก IgM 17 ราย (ร้อยละ 45.9) ผลการตรวจด้วยวิธี real-time PCR พบสายพันธุ์ของ DENV-1 จำนวน 2 ราย (ร้อยละ 5.4) DENV-2 จำนวน 16 ราย (ร้อยละ 43.2) DENV-3 จำนวน 7 ราย (ร้อยละ 18.9) และ DENV-4 จำนวน 12 ราย (ร้อยละ 32.4) จากการศึกษาครั้งนี้พบว่ามีการกระจายของเชื้อไวรัสเดงกีทุกสายพันธุ์ และการตรวจหาสายพันธุ์ของเชื้อเดงกีด้วยวิธีอณูชีวโมเลกุลให้ผลวินิจฉัยที่ชัดเจน และสอดคล้องกับผลการตรวจแอนติเจน DENV NS1 การศึกษานี้แสดงว่าการจำแนกสายพันธุ์ด้วยวิธีอณูชีวโมเลกุลมีประโยชน์ต่อการศึกษาความสัมพันธ์ของความหลากหลายของไวรัสและการตอบสนองทางภูมิคุ้มกันของผู้ติดเชื้อไวรัสเดงกี