EFFICACY OF NS1 ANTIGEN DETECTION FOR EARLY DENGUE INFECTION DIAGNOSIS IN INDONESIA

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Abstract. Diagnosing dengue is challenging and problematic as clinical symptoms of dengue show similar clinical presentations with another vector-borne diseases, such as chikungunya and malaria particularly in the initial stages of infection. There are several laboratory methods available for dengue diagnosis, however, some of them are costly, time-consuming and need specialized laboratory staff, who may not be widely available in hospital in challenging settings. This study was a cross-sectional study that evaluated the sensitivity and specificity of SD Bioline Dengue Duo for primary and secondary DENV infection in Jakarta, Indonesia between 2010 and 2011. In addition, we investigated the kinetics of NS1 as well as anti-DENV antibodies. A panel of sera samples from 102 patients experiencing fever within the last 48 hours was tested. DENV infection was confirmed using gold standard tests, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) or virus isolation in C6/36 cell line or increased antibody titer by ELISA. The type of immune response towards the infection was determined by measurement of antibody titers on convalescent phases by HI test. The sensitivity of SD Bioline Dengue Duo was higher in primary infection [93.11% (95% CI: 92.7-100)] than in secondary infection [86.11% (95% CI: 82.7-100)]. The specificity of NS1 antigen detection kit was 100%. NS1 detection rate reduced over the course of the illness and was lowest on day 7 in both primary and secondary infection. However, decreased of NS1 detection was recovered by increased antibody detection. Thus, a combination of DENV NS1 antigen test and IgM and IgG test in the early stages of dengue infection should be conducted to obtain a more accurate early diagnosis, thus allowing appropriate treatment to be made and reducing mortality if the disease is allowed to progress to a severe stage.

Keywords: dengue, early diagnosis, NS1 antigen, RT-PCR

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INTRODUCTION

Dengue is a mosquito-borne disease affecting particularly people in tropical and subtropical areas. The disease is caused by infection with dengue virus, which consists of serotype 1, 2, 3, 4, and 5 (Mustafa *et al*, 2015) transmitted through bites of *Aedes aegypti*, the primary vector, and *Ae. Albopictus*, the secondary vector (Higa, 2011). Clinical manifestation of dengue varies from asymptomatic to severe forms such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (WHO, 1997; WHO, 1999). Globally mortality from dengue infection averages 9,221 cases per year between 1990 and 2013 (Stanaway *et al*, 2016). Although the number of cases is still increasing, case fatality rate (CFR) is decreasing due to improved treatment management.

However, dengue diagnosis still faces challenges and problems. Clinical symptoms of dengue shows similar clinical presentations with other vector-borne disease such as chikungunya and malaria especially in the initial stages of infection (Mardekian and Roberts, 2015). Concurrent infection with another vector-borne disease also poses another challenge for diagnosis because of overlapping clinical symptoms (Raut *et al*, 2015; Edwards *et al*, 2016). Accurate diagnosis is imperative as outcomes and management strategies for each disease are vastly different. In addition, diagnosis is crucial for surveillance, control of outbreak and research related to vaccine and drug development (Salam *et al*, 2018).

There are several laboratory methods available for dengue diagnosis, such as virus isolation, detection of virus RNA through RT-PCR and antibody detection tests (IgM and IgG MAC-ELISA) (Kumarasamy *et al*, 2007). Dengue diagnosis based on virus isolation and RT-PCR is costly and requires specialized laboratory staff, which may not be widely available in hospitals with limited resources (Peeling *et al*, 2010). Those methods also are time-consuming and may not provide prompt confirmation of diagnosis particularly at early stages of illness. In addition, diagnostic tests of the early stages of dengue infection have their own weakness. In total of 112 clinically suspected dengue, the positive detection rate of NS1 antigen ELISA and RT-PCR are only 80.9% and 68.1%, respectively (Anand *et al*, 2016). It is important to obtain an accurate and sensitive diagnosis of dengue at the early stages of infection to reduce CFR if the disease is allowed to progress to DHF/DSS form (Mat Jusoh and Shueb, 2017).

