Laboratory Diagnosis of Dengue Virus Infection

Tri Wibawa[1]
[1]Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Corresponding author: twibawa@ugm.ac.id

ABSTRACT

Dengue virus (DENV) infection was reported by more than 100 countries in the world. DENV was estimated infects 2.5 billion people living at tropical and subtropical countries annually. Laboratory diagnosis of DENV infection is important for the best clinical management of the patients. There are many tools and methods have been developed and dedicated to serve accurate diagnostic of DENV infection. In hitherto, laboratory diagnosis of DENV infection may be done by several methods, i.e. serology, viral culture, and viral genome-based detection. However, there is no convincing diagnosis procedures that easy to be performed and user friendly. This review will address the principle and characteristic of the laboratory diagnostic procedures that is available at this moment.

Keywords: Dengue virus, infection, laboratory assay, diagnosis

INTISARI

Infeksi virus Dengue (DENV) dilaporkan oleh lebih dari 100 negara di dunia. Setiap tahunnya, DENV diperkirakan menginfeksi 2,5 miliar orang yang tinggal di negara-negara tropis dan subtropis. Diagnosis laboratorium infeksi DENV penting bagi pengelolaan klinis terbaik bagi pasien. Terdapat banyak alat dan metode yang telah dikembangkan dan didedikasikan untuk memberikan pelayanan diagnostik infeksi DENV yang akurat. Sampai sekarang, diagnosis laboratorium infeksi DENV dapat dilakukan dengan beberapa metode, yaitu serologi, kultur virus, dan deteksi berbasis genom virus. Namun, tidak ada prosedur diagnosis yang meyakinkan, mudah untuk dilakukan dan *user friendly*. Review ini akan membahas prinsip dan karakteristik dari prosedur diagnostik laboratorium yang tersedia pada saat ini.

Kata kunci: virus Dengue, infeksi, uji laboratorium, diagnosis

INTRODUCTION

Dengue virus (DENV) infection was reported by more than 100 countries in the world. DENV was estimated infects 2.5 billion people living at tropical and subtropical countries annually. The DENV infection also involves 120 million travelers to these particular countries[1]. WHO claimed that 100 million peoples infected by DENV annually. As much as 500.000 people have developed dengue hemorrhagic fever which were need medical attention, most of them are children[2].

DENV infection has broad spectrum of clinical manifestation. Traditionally, it was

classified into three major group of clinical manifestation: i.e. dengue fever, dengue hemorrhagic fever and dengue shock syndrome[3]. Later on, this classification was revised in 2009. There are many debates about the effectiveness of the two classification of DENV infection clinical classification in the field and clinic[4].

Dengue fever (DF) is a mildest syndrome which characterized by biphasic fever, myalgia or athragia, rash, leucopenia and lymphadenophaty. Dengue hemorrhagic fever (DHF) is a worst clinical manifestation compare to DF. DHF may be fatal that shown haemostatic and capillary permeability disturbance. DHF may be worsen and become dengue shock syndrome (DSS)[5]. DSS contributes to 1-2.5% mortality rate with proper medical attention and dramatically increase to 20% in poor patient management setting[3].

Dengue virus (DENV) infection is remained the most frequent arboviral infection globally. DENV is consisting of four serotypes: i.e. DENV1, DENV2, DENV3, and DENV4. These four serotypes are circulating globally. There is no specific geographical region associated with particular serotypes. Indonesia as well as other country has been reported all those four serotypes as causative agents of dengue virus infection[2].

DENV is included into flaviviridae family, genus flavivirus. Though it is common to be describe as four serotypes DENV1, DENV2, DENV3, and DENV4, some researcher think that this nomenclature is not completely describe the fact. The four serotypes of DENV are antigenic and genetically distinct among each other. It is unlike polio virus with three serotypes. DENV may be described as four different viruses that potentially cause similar diseases in the human being[6].

