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A relationship between dengue virus serotype and the clinical severity in paediatric patients from Gondokusuman region, Yogyakarta between 1995 and 1999

Zależność między serotypem wirusa dengi a stopniem nasilenia objawów klinicznych u dzieci pochodzących z dzielnicy Gondokusuman w mieście Yogyakarta w latach 1995–1999

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Abstract

Aim of the study: Dengue infection occurs almost all over subtropical and tropical countries. Dengue pathogenesis explaining its clinical manifestations is still unclear. Indonesia is a country with several hyperendemic regions. The study was aimed to investigate the incidence rate, sero-epidemiology, and the relationship between the serotype and the clinical severity of dengue viral infection in paediatric patients from Gondokusuman, Yogyakarta. **Material and methods:** It was an epidemiological research with prospective observational design reviewing febrile paediatric patients involved in "A Prospective Sero-epidemiology Study on Dengue Children Infection in Yogyakarta, Indonesia, 1995–1999 cohort study." Febrile paediatric patients were diagnosed for dengue fever, dengue haemorrhagic fever, or dengue shock syndrome based on World Health Organization 1997 criteria. Serological diagnosis was performed using PRNT and serotype identification was performed by viral culture isolation and RT-PCR. **Results:** Laboratory data (PRNT, ELISA, RT-PCR and Isolation) showed that there were 220 children (130 males and 90 females) from 509 febrile patients among 2,149 paediatric subjects who were infected with dengue virus. Based on serotype identification, the following dengue virus serotype distributions were identified: DEN-1 26.81%, DEN-2 23.18%, DEN-3 22.72%, DEN-4 8.63%, and unidentified 18.63%. Clinical severities observed were as follows: dengue fever 78.6%, dengue haemorrhagic fever 18.2%, and dengue shock syndrome 3.2%. In the case of primary infection, only DEN-3 could cause severe clinical manifestations. **Conclusions**: Gondokusuman region in Yogyakarta could be classified as a hyperendemic region between 1995 and 1999, with the highest risk of severe clinical manifestations shown for DEN-3 during both, primary and secondary infection.

Key words: dengue virus, serotype, dengue fever, dengue haemorrhagic fever, dengue shock syndrome

Streszczenie

Cel badań: Zakażenie wirusem dengi występuje niemal we wszystkich krajach subtropikalnych i tropikalnych. Patogeneza dengi oraz przyczyny wystąpienia objawów klinicznych pozostają niejasne. Indonezja jest krajem, w którym znajduje się kilka obszarów hiperendemicznych. Celem badania było ustalenie częstości występowania, seroepidemiologii oraz zależności między serotypem a nasileniem objawów klinicznych zakażenia wirusem dengi u dzieci pochodzących z dzielnicy Gondokusuman w mieście Yogyakarta. **Materiał i metody:** Przeprowadzono prospektywne epidemiologiczne badanie obserwacyjne oceniające dzieci z gorączką uczestniczące w badaniu zatytułowanym „A Prospective Sero-epidemiology Study on Dengue Children Infection in Yogyakarta, Indonesia, 1995–1999 cohort study". U dzieci z gorączką rozpoznano gorączkę denga, gorączkę krwotoczną denga lub zespół wstrząsu dengi, zgodnie z kryteriami Światowej Organizacji Zdrowia z 1997 roku. Rozpoznanie serologiczne oparto na metodzie PRNT, serotyp identyfikowano poprzez izolację RNA z hodowli i analizę RT-PCR. **Wyniki:** Z danych uzyskanych w badaniach laboratoryjnych (PRNT, ELISA, RT-PCR i izolacja) wynika, że spośród 509 pacjentów z gorączką z grupy 2149 badanych osób 220 dzieci (130 chłopców i 90 dziewczynek) było zakażonych wirusem dengi. Na podstawie identyfikacji serotypów wirusa dengi określono rozkład dla każdego serotypu: DEN-1 26,81%, DEN-2 23,18%, DEN-3 22,72%, DEN-4 8,63% oraz niezidentyfikowany 18,63%. Nasilenie objawów klinicznych kształtowało się w następujący sposób: gorączka denga 78,6%, gorączka krwotoczna denga 18,2%, zespół wstrząsu dengi 3,2%. W przypadku zakażenia pierwotnego jedynie serotyp DEN-3 powodował wystąpienie ciężkich objawów klinicznych.

Wnioski: W latach 1995–1999 dzielnicę Gondokusuman w mieście Yogyakarta można było klasyfikować jako obszar hiperendemiczny, charakteryzujący się najwyższym ryzykiem wystąpienia ciężkich objawów klinicznych wywołanych serotypem DEN-3 podczas zakażenia pierwotnego i wtórnego.