Enzyme-linked immunosorbent assays (ELISAs) directed against dengue virus (DENV) non-structural glycoprotein (NS1 antigen) provides a (purported) sensitive and specific dengue diagnostic test at the early stages of disease as NS1 antigen is present at high concentrations at this period of the disease (Hermann *et al*, 2014). However, the performances of NS1 ELISA kits vary depending on patient serological status (primary versus secondary dengue infection), time of specimen collection and dengue serotype (Hermann *et al*, 2014; Anand *et al*, 2016). Its sensitivity and specificity also are related to different geographical regions (Guzman *et al*, 2010; Aryati *et al*, 2013), suggesting the need for further assessment.

The efficacy of NS1 antigen detection as an early dengue infection diagnosis was evaluated in Jakarta, Indonesia during 2010-2011. Information from this study could be adopted as a recommendation of an early dengue diagnosis method, thereby providing appropriate and timely

treatment, which should reduce mortality stemming from the severe forms at the later stages of disease. Accurate diagnosis also will provide early information for public health control of a dengue outbreak.

MATERIALS AND METHODS

Patients' samples collection

Study locations were in dengue-endemic communities of East Jakarta, Indonesia. East Jakarta is one of the five administrative cities forming Special Capital Region of Jakarta, Indonesia with population of 2,687,027 in 2010 (BPS, 2011). This was a cohort study conducted from April 2010 to December 2010 involving subjects ≥14 years of age with fever >38.0ºC within the previous 48 hours. Clinical (suspected) symptoms for dengue and disease severity were based on WHO (1997) criteria: fever, hemorrhagic manifestations, thrombocytopenia (platelets ≤100,000 /mm^3), and evidence of plasma leakage (pleural effusion, ascites, hemoconcentration ≥20% or hypoproteinemia). Then, 10 ml of blood was taken for dengue serologic test, culture and assays of other laboratory markers (Dewi *et al*, 2014; van de Weg *et al*, 2015). DENV NS1 antigen and IgM/IgG tests (SD Bioline Dengue Duo, Standard Diagnostic, Kyonggi, Korea) were performed for first screening of dengue infection. Subjects positive for putative dengue infection were admitted to Cipto Mangunkusumo General Hospital, Jakarta. The gold standard for dengue virus infection was RT-PCR (Lanciotti *et al*, 1992 with slight modification by Dewi *et al*, 2014), virus isolation employing a C6/36 cell line (Standard Operational Procedure, Department of Microbiology, FKUI) or IgG ELISA (Focus, Diagnostics, CA) on acute and convalescence sera. A case is considered as having dengue infection when positive for one or more of these tests performed at the Department of Microbiology, Medical Faculty, Universitas Indonesia, Jakarta. Hemagglutination inhibition test was performed to determine whether infection was primary or secondary (Clarke and Casals, 1958).

Study protocols were approved by the Ethical Committee, Faculty of Medicine, University of Indonesia No. 71/PT02. FK/ETIK/2009. After detection of fever, patients or their parents or legal guardians if <18 years of age provided prior written informed consent before recruitment into the study.

RT-PCR of DENV

We used semi-nested RT-PCR as previously described (Lanciotti *et al*, 1992). In brief, RNA was extracted from 140 µl of plasma or culture supernatant of infected C6/36 cells using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). In addition to confirming DENV infection, this RT-PCR also can be used to determine DENV serotype. RNA isolation and PCR were performed in a strict containment area to avoid sample cross-contamination. RNase free water as negative control was included in every experiment. First step PCR mixture (40 µl) contained 4 µl of 10X PCR buffer containing 1.5 mM $MgCl_2$, 0.8 µl of 10 mM each dNTP, 0.4 µl of SuperScript II RTase (Invitrogen, Carlsbad, CA), 0.15 µl of 5 U/µl Platinum *Taq* DNA polymerase (Invitrogen), 0.8 µl of 10 µM Land D1: 5'-TCA ATA TGC TGA AAC GCG CGA GAA ACC G-3' and Land D2: 5'-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3' primers (Lanciotti *et al*, 1992), and 8 µl of RNA. Thermocycling (Applied Biosystem