DENV genetically is close related with other flavivirus, i.e. Japanese encephalitis and tick-borne encephalitis serogroups, the non-vector-borne viruses Rio Bravo, Montana myotis leukoencephalitis, Modoc, Tamana bat and Apoi. Japanese encephalitis serogroup may cause similar disease in human, encephalitis. This group includes Murray Valley encephalitis virus (MVEV) which is circulating in Australia, St. Louis encephalitis virus (SLEV) in America, Japanese encephalitis virus (JEV) in Asia, and West Nile virus (WNV) that is broadly circulating in Africa, America, Europe and Middle East[6]. Box 1 shows the representative arboviruses of flaviviridae that commonly cause human diseases.

BOX 1: Representative *Flaviviridae* that cause human diseases

- Dengue serotype 1
- Dengue serotype 2
- Dengue serotype 3
- Dengue serotype 4
- West Nile
- Japanese encephalitis
- Kyasanur Forest
- Murray Valley
- Rocio
- St. Louis encephalitis
- Tick-borne encephalitis
- Omsk hemorrhagic fever
- Yellow fever

DENV virion consists of single strand RNA which is encapsulated with icosahedral or isometric nucleocapsid. The most outer part of the DENV virion is envelope structure. DENV has approximately 50 nm in diameter. The genome of DENV consists of single open reading frame (ORF) gene which encodes three structural protein (PrM, C, and E) and seven non structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). The whole genome is translated into a polyprotein, which is processed co- and post-translationally by host and viral proteases (Figure 1)[7].

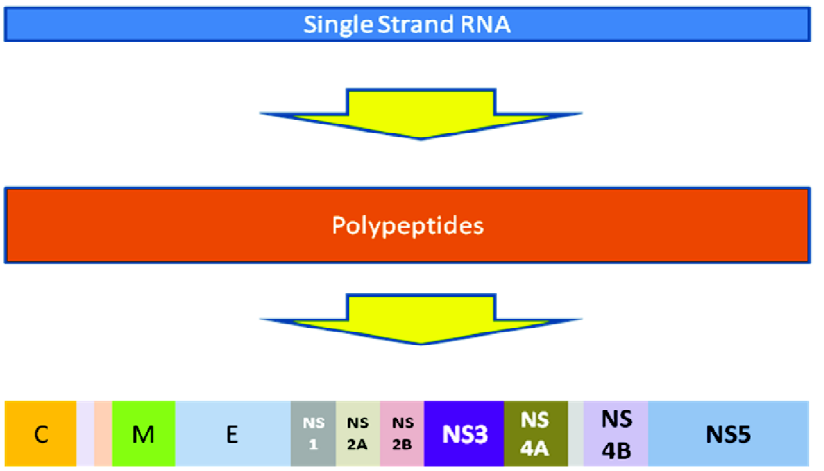

Figure 1. The schematic structure of DENV genome and translation process which will produce single polypetptide that eventually cleaved by signal peptidase, golgy protease and NS3 protease. The different colors denote the putative cleavage sites of proteases (modified from http://viralzone.expasy.org/all_by_species/43.html).

Laboratory diagnosis of DENV infection is not conclusive yet. There are many tools and methods have been developed and dedicated to serve accurate diagnostic of DENV infection. In hitherto, laboratory diagnosis of DENV infection may be done by several methods, i.e. serology, viral culture, and viral genome-based detection. However, there is no convincing diagnosis procedures that easy to be performed and user friendly. This review will address the principle and characteristic of the diagnostic procedures that is available at this moment.

Diagnosis of DENV Infection

DENV is present in the saliva of mosquito and injected into the skin tissue of human being. Injected DENV virion binds and replicates in the dendritic cells of the skin. DENV further replicates and transfer into the lymphoid tissues and viremia will be occur 3-5 days later. Viremia will be finished at the 4^{th} - 5^{th} day. The symptoms, including fever, start to appear 24

hours after viremia onset. The time frame of DENV infection is important to understand the laboratory diagnostic methods that are applicable to detect DENV infection[8]. Box 2 shows the current diagnostic tools that available to detect DENV infection.