Słowa kluczowe: wirus dengi, serotyp, gorączka denga, gorączka krwotoczna denga, zespół wstrząsu dengi

INTRODUCTION

Dengue infections are found in almost all tropical and subtropical regions and are difficult to eradicate. More than 2.5 million people are at risk of developing this disease and more than 100 countries are endemic. Dengue virus belongs to the *Flaviviridae* family, *Flavivirus* genus, which has four known genetic serotypes (DEN-1, DEN-2, DEN-3 and DEN-4). This virus has a positive-sense single strand RNA, approximately 11 kb long, encoding structural proteins (capsid, Caps; pre-membrane, PRM; and envelope, E) and seven non-structural proteins (NS) (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). The clinical manifestations of the disease caused by dengue virus infection are often not typical, for example they can resemble the flu, typhoid fever, leptospirosis, and malaria. The course of infection may be diverse, ranging from asymptomatic, with low-grade fever that is not specific (undifferentiated febrile illness), dengue fever (DF), to a more severe form, i.e. dengue haemorrhagic fever (DHF) with or without dengue shock syndrome (DSS)[1–3]. Due to the difficulty in using strict criteria for DHF, while many cases of severe DF occur at the same time, there is a need for reconsideration of this classification. A clinical prospective multicentre study in endemic areas supported by the World Health Organization/Special Programme for Research and Training in Tropical Diseases (WHO/TDR) reviewed the classification criteria for dengue. These studies indicate that there evidently are considerable differences in clinical and laboratory data between severe and non-severe dengue cases. However, it has been suggested to use new classification which divides non-severe cases into two groups: patients with and without warning signs, in order to determine observation, care, treatment and monitoring intensity as well as for the purpose of clinical trials on vaccines or medications. Both of these groups are likely to develop into a more severe cases[4].

The pathogenesis of the clinical manifestations of dengue virus infection is still unclear. This is evidenced by many hypotheses that try to explain, for example, the virulence, secondary infection (secondary heterologous infection) with antibody-dependent enhancement (ADE), endothelial cells and thrombocytopenia, the role of endotoxin, the role of cytokines, apoptosis, the bond between the virus and the host cell, as well as the role of Langerhans cells (LCs) and dendritic cells (DCs)[5–12].

In general, epidemiological studies in Indonesia after 1990 show that although all four serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) are present in Indonesia, dengue virus infections due to DEN-3 serotype are more prevalent. A study conducted by Malavige *et al.* shows, based on serological survey in Indonesia continued till the end of the 1980s, that the DEN-1 and DEN-2 are the main causes of infection. However, after the end of the decade of the 1980s, DEN-3 was the main cause of infection and the clinical manifestations of dengue virus infection[9]. A research conducted by Suwandono *et al.* in Jakarta, which investigated 53 DHF samples by means of PCR showed that all four serotypes were present in Jakarta with the following rate: DEN-3 57% (30/53), DEN-4 20.7% (11/53), DEN-2 13% (7/53), and DEN-1 5.6% (3/53)[13]. The results in Jakarta are in contrast with the findings of Aryati who analysed 25 samples with positive PCR observed in Surabaya, with a dominance of DEN-2 by 80% (20/25), followed by DEN-3 by 16% (4/25), and DEN-4 by 4% (1/25). A study conducted by Porter (2003) in Bandung shows that most infections caused by DEN-2 are accompanied by dengue fever as a clinical manifestation, while the clinical manifestations of DHF are caused by DEN-3[1]. This study aims to assess dengue virus infection sero-epidemiologic profile based on clinical features in febrile children with suspected dengue virus infection in Gondokusuman, Yogyakarta in 1995–1999.

MATERIAL AND METHODS

Research design

We have conducted an epidemiological prospective study with observational design. This research continued a cohort study entitled *A prospective seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia I. Studies in 1995–1996* by Graham *et al.*, 1999[14], which aimed to investigate the incidence rate and the degree of clinical manifestations in patients with dengue virus infection. This study re-assessed and re-validated patient data and samples collected between 1995 and 1999. These data and samples were stored at Naval Medical Research Unit No. 2 (NAMRU-2), Jakarta, Indonesia, and the study was performed between August and October 2011. Serological examination (PRNTs and RT-PCR) was also performed again, using liquid nitrogen tank-stored samples collected before. This research was approved by The Committee on Health Research Ethics, Ministry of Health, Indonesia, No. KS.02.01.2.1.1661.

Study area

Yogyakarta was a capital city of Yogyakarta Province with about 421,000 citizens. Province was divided into smaller

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territories called Kabupaten/city, which were again divided into smaller subdistricts called Kecamatan. This study was conducted in Kecamatan Gondokusuman, Yogyakarta City, and Yogyakarta Province, Indonesia. There were 2,772 dengue cases identified between 1990 and 1994 in Yogyakarta Province and 2,082 of these were diagnosed in children less than 15 years old. Kecamatan Gondokusuman was chosen because it had high incidence of children hospitalised due to dengue cases. Two primary health care services/Puskesmas (Puskesmas Gondokusuman I and II) and four city hospitals (Dr. Sardjito General Hospital, Bethesda, Panti Rapih and Muhammadiyah Private Hospital) were involved in this study[14].