Programmable 9700 Thermal Cycler; Applied Biosystem, Foster City, CA) was performed as follows: 53ºC for 30 minutes; 95ºC for 5 minutes; 30 cycles of 95ºC for 45 seconds, 60ºC for 30 seconds and 72ºC for 90 seconds; and a final heating at 72ºC for 7 minutes. Second step PCR (25 µl) contained 2.5 µl of 10X PCR buffer containing 1.5 mM $MgCl_2$, 0.5 µl of 10 mM each dNTP, 0.15 µl of 5 U/µl of Platinum *Taq* DNA polymerase (Invitrogen), 1 µl of 10 µM D1, TS1: 5'-CGT CTC AGT GAT CCG GGGG-3', TS2: 5'-CGC CAC AAG GGC CAT GAA CAG-3', TS3: 5'-TAA CAT CAT CAT GAG ACA GAG C-3', and TS4: 5'-CTC TGT TGT CTT AAA CAA GAG A-3' primers (Lanciotti *et al*, 1992), 2 µl of first PCR product. Thermocycling (Applied Biosystem Programmable 9700 Thermal Cycler) was carried out as follows: 95ºC for 5 minutes; 35 cycles of 95ºC for 45 seconds, 60ºC for 30 seconds and 72ºC for 60 seconds; with a final step at 72ºC for 7 minutes. Amplicons were 482, 119, 290 and 392 bp for DENV-1, DENV-2, DENV-3 and DENV-4, respectively. The amplicons were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. For specimens with PCR results showing co-infection with more than one serotype, second step PCR was conducted separately using primers specific for each serotype.

Virus Isolation in C6/36 cell line.

Virus isolation were performed according to standard operating procedure of the Department of Microbiology Faculty of Medicine, University of Indonesia. After monolayer (24 hours after seeded), C6/36 cell in 24 well were infected with various dilution of patients' plasma. One hundred µl of plasma with 1/10, 1/50 and 1/100 dilutions in BS-BPA were added in each well. After 2 hours of incubation time, 1 ml of MEM with 10%FBS were added. Cell were incubated for 5-7 days. The apparance of CPE and any contaminations were observbed daily. After three times passage in C6/36, the presence of DENV in the supernatant was detected by RT-PCR.

IgG ELISA

Level of IgG from patients at acute and convalescence were performed to confirm DENV infection. IgG ELISA from Focus, Diagnostic, USA was carried out according to manufacturer's instruction. After allowing all reagents to warm to room temperature, we added 100 µl of diluted sample (1/100) to each well in duplicate. After incubation for 60 minutes at room temperature and washed, we added reagent peroxidase-conjugated goat anti-human IgG. Substrate was added and incubated, followed by addition of stop solution. Absorbance of each well was measured within one hour of stopping the assay at a wavelength of 450 nm. The increase of absorbance of convalescence phase was used as additive of DENV infection confirmation.

NS1and IgM/IgG tests by SD Bioline Dengue Duo.

In this study, the sensitivity, specificity and kinetics of NS1 antigen, IgM and IgG of NS1 and IgM/IgG tests were evaluated by SD Bioline Dengue Duo, Standard Diagnostic, Kyonggi, Korea. The detection of NS1 antigen was performed according to manufactirer's intruction as well as IgM and IgG detection (SD Bioline Dengue Duo, Standard Diagnostic, Kyonggi, Korea). To check for NS1 antigen, 100 µl of patient's plasma was added into the sample well labeled letter "S" on the left side of kits. For detection of IgM/IgG antibodies, 10 µl of blood specimen was added into the sample well, labeled "S"

on the right side of the kit using 10 μl capillary pipette. Subsequently, 4 drops (90-120 μl) of assay diluents were added into the well. Results were interpreted 15-20 minutes afterwards.