1. DENV Isolation

For viral isolation purpose, the specimen should be obtained in the very beginning of infection, since viremia occurs before the 5th day post infection. DENV may be isolated from serum, plasma or peripheral blood mononuclear cells (PBMCs). Specimen for viral isolation in certain cases may be obtained from post mortem tissue biopsy i.e. liver, lung, lymph nodes, thymus, and bone marrow[9]. Since DENV is sensitive to temperature, specimen must be transfer to the laboratory in ice pack to maintain the cold chain. It may be stored at 4p C-8p C for less than 24 hours. For longer preservation, it may be stored at -70p C or in the liquid nitrogen. It is not recommended to store at -20p C[3].

The most common method to isolate DENV is by infected to cells. The mosquito origins cell line which is routinely used for this purpose is C6/36, a cell line that obtained from *Ae albopictus*[10]. Another cell line is from *Ae. pseudoscutellaris*, namely AP61[11]. Morphological changes in cells caused by viral infection are called cytopathic effects (CPE). The presence of DENV in the clinical specimen can be seen by observing the cytopathic effect in the cell lines. The degree of visible damage to cells caused by viral infection varies with type of virus, type of host cells, multiplicity of infection (MOI), and other factors. Some cell will die because of being infected by DENV. However, Cytopathic effect of DENV to the mosquito origin cell line sometimes not easy to be seen. To detect DENV in the cell lines we may use immune-fluorescence techniques. Monoclonal antibody against specific DENV serotype will able to recognize the presence of DENV in the cell culture. Mammalian cell which is commonly used for DENV isolation is Vero, LLCMK2, and BHK21. However, the mammalian cell is less superior compare to mosquito cell lines[12].

Specimen from the patient may directly being inoculated intracerebrally to suckling mice. The presence of DENV can be detected by behavioral change of mice. Immunofluorescence detection of suckling mice brain is also possible to visualize the DENV. This method has several disadvantages, including high cost, long time for isolation, and low sensitivity. These disadvantages have prevented further recommendation of this method for DENV isolation[12].

The last method available to isolate DENV is direct inoculation into live mosquito. Specimen can be injected intra-thorax into the mosquito. Several mosquito are commonly used for this purpose i.e. *Toxorhynchites splendens*, *Aedes albopictus*, and *Aedes aegypti*. *Toxorhynchites splendens* is the most common use because of its extra large size, easy to be inoculated, and it does not need blood for live. To detect the inoculated DENV in the mosquito we can use the head squash immunostaining using monoclonal antibody against DENV. This method is superior compare to the cell culture inoculation and even comparable to the RT-PCR method[2,8,13].

2. Nucleic acid based detection
RT-PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) for dengue virus detection was developed since 1990. RT- PCR is a useful

method for dengue diagnosis, but it still needs to be better standardized. There are various methods and primer correspond to certain specific region of DENV genome had been developed. These methods need to be standardized to have optimum validity. The basic principle of the method is conversion of RNA of DENV to cDNA by using reverse transcripatase enzymes. The cDNA then is subjected to PCR amplification. If specific nucleotide sequence succeeds to be amplified using specific primer, it would be considered as positive result.

RNA based detection of DENV was started by using nested RT-PCR approach[14]. Universal primers correspond to C/prM gene of DENV genome were employed for the first round amplification. Then, specific serotype amplification was performed using nested PCR strategy. One step multiplex RT-PCR was developed by Harris *et al.*[15]. One universal primer and four serotypes specific primers were employed for DENV serotypes detection purpose. DENV Serotypes were recognized by different band expected size in electrophoresis assay after amplification.