Research population

Research subjects were children who participated in a cohort study *A prospective seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia I. Studies in 1995–1996* by Graham *et al.*, 1999, but with several inclusion criteria and sampling time differences as described below. A total of 2,149 children in Kecamatan Gondokusuman were involved in the study. The inclusion criteria were as follows: age between 4 and 13 years, children from Gondokusuman District with fever and a clinical diagnosis of DHF, DSS, or DF accompanied by evidence of dengue virus infection confirmed based on virus isolation from patient specimen. Exclusion criteria were as follows: a subject with DHF and DSS without evidence of infection based on virus isolation. Informed consent was obtained from each parent.

Cohort serum collection

Serological and molecular examinations were performed through serum collection. Venous blood was collected four times: 1) initial venous blood was collected in October 1995 (S1); 2) second collection was performed in October 1996 (S2); 3) third collection was performed in October 1997 (S3); and 4) fourth collection was performed in October 1998 (S4). According to Graham *et al.*, venous blood samples were collected in 5-mL heparin-containing Vacutainer tubes. Plasma was then separated by 1,600 × g centrifugation for 15 minutes. Plasma was stored at −20°C inside cryovial tubes[14].

Plasma for virus isolation and serology

Plasma sample preparation for virus isolation and serology has already been described before[14]. Acute-phase blood samples were collected in 5-mL citrated Vacutainer tubes (Becton Dickinson, Rutherford, NJ) from children who visited clinical facilities. Tubes were placed in 4°C refrigerator and delivered to Dr. Sardjito Hospital inside a cold chest. In laboratory, plasma was collected after 1,600 rpm centrifugation for 10 minutes. Plasma was placed in 2 sterile cryovials. Buffy coat and 0.5 mL plasma above the buffy coat were also collected and placed inside 2 sterile cryovials. Then, all samples were stored in liquid nitrogen. Convalescent phase plasma sample was also collected in Vacutainer tubes and processed as above. Liquid nitrogen tank was delivered to NAMRU-2 once per month[14].

Diagnosis of dengue and DHF/DSS

Blood samples were collected from participants when they had fever 38°C or above, suggesting dengue infection. Determination of patients with DHF, DSS, and DF were based on the diagnosis performed by a doctor in the clinic or hospital caregivers with reference to the WHO criteria (1997). All laboratory examination and data documentation was performed at the Laboratory of NAMRU-2 Jakarta, except for the evaluation of haematocrit and platelet count as a supporting clinical diagnosis performed in a Hospital involved in the research by a quantitative method using Coulter (Hialeah, FL) counter or the manual method.

All suspected DHF cases were reviewed by a paediatric specialist who was not involved in this study. DHF/DSS diagnosis was based on WHO 1997 criteria: two or more haematocrit assays that were 20% higher than the convalescent phase value and two or more platelet counts that were at or below 100,000/mm^3. Haemoconcentration due to plasma leakage was also determined by the identification of pleural effusion on upright chest radiograph. Cases that were not meeting the above criteria were defined as dengue fever[14].

Virus isolation

Virus isolation methods have already been described before by Graham *et al.* Briefly, acute phase plasma and buffy coats were used for virus culture in several types of medium: C6/36, Vero, baby hamster kidney (BHK)-21 (clone 13) tissue culture cells and mosquito intra-thoracic inoculation of *Toxorhynchites splendens.* Samples were diluted and dispensed in 24-well plates for three to five days. Then, the inoculated cells were transferred into a 15-mL plastic tube containing appropriate cell line for seven days. Fluorescence staining using specific monoclonal antibody was applied over glass slide. For *T. splendens* inoculation, 0.85 ml of undiluted plasma was inoculated for 14 days. The process was continued as described by Graham *et al.*, 1999[14].

Polymerase chain reaction (PCR)

Acute phase plasma and buffy coats were also sampled for PCR examination. PCR methods have already been clearly described before[14,15]. Viral RNA was extracted and reverse-transcribed resulting in cDNA. The obtained PCR product was subsequently subjected to a standard nested PCR using serotype specific oligonucleotides. The products were electrophoresed in 2% agarose gel and the amplicons were compared to standards.

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Plaque reduction neutralization tests (PRNTs)

PRNT methods have already been clearly described before[14,16]. The seropositive threshold for PRNT examination was 1:40 titres for blood samples taken on days 2–4 of fever and 1:80 titres for blood samples taken on day 5 of fever and later[2,3].

RESULTS

Epidemiology of dengue virus infection in Gondokusuman Yogyakarta between 1995 and 1999

The research involved 2,149 children, including 509 children with fever. Clinical examination performed by competent general practitioners, based on WHO 1997 criteria, identified 246 children (148 boys, 97 girls, and 1 child with unclear identity) which suspected dengue virus infection. The degree of clinical severity in children with suspected dengue virus infection was as follows: DF in 197 children (80.18%), DHF in 42 children (17.08%), and DSS in 7 children (2.84%) (Fig. 1 A).