Data analysis

Diagnostic accuracy of DENV NS1 antigen and IgG-IgM test were evaluated for percent sensitivity (a/(a+c) ×100) and percent specificity (d/(d+b)×100) where a = number of true positives, b = number of false positives, c = number of false negatives, and d = number of true negatives. DENV NS1 antigen and IgG and IgM test were compared with gold standards.

RESULTS

Characteristic of subjects

Of the suspected dengue patients (n = 102), 68 (68.3%) were confirmed positive. The majority of patients being 14-23 years of age (Table 1). Patient were determined as primary infection when the titer of antibody ≤1:1280 at convalescent phase. Secondary DENV infection accounts for the highest percentage (60.3%).

Table 1
Demographics of participating dengue patients at Cipto Mangunkusumo General Hospital, Jakarta, Indonesia (April 2010 to December 2010).

Characteristics	*n* (%)
Dengue infection	
Positive	68 (68.3%)
Negative	34 (31.7%)
Age (Years)	
14-23	51 (50.0%)
24-33	25 (24.5%)
34-43	8 (7.8%)
44-53	7 (6.9%)
>53	1 (1.0%)
Unknown	10 (9.8%)
Type of Infection	
Primary	27 (39.7%)
Secondary	41 (60.3%)

Sensitivity and specificity of NS1 in primary and secondary DENV infection cases

The performance of SD Bioline Dengue Duo was evaluated by comparing the NS1 Ag detection test of the kit with gold standard diagnostic methods, RT-PCR and virus isolation. This study unveiled the sensitivity and specificity of SD Bioline Dengue Duo for detecting NS1 antigen in patients who had primary DENV infection in Jakarta were 93.3% and 100%, respectively (95% CI: 92.7-100). In secondary DENV infection, it turned out that SD Bioline Dengue Duo had a lower sensitivity rate, which was 86.1% (95% CI: 82.7-100). The specificity of NS-1 test was 100%. This result indicates that SD Bioline Dengue Duo had a better ability to detect NS1 antigen in primary DENV infection rather than secondary DENV infection.

Kinetics of NS1, IgM and IgG in primary DENV infection

Optimal performance of NS1 antigen detection by SD Bioline Dengue Duo was obtained on the first three days of fever. At Day 1 of fever, all samples (100%: 4 patients) were of NS1-positive. On the next 2 days of fever, all dengue-confirmed samples were also tested positive for NS1 antigen. After the third day of illness, the proportion of samples in which NS1 antigen could be detected began to decline, 96.4% on Day 4, 80.7% on Day 5, 74.1% on Day 6, and finally reaching its lowest level (70.4%) on Day 7 (Fig 1). In contrast to NS1, there was an opposite trend of IgM and IgG profiles. IgM antibodies became detectable by SD Bioline Dengue Duo as early as the second

day after the onset of fever. The number of IgM positive samples then gradually increased and peaked on the seventh day of illness. Meanwhile, IgG antibodies did not appear until Day 6. This concurs well with previous studies.

Kinetics of NS1, IgM and IgG in secondary DENV infection

The pattern of NS1 antigen kinetics in secondary DENV infection (Fig 2) was almost similar with that observed in primary DENV infection on the early fever. NS1 could be detected in all DENV confirmed samples (100%). However, the percentage of NS1 positive started to decrease on Day 3 and fell dramatically after Day 5 of fever. In comparison to primary DENV infection, IgG antibodies could be detected from the second day of fever and ealier than IgM in patients with secondary dengue infection. On the seventh day of fever, IgG antibodies were present in 29 out of 30 samples tested. Although some samples were IgM positive from Day 4 to Day 7, their numbers are not as high as in primary DENV infection.