The DENV RT-PCR assay provides a rapid, sensitive, diagnostic tool for detecting DENV in specimens from infected individuals. There are several limitation on the application of RT-PCR to the diagnosis of DENV: RT-PCR only suitable to detect the acute phase samples. RT-PCR need specific well equipped laboratory, such as ultracentrifuge, thermocycler and electrophoresis apparatus[8]. This technique also needs well trained laboratory technician.

RT-PCR sensitivity was 80%-100% compared to virus isolation method. The variable sensitivity might be caused by different primer being used and target genome of the DENV. There are two variations of RT-PCR methods: one step RT-PCR and two step RT-PCR[2].

Real Time RT-PCR

Standard RT-PCR will indicate positive if a particular nucleotide sequence is present in the clinical specimens. However, sometimes the clinician is also interested in the amount of the target sequence present in the specimen[16]. Real time RT-PCR is a technique that is sufficient to detect and count the RNA of virus in particular specimen.

Box 2: Laboratory diagnosis of DENV infection

- Virus isolation
  - Mosquitos cell lines : C6/36, AP61
  - Mamalian cell lines: Vero, LLCMK2, and BHK21
- Molecular techniques
  - Blot hybridization
  - RT-PCR
  - Real time RT-PCR
  - NASBA
- Serology
  - Hemagglutination inhibition (HI) assay
  - Plaque reduction neutralization test (PRNT)
  - Indirect fluorescent antibody (IFA) test
  - western blott
  - rapid detection assay
  - IgG antibody detection
  - IgM antibody detection
  - Antigen detection: NS1

There are many RT-PCR protocols have been developed. The protocols are using Taq Man real time RT-PCR or SYBRGreen real time RT-PCR. The Taq Man real time RT-PCR is considered more specific compare to SYBRGreen real time RT-PCR[2, 17]. Real time RT-PCR may be used singleplex or multiplex approach. The first is using only primer which corresponds to one DENV serotypes. The multiplex approach is employed several primers which correspond to more than one DENV serotypes[2,18,19].

The advantage of real time RT-PCR is superior in diagnosis of DENV infection in clinical setting. Real time RT-PCR Assay had been shown sensitivity of 97.65%, specificity was 92.59% and accuracy was 95.82% when compared to conventional RT-PCR. Moreover, there was no cross-reaction between chikungunya virus (CHIKV), DENV, Japanese encephalitis virus, hepatitis C, hepatitis A or hepatitis E virus. The data were obtained from 290 suspected individuals[20]. These data indicated that real time RT-PCR method provides a reliable diagnostic for DENV infection. However, the real time RT-PCR method is not recommended in the institution with limited resources. This technology is demanded high cost-sophisticated equipments and reagents.

3. DENV Antigen Detection

Detection of DENV infection may be made use of surrogate biomarker such as viral antigen. One of remarkable succeed of biomarker identification is the NS1 of DENV which is a non structural protein. NS1 can be utilized to detect DENV in viremia stage, since cellular infection with ENV results in the production and release of the nonstructural protein NS-1. A series of 1,055 samples were analyzed by the NS1 test and using virus isolation as gold standard. As much as 790 samples were NS1-positive and 726 of these were positive for virus isolation. The agreement between these two assays was 91.9%. Among the 265 NS1-negative samples, there were 13 positive virus isolations (4.9% of false NS1-negative)[21]. This method is a promising for further development. NS1 detection assay has been shown to be inexpensive, high throughput and easy to performed in the field.

NS1 is produced by DENV and localized at the cell surface, which eventually released in to the blood stream. The half-life of NS1 antigen in the blood of infected patients is longer than that of intact virus and has been detected in sera up to18 days after the onset of symptoms, with peak antigen detected at days 6–10 after the onset of fever[8, 22]. Enzyme link immunosorband assay (ELISA) and rapid test immunochromatography based technology have been develop for NS1 antigen detection assay. These two technology offer good approach for DENV infection detection[23]. The accuracy of the Platelia NS1 ELISA assay and an NS1 lateral flow rapid test (LFRT) were compared against a gold standard reference diagnostic algorithm in 138 Vietnamese children and adults. The Platelia NS1 ELISA was modestly more sensitive (82%) than the NS1 LFRT (72%) in confirmed DENV infection cases[24].