Laboratory examination in 246 febrile paediatric patients suspected of having dengue virus infection was performed using PRNT in 244 children, RT-PCR in 146 children, and isolation/culture with C6/36, Vero, BHK-21 media, and intrathoracic inoculation of mosquitoes in 146 children, respectively (Tab. 1). These four techniques revealed that 26 children were not infected by dengue virus and 220 children were definitively diagnosed as suffering from dengue virus infection (later in the article they will be referred to as patients with dengue virus infection, Fig. 1 B). Based on gender classification, there were 130 (59.1%) boys and 90 (40.9%) girls, with boys/girls ratio of 1.45:1 (Fig. 1 C) among patients.

As shown in Tab. 1, positive results of each laboratory examination in 220 children with dengue virus infection were obtained in 89.49% by means of PRNT (196 of 219 tests), 50% using PCR (70 of 140 tests), and 29.29% using virus isolation (combined, 41 of 140 tests). The distribution of clinical severity in 220 patients with dengue virus

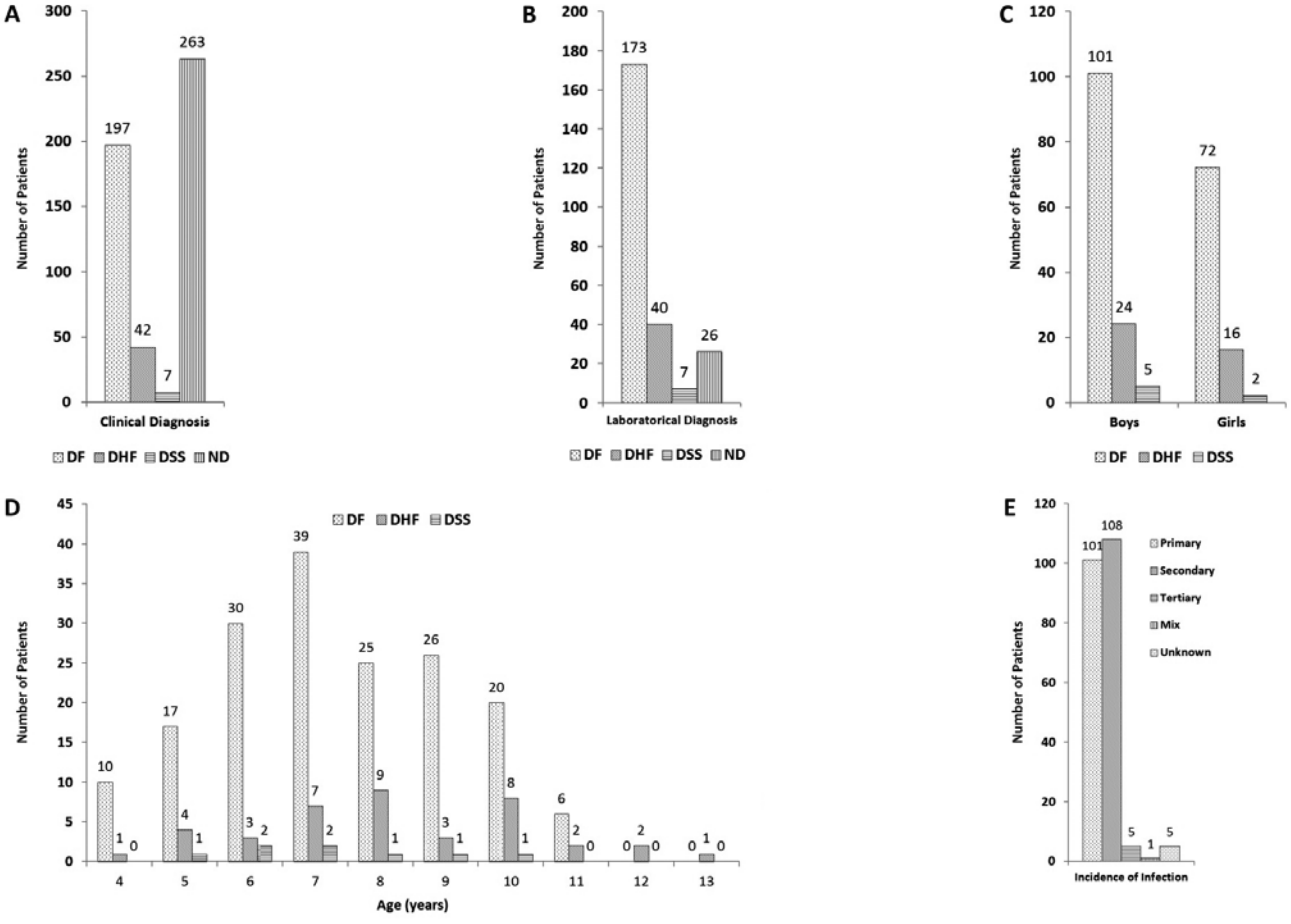

Fig. 1. A graph illustrating incidence of dengue virus infection and clinical manifestations developed by patients. **A.** *A graph shortening a number of febrile patients suspected with dengue virus infection based on clinical diagnosis.* **B.** *The incidence of clinical severities due to dengue virus infection after laboratory confirmation.* **C.** *Definite dengue patients and clinical severities according to sex (boys/girls) distribution.* **D.** *Clinical severities according to patient age groups.* E. *Dengue virus infection based on the incidence of virus infection.* **DF** *– dengue fever;* **DHF** *– dengue haemorrhagic fever;* **DSS** *– dengue shock syndrome;* **ND** *– not determined/undetermined*