DISCUSSION

It is crucial to diagnose dengue rapidly during the early phase to enable appropriate management and evade the complications of the disease in the later phase. Sensitivity and specificity of any rapid test are essential for accurate laboratory diagnosis of suspected dengue patient. In the early phase, specificity of DENV NS1 antigen assay was higher than that of IgG and IgM ELISAs, however, its sensitivity was lower than that of IgG ELISA. This finding is in concordance with several earlier studies that showed DENV NS1 antigen strip test has a consistent high specificity, but sensitivity varies from 34 to 96% and is significantly lower in secondary compared with primary dengue infections (Chaterji *et al*, 2011; Hermann *et al*, 2014). The latter phenomenon is pertinent as 91% of the samples from the present study were from secondary infection. The lower sensitivity of DENV NS1 antigen strip test in secondary infection is believed to be due a rapid rise of pre-existing NS1 antibodies in serum, resulting in formation of immune complexes with IgG antibody, thereby compromising detection of NS1 antigen (Tricou *et al*, 2010; Hermann *et al*, 2014). Variations in sensitivity could also be influenced by dengue serotype (Chaterji *et al*, 2011; Hermann *et al*, 2014).

Sensitivity of DENV NS1 was higher on Day 4 than IgG and IgM ELISAs. Falconar *et al* (1997) reported NS1 antigen can be detected up to Day 9 after onset of dengue symptoms and is in a free or soluble form in serum of patients. Surprisingly, the sensitivity and specificity of IgG and IgM ELISAs in the present study were low as compared to DENV NS1 antigen assay on Day 4. In general, IgG and IgM ELISAs have higher sensitivity in the later stages of dengue infection as antibodies against DENV develop a few days after infection (Changal *et al*, 2016; Hunsperger *et al*, 2017). Thus, in the early stages of the dengue, virus isolation or nucleic acid or antigen detection is recommended as diagnostic tests, while serologic assays are the methods of choice for diagnosis in the later stages of infection (Muller *et al*, 2017). Although serologic tests have problems of cross-reactivity particularly in dengue-endemic areas where there is co-circulation of more than one flavivirus (Lee *et al*, 2015). The high NS1 levels detected within the first 72h of fever is also a strong predictor of progression to

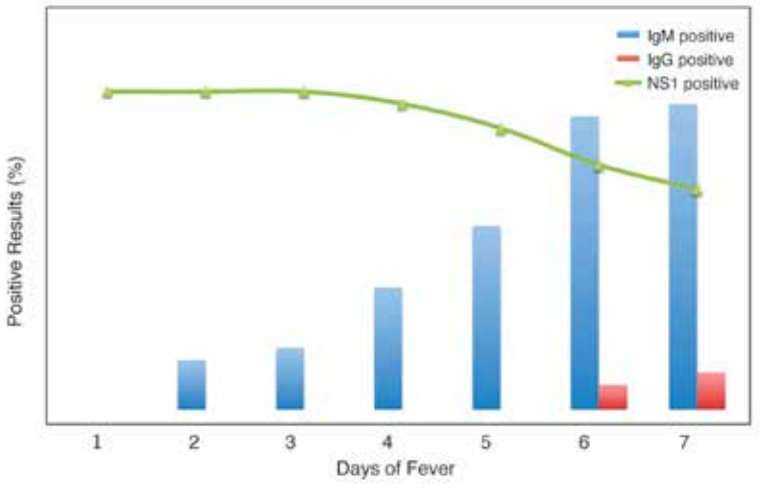

Fig 1-Kinetics of NS1 antigen, IgM, and IgG in primary DENV infection.