4. Serology

Serological assays for DENV infection includes: Hemagglutination inhibition (HI) assay, Plaque reduction neutralization test (PRNT), Enzyme immunosorbent assays (EIA's), Indirect fluorescent antibody (IFA) test, western blott, and rapid detection assay

Hemagglutination inhibition (HI) assay

The haemagglutination-inhibition (HI) assay is based on the ability of DENV antigens to agglutinate red blood cells (RBC) of ganders or trypsinized human O RBC. DENV antigen is able to agglutinate normal erythrocytes and the complex will precipitate at the bottom of wells after 30-60 minutes[8]. Anti-dengue antibodies in sera of the patients can inhibit this agglutination and the potency of this inhibition is measured in an HI assay[2]. Inhibition by the antibody in the sera will give specific bottom like appearance in the wells (Figure 2). The titer of antibody is defined by the lowest concentration which is shown to have inhibition of hemaglutination. WHO has released the guideline for interpretation of the HI assay in dengue virus infection cases (table 1)[3].

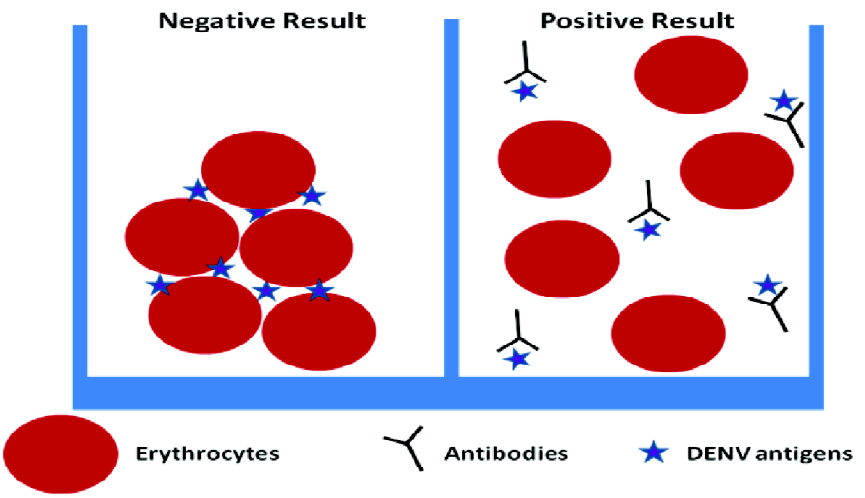

Figure 2. (A) Negative result: DENV antigens able to agglutinate the red blood cells and precipitates in the bottom of the wells. (B) Positive result: The anti-DENV antibodies which is presence in the patients sera inhibit the agglutination process

HI assay has been the most widely used method for the serological diagnosis of dengue infection in the past. However, due to the nature of HI assay: extensive cross-reaction of all flaviviruses[25], time consuming, also the requirement of both acute and convalescent sera collected at least seven days apart have reduced the general applicability of this assay[2].

HI assays is recommended to distinguish the primary and secondary DENV infection[3]. However, recently there are many novel techniques available for this purpose[26].

Tabel 1. WHO guideline for HI assay interpretation in dengue virus infection cases[3]

Change in antibody titer	Sample interval	Antibody titer at time of convalescence	Interpretation
≥ to a 4-fold rise	Paired sera with ≥ 7 days of separation	≤ 1:1280	Acute primary flavivirus infection
≥ to a 4-fold rise	With any specimen	≥ 1:2560	Acute secondary flavivirus infection
≥ to a 4-fold rise	Paired sera with < 7 days of separation	≤ 1:1280	Acute flavivirus infection, indeterminate primary/ secondary
No change in titer	With any specimen	> 1:2560	Recent secondary flavivirus infection
No change in titer	Paired sera with ≥ 7 days of separation	≤ 1:1280	Not dengue
No change in titer	Paired sera with < 7 days of separation	≤ 1:1280	None
Uncertain	One seraum specimen	≤ 1:1280	None