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	Serology	PCR	Isolation				Total results
	PNRT		C6/36	Vero	BHK-21	MOSQ	
Examined	244	146	146	146	146	146	
(+)	196	70	33	9	5	27	220
(–)	48	76	113	137	141	119	26
Not examined	2	100	100	100	100	100	
Total	246	246	246	246	246	246	246

PRNT – plaque reduction neutralization tests; **PCR** – polymerase chain reaction; **BHK** – baby hamster kidney; **MOSQ** – mosquito.

Tab. 1. Laboratory examination in patients clinically suspected of dengue virus infection

infection was as follows: 173 (78.6%) DF cases, 40 (18.2%) DHF cases, and 7 (3.2%) DSS cases (Fig. 1 B). Data on the degree of clinical severity showed that the percentage of patients with mild clinical symptoms (DF) was higher in younger (4–9 years) age group than in older (≥10 years) age group as shown in Fig. 1 D. Patients aged 4–9 years accounted for 81.66% (147/180 patients) of those suffering from DF with the highest percentage of 4-year-old children (90.9%), and 65% in the 10–13 years age group (26/40 patients). Older children had higher risk of suffering from severe dengue infection (DHF and DSS) compared to younger children. The incidence of DHF and DSS was 35% in children aged 10–13 years (14/40 patients, all children aged 12 and 13 years suffered from DHF), whereas the incidence of DHF and DSS was18.33% in the group of children aged 4–9 years(33/180 patients), as shown in Fig. 1 D.

The incidence of dengue virus infection

From 220 patients with dengue virus infection, 101 children (45.9%) were found to be suffering from primary dengue infection, 108 children (49.1%) had secondary infection, 5 children (2.27%) were diagnosed with tertiary infection, 1 child (0.45%) had mixed infection, while the type of infection remained unknown in 5 patients (Fig. 1 E). The percentage of primary infection in the 4–9 years age group was 48.33%, and 35% in 10–13 years age group. The percentage of secondary infection in the two groups was 46.67% and 60%, respectively. These data indicate that the percentage of primary infection was higher in younger age group of children than in the older age group, while the percentage of secondary infection was higher in the older age group of children compared to younger children. Studies on the incidence of infection in relation to the degree of clinical manifestations in patients showed that the percentage of patients with mild clinical degree was higher in younger children than in older children (81.66% *vs.* 65%), while the percentage of patients with severe clinical degree (DHF and DSS) was lower in younger children compared to older children (18.33% *vs.* 35%).

The relationship between dengue serotypes and the clinical severity

Dengue virus serotypes that infected 220 patients included: DEN-1 in 59 children (26.81%), DEN-2 in 52 (23.63%), DEN-3 in 50 (22.72%), DEN-4 in 19 (8.63%), and unidentified serotype in 41 (18.55%) children (Tab. 2). As many as 59 patients who were infected by DEN-1 virus included 39 children (66.10%) with primary infection, 19 children (32.20%) with secondary infection, and 1 child (1.69%) with unknown nature of infection (Tab. 2). All patients with primary infection had mild clinical symptoms (DF). Meanwhile, patients with secondary infection included 10 (52.63%) DF, 7 (36.84%) DHF, and 2 (10.52%) DSS cases. In 8 (42.10%) patients, severe clinical symptoms occurred when secondary infection caused by DEN-1 was preceded by DEN-2 as primary infection. This study also found one case of DEN-1 secondary infection which was preceded by DEN-1 primary infection (Tab. 2).

Fifty-one patients with DEN-2 infections included 37 (72.55%) patients suffering from primary infection, 14 (27.45%) patients with secondary infection, and 1 (1.96%) patient with mixed DEN-2/DEN-3 infection. All patients with primary DEN-2 infection had mild clinical manifestations, whereas in secondary infection, 11 of 14 (78.47%) patients had mild clinical symptoms and 3 (21.42%) patients had severe clinical symptoms (Tab. 2). In this study, the percentage of patients with severe clinical manifestations in secondary infections caused by DEN-2 (21.42%) was lower than that in secondary infections caused by DEN-1 and DEN-3 (47.36% and 41.37%, respectively).

There were 50 subjects suffering from DEN-3 infection, including 20 (40%) patients with primary infection, 29 (58%) patients with secondary infection, and 1 (2%) patient with DEN-2/DEN-3 mixed infection (Tab. 2). The clinical manifestations in 20 patients with DEN-3 primary infection were as follows: 18 of 20 (90%) patients with DF and 2 (10%) patients with DHF. Meanwhile, 29 patients with secondary infection included 17 of 29 (58.62%) patients with DF, 11 (37.93%) patients with DHF, and 1 (3.44%) patient with SSD. One patient infected by a combination of DEN-2 and DEN-3 developed DHF (Tab. 2). These data indicate that among DEN-1, DEN-2, DEN-3, and DEN-4, only DEN-3 virus could cause DHF during primary infection.