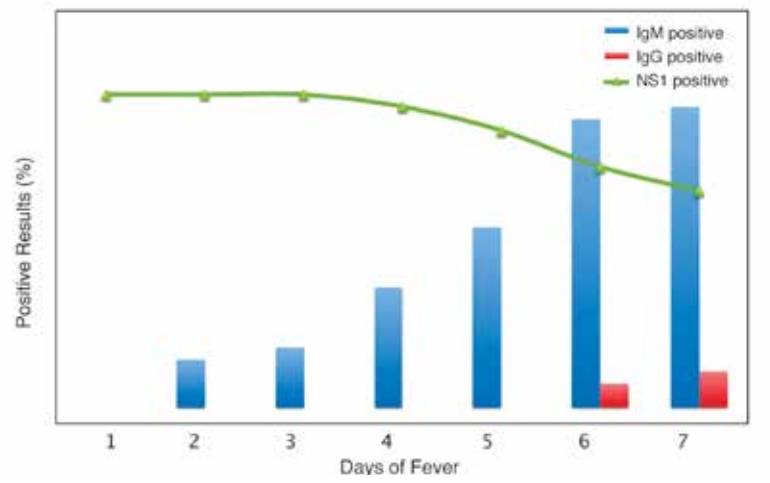

Fig 2-Kinetics of NS1 antigen, IgM, and IgG in secondary DENV infection.

the more severe disease (Simmons *et al*, 2012). However, the kinetics of NS1 in secondary infections is shorter than that of primary infections due to the rapid anamnestic rise in NS1 cross-reacting antibodies during the acute phase of disease (Libraty *et al*, 2002). The sensitivity and specificity of NS-1 detection kits vary. The In Bios NS1 ELISA Ag kit had a higher overall sensitivity (86% *vs* 72.8%) but equal specificity (100%) compared to the BioRad Platelia kit (Hermann *et al*, 2014).

Our study reveals that SD Bioline Dengue is more robust in detecting primary dengue infection. This result is in the contrary with earlier study by Andries *et al* (2012). The sensitivity of NS1 Ag test component of SD Bioline Dengue Duo is significantly higher in the population of patients who had primary infection than in those with secondary infection (89.5%, *n*=17 *vs* 43.4%, *n*=83) during an epidemic

in Cambodia in 2011 (Andries *et al*, 2012).

An investigation by Tricou *et al* (2010) also demonstrated the same pattern of results, in which SD Bioline Dengue Duo had a far less sensitivity for NS1 detection in secondary DENV cases (55.1%, *n*=176) compared to primary DENV cases (80.3%, *n*=66). In this present study, however, the difference of NS1 sensitivity between primary and secondary dengue groups is not as large as those observed by Andries *et al* (2012) and Tricou *et al* (2010). This might result from the stricter inclusion criteria adopted in our study, *ie* the subjects enrolled were dengue-suspected patients whose fever had lasted for or less than 48 hours, and thus the samples included for the calculation of the kit's sensitivity were sera samples obtained from the patients' blood taken on Day 1 or Day 2 after fever onset. The fact that NS1 detection was performed early in the course of the illness led to a better sensitivity for secondary DENV infection compared to what was found in the two studies mentioned previously.

The explanation to why the NS1 sensitivity rate of the kit is lower for secondary DENV infection is because NS1 detection is influenced by some extrinsic factors. Those factors include: (1) the presence of IgG antibodies, which are highly reactive to different species of flavivirus and are the main antibodies whose concentration increases significantly during a subsequent exposure to DENV; and (2) the level of viremia (Duong *et al*, 2011). The relationship between NS1 detection rate and the concentrations of anti-DENV antibodies in sera has been well described by Wang and Sekaran through their research in Malaysia, saying that the NS1 test component of SD Bioline Dengue Duo has better performance when the levels of anti-DENV antibodies as measured by HI method are low (Wang and Sekaran, 2010a). Nevertheless, several rapid NS1 tests other than SD Bioline Dengue Duo also have lower sensitivity when used for secondary infection (Lima *et al*, 2010; Wang and Sekaran, 2010b).

In conclusion, DENV NS1 antigen test was highly specific in the early stages of dengue infection but the sensitivity was poorer than IgG ELISA. Thus, a combination of DENV NS1 antigen test and IgG ELISA in the early stages of disease presentation provides optimal detection of dengue infection allowing timely appropriate treatment to be taken and thereby reduce the risk of mortality due to severe dengue manifestations that can occur in the later stages of dengue progression if left untreated.

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