DEN-4 virus infection affected 19 patients, including 5 (26.32%) subjects with primary infection, 11 (57.89%) subjects with secondary infection, 2 (5.6%) subjects with tertiary infection, and 1 (7.63%) patient with unknown type of infection (Tab. 2). Clinical manifestations of DEN-4 primary infection included only DF, while DEN-4 secondary infection caused DF in 10 of 11 (90.9%) cases and DHF

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Serotype	Primary			Secondary				Mixed				Tertiary				Unknown		
	DF	DHF	DSS	Combination	DF	DHF	DSS	Combination	DF	DHF	DSS	Combination	DF	DHF	DSS	DF	DHF	DSS
DEN-1	39	0	0	DEN 1,1	1	0	0									0	1	0
				DEN 2,1	4	6	2											
				DEN 3,1	4	1	0											
				DEN 4,1	1	0	0											
Total	39	0	0		10	7	2									0	1	0
DEN-2	37	0	0	DEN 1,2	10	1	0											
				DEN 2,2	0	0	0											
				DEN 3,2	0	1	1	DEN 2-3	0	1	0							
				DEN 4,2	1	0	0											
Total	37	0	0		11	2	1		0	1	0							
DEN-3	18	2	0	DEN 1,3	12	6	0											
				DEN 2,3	4	5	1		0	1	0							
				DEN 3,3	0	0	0											
				DEN 4,3	1	0	0											
Total	18	2	0		17	11	1		0	1	0							
DEN-4	5	0	0	DEN 1,4	4	0	0									1	0	0
				DEN 2,4	5	0	0					DEN 1-2-4	1	0	0			
				DEN 3,4	1	1	0					DEN 1-3-4	1	0	0			
				DEN 4,4	0	0	0											
Total	5	0	0		10	1	0						2	0	0	1	0	0
Unidentified					17	15	3		0	0	0		3	0	0	3	0	0

DF – dengue fever; **DHF** – dengue haemorrhagic fever; **DSS** – dengue shock syndrome.

Tab. 2. Serotype distributions and clinical severities in patients suffering from dengue virus infection

in 1 (9.1%) case. Clinical manifestations in the form of DF developed in two cases of DEN-4 tertiary infection as the latest infection (current infection).

DISCUSSION

Gender analysis of patient data, which was performed by Fried *et al.*, between 1994 and 2006 in Bangkok (Thailand) produced similar results. Fried *et al.* found that patient's gender distribution was 55.5% males and 44.5% females[17]. A study of dengue virus infection outbreak in Bangladesh 2002 also showed higher prevalence in men than in women with 2.7:1 ratio[18]. However, several other studies showed no difference in the risk of infection between boys and girls[19–22].

The pattern of clinical manifestation distribution in this study is similar to the one reported by Suwandono *et al.* in Jakarta, who found that DF patients were more common among young children, while DHF patients were more common among older or mature children[13]. Similar results were also obtained by Balmaseda *et al.*, who conducted their study during an epidemic of Dengue virus infection in Nicaragua between 1999 and 2004. Children aged 10–14 years were more likely to suffer from severe clinical manifestations compared to children aged 1–9 years[21]. Shepherd *et al.* also concluded that Dengue infection could affect all ages, but dengue fever usually occurred in children in the range of 15 years in Southeast Asia which is a hyperendemic area[23].

Infection incidence analysis related to the degree of clinical manifestations indicates that patients with primary infection are more likely to develop mild clinical symptoms, while patients with secondary infection have higher risk of developing severe clinical symptoms. It shows that the infection pattern and the clinical degree in children in Gondokusuman (1995–1999), according to previous studies as well as secondary infection theory on the risk of severe clinical symptoms, is higher in patients with heterologous secondary infection. A research conducted by Fox *et al.* in Hanoi, Vietnam, in 2008 produced similar results. The percentage of patients with DHF secondary infection was higher than that of patients with primary infection alone (32%:18%)[22].

The serotype distribution indicates that Gondokusuman in Yogyakarta is a hyperendemic region due to the simultaneous presence of all dengue virus serotypes[2,4,24,25]. Eight (42.10%) patients developed severe clinical manifestations when secondary DEN-1 infection was preceded by primary DEN-2 infection (Tab. 2). This finding is in contrast to the study conducted by Corwin *et al.* in South Sumatra, which showed that DEN-1 infection is associated with mild clinical manifestations[26]. Endy *et al.* (Thailand) also concluded that in the case of DEN-1 and DEN-2 secondary infection, antibodies formed during primary infection did not affect the risk of DHF occurrence[19].

This study shows that the high risk of developing severe clinical manifestations in patients with secondary DEN-1 infection preceded by primary DEN-2 infection may be

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caused as follows: first, mutation or difference in the genetic structure between DEN-1 in Gondokusuman (Yogyakarta) compared to DEN-1 in Sumatra and Thailand occurs; second, antibody titres induced by DEN-2 during primary infection are not sufficient to act as a neutralizing antibody against DEN-1 but rather act as an ADE (antibody-dependent enhancement)-trigger mechanism; third, antibodies formed during primary DEN-2 infection have low binding affinity or are unsuitable for the neutralization epitope of DEN-1.

This study also found one case of secondary DEN-1 infection preceded by primary DEN-1 infection. Cases of recurrent infections were also found by other researchers. Endy *et al.* reported one case of DEN-2 recurrent infection in Thailand. Anoop *et al.* also found DEN-3 recurrent infections in India. Secondary infection preceded by primary infection caused by the same serotype may be due to antibody titres that are insufficiently formed during primary infection or due to genetic structure differences between the serotypes circulating in the region, which is caused by evolutionary changes or perhaps there are more than one genotype of the same serotype in same period[19,27].

The study showed that the clinical manifestations in all patients suffering from DEN-2 infection were mild. A research conducted by Fried *et al.* in Bangkok between 1994 and 2006 also showed that no severe clinical manifestations occurred in DEN-2 primary infection[17]. In this study, the percentage of patients with severe clinical symptoms developed during secondary infections caused by DEN-2 (21.42%) was lower compared to secondary infection due to DEN-1 and DEN-3 (47.36% and 41.37%, respectively). This may be due to the fact that the binding affinity of neutralizing epitope and neutralizing antibody activity/potency against DEN-2 is much stronger than against DEN-3 (16–17 times and 20–71 times, respectively)[28]. Researchers speculate that, in addition to genotype virulence differences in DEN-2 circulating in Indonesia (Genotype IV/Cosmopolitan), as also found by Matsui *et al.* above, differences between this study and research results in Thailand indicate a high risk of severe clinical manifestations in secondary DEN-2 infection at the start of DEN-2 genotype I invasion in that region[11,20,21,25].

Data on the severity of clinical manifestations in patients with primary and secondary DEN-3 infection show a higher risk of severe clinical symptoms compared to other serotypes causing infection in Gondokusuman in Yogyakarta. Results obtained in this study are consistent with the opinion of Suwandono *et al.* that the risk of dengue fever is higher in DEN-3 virus infection compared to infections caused by other viruses[13]. Gubler *et al.*, 1979, quoted by Nogueira *et al.*, 2008, mentioned that the number of fatalities due to DEN-3 infection in Jakarta was 3 times higher compared to other serotypes[20]. Endy *et al.* also reported that during secondary DEN-3 infection, a cross-reaction between antibodies formed during DEN-1, DEN-2 and DEN-4 primary infection and neutralizing epitopes located in DEN-3 E and PrM region did not cause neutralization[19]. Researchers suspect this finding also underlies the high risk of severe clinical manifestations during DEN-3 secondary infection in Gondokusuman (Yogyakarta).

In this study, there was one case of DEN-2 and DEN-3 mixed infection. This finding had also been reported by Loroño-Pino *et al.* Anoop *et al.* also reported the occurrence of mixed infection during the epidemic in South India in 2009. RT-PCR examination of 75 patients suspected of suffering from dengue virus infection revealed 37 (49.63%) positive patient blood samples. A total of 21 (56.8%) of these were identified as mixed infection caused by DEN-2 and DEN-3 (18 cases), DEN-1/DEN-2 (one case), and DEN-1, DEN-2 and DEN-3 (one case). The occurrence of mixed infection in a hyperendemic region may be due to *Aedes aegypti* and *Aedes albopictus*, which are vectors of dengue virus and tend to bite and suck human blood several times for the egg maturation during one gonotropin cycle. Thus, these vectors can carry multiple dengue virus serotypes when biting and sucking blood from patients who become infected by different dengue virus serotypes[27,29].

Two cases of tertiary infection with DEN-4 as the latest infection (current infection) produced clinical manifestations in the form of DF. Determination of tertiary infection in these patients was based on their past medical history confirmed by serological test as viral RNA detection by RT-PCR and virus isolation gave negative results. Both, a mild clinical degree as well as negative detection of DEN-4 tertiary infection may be caused by rapid viral RNA cleaning up and shortened viral lifetime in patients' blood due to the presence of IgG which is triggered repeatedly during the two previous infections.

Studies on the sero-epidemiological data collected between 1995 and 1999 in Gondokusuman Yogyakarta provide some characteristics that can be used for surveillance and as an input for policy makers in the field of health such as: 1) Gondokusuman area is hyperendemic dengue virus infection area; 2) infection due to DEN-3 involves the highest risk of severe clinical manifestations; 3) secondary infection caused by DEN-1, which is considered to induce mild clinical symptoms, involves the risk of severe clinical symptoms, especially when DEN-2 the cause of primary infection.

Conflict of interest

The authors do not report any financial or personal affiliations to persons or organisations that could negatively affect the content of or claim to have rights to this publication.

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References

1. Aryati: Epidemiologi Molekuler Virus Dengue di Indonesia. Airlangga University, Surabaya 2006.
2. World Health Organization: Dengue Haemorrhagic Fever : Diagnosis, Treatment, Prevention and Control. 2nd ed., World Health Organization, Geneva 1997.
3. Gubler DJ: Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 1998; 11: 480–496.
4. World Health Organization: Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control. 1st ed., World Health Organization, Geneva 2009.
5. Halstead SB, O'Rourke EJ: Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J Exp Med 1977; 146: 201–217.
6. Kurane I: Dengue hemorrhagic fever with special emphasis on immunopathogenesis. Comp Immunol Microbiol Infect Dis 2007; 30: 329–340.
7. Weaver SC, Vasilakis N: Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infect Genet Evol 2009; 9: 523–540.
8. Henchal EA, Henchal LS, Schlesinger JJ: Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol 1988; 69:2101–2107.
9. Malavige GN, Fernando S, Fernando DJ *et al.*: Dengue viral infections. Postgrad Med J 2004; 80: 588–601.
10. Klungthong C, Putnak R, Mammen MP *et al.*: Molecular genotyping of dengue viruses by phylogenetic analysis of the sequences of individual genes. J Virol Methods 2008; 154: 175–181.
11. Leitmeyer KC, Vaughn DW, Watts DM *et al.*: Dengue virus structural differences that correlate with pathogenesis. J Virol 1999; 73: 4738–4747.
12. Raekiansyah M, Pramesyanti A, Bela B *et al.*: Genetic variations and relationship among dengue virus type 3 strains isolated from patients with mild or severe form of dengue disease in Indonesia and Thailand. Southeast Asian J Trop Med Public Health 2005; 36: 1187–1197.
13. Suwandono A, Kosasih H, Nurhayati *et al.*: Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004. Trans R Soc Trop Med Hyg 2005; 100: 855–862.
14. Graham RR, Juffrie M, Tan R *et al.*: A prospective seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia I. Studies in 1995–1996. Am J Trop Med Hyg 1999; 61: 412–419.
15. Lanciotti RS, Calisher CH, Gubler DJ *et al.*: Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 1992; 30: 545–551.
16. Sangkawibha N, Rojanasuphot S, Ahandrik S *et al.*: Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 1984; 120: 653–669.
17. Fried JR, Gibbons RV, Kalayanarooj S *et al.*: Serotype-specific differences in the risk of dengue hemorrhagic fever: an analysis of data collected in Bangkok, Thailand from 1994 to 2006. PLoS Negl Trop Dis 2010; 4: e617.
18. Islam MA, Ahmed MU, Begum N *et al.*: Molecular characterization and clinical evaluation of dengue outbreak in 2002 in Bangladesh. Jpn J Infect Dis 2006; 59: 85–91.
19. Endy TP, Nisalak A, Chunsuttitwat S *et al.*: Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. J Infect Dis 2004; 189: 990–1000.
20. Nogueira MB, Stella V, Bordignon J *et al.*: Evidence for the co-circulation of dengue virus type 3 genotypes III and V in the Northern region of Brazil during the 2002–2004 epidemics. Mem Inst Oswaldo Cruz 2008; 103: 483–488.
21. Balmaseda A, Hammond SN, Pérez L *et al.*: Serotype-specific differences in clinical manifestations of dengue. Am J Trop Med Hyg 2006; 74: 449–456.
22. Fox A, Le NMH, Simmons CP *et al.*: Immunological and viral determinants of dengue severity in hospitalized adults in Ha Noi, Viet Nam. PLoS Negl Trop Dis 2011; 5: e967.
23. Shepherd SM, Hinfey PB, Shoff WH: Dengue. Available from: http://emedicine.medscape.com/article/215840-overview [cited 15 July 2016].
24. Kumaria R: Correlation of disease spectrum among four Dengue serotypes: a five years hospital based study from India. Braz J Infect Dis 2010; 14: 141–146.
25. Vázquez-Pichardo M, Rosales-Jiménez C, Núñez-León A *et al.*: Serotipos de dengue en México durante 2009 y 2010. Bol Med Hosp Infant Mex 2011; 68: 103–110.
26. Corwin AL, Larasati RP, Bangs MJ *et al.*: Epidemic dengue transmission in southern Sumatra, Indonesia. Trans R Soc Trop Med Hyg 2001; 95: 257–265.
27. Anoop M, Issac A, Mathew T *et al.*: Genetic characterization of dengue virus serotypes causing concurrent infection in an outbreak in Ernakulam, Kerala, South India. Indian J Exp Biol 2010; 48: 849–857.
28. Matsui K, Gromowski GD, Li L *et al.*: Characterization of dengue complex-reactive epitopes on dengue 3 virus envelope protein domain III. Virology 2009; 384: 16–20.
29. Loroño-Pino MA, Cropp CB, Farfán JA *et al.*: Common occurrence of concurrent infections by multiple dengue virus serotypes. Am J Trop Med Hyg 1999; 61: 725–730.