Plaque reduction neutralization test (PRNT)

PRNT was introduced in 1967 by Russel and Nisalak and able to measure the antibodies serotypes specific titers[27]. PRNT is considered as the most specific serology method to detect DENV infection. This method may be employed to detect DENV serotype in the sera at the convalescent period. PRNT measures the titer of the neutralizing antibodies in the sera of DENV infected patients and determines the level of protective antibodies of the particular individual. towards the virus. The assay is a biological assay based on the principle of interaction of virus and antibody resulting in inactivation of virus. The virus ability to infect and replicate in cell culture was diminished[28].

PRNT is a gold standard for antibody against DENV titer measurement. Since the first method developed by Russell and Nisalak, there are many modification which is employed different cell lines, virus preparation, and the presence or absence of complement. Modification of these conditions had significant effects on the PRNT titers measured in a particular serum sample. Significant associations were observed between testing conditions and titers measured from different tests on the same serum sample[29]. Base on this finding, very careful standardization PRNT method is highly demanded in clinical microbiology laboratory serving for dengue virus infection diagnostic tests

MAC-ELISA

IgM antibody-capture enzyme immunosorbent assay (MAC-ELISA) can be used to measure IgM total in patients serum. MAC-ELISA is the most common method employed in diagnostic laboratories and commercial available diagnostic kits. The assay is based on capturing human IgM antibodies on a microtiter plate using anti-human-IgM antibody followed by the addition of dengue virus specific antigen (DENV1-4). The antigens used for this assay are derived from mainly envelope protein of the virus. Specific antigen of DENV1, DENV2, DENV3, or DENV4 will

bind to the IgM anti dengue which is present in the patients serum[28].

MAC-ELISA can be used for IgM against DENV detection from serum, whole blood on filter paper, and saliva, but not the urine, which obtained more than five days from first onset of fever. MAC-ELISA has high specificity and sensitivity. Different commercial kits are available but have variable sensitivity and specifi city[2].

The pitfall of this method is the cross reactivity between DENV and other diseases i.e. malaria, leptospirosis and previous DENV infection, though cross-reactivity with other circulating flaviviruses such as Japanese encephalitis, St Louis encephalitis and yellow fever, does not seem to be a problem[2].

IgG ELISA

The IgG ELISA test is being used for the detection of a past infection of DENV. The antigens of DENV are employed in this assay, exactly similar with MAC-ELISA. The assay is usually performed with serial dilutions of the sera of the patients. The aim is to determine an end-point dilution. In general, IgG ELISA has low specificity within the flavivirus sero-complex groups[9].

IgG against DENV may be presence lifelong in the patients serum. However, fourfold or greater elevation of IgG titer between the convalescent and acute phase may be indicated of present DENV infection[2]. (WHO, 2009). IgG-ELISA is comparable to the HI assay in term of it can be used for the differentiation of primary and secondary DENV infections[30].

CONCLUSION

The available laboratory assays have been shown to be good to provide platform of the DENV infection diagnosis in the clinical and field setting. However, laboratory assays as tools for DENV infection detection needs to be further developed. The ideal laboratory diagnosis method which is user friendly, cost effective, accurate and robust is needed for DENV infection detection.

REFERENCES

1. Guzman MG, Kouri G. Dengue: an update. *Lancet Infect Dis*, 2002;2:33-42.
2. WHO. Dengue: guidelines for diagnosis, treatment, prevention and control. New edition, WHO Geneva, 2009.
3. WHO. *Dengue haemorrhagic fever. Diagnosis, treatment, prevention and control.* Geneva: WHO, 1997;12-23.
4. Hadinegoro SRS. The revised WHO dengue case classification: does the system need to be modified? *Paediatrics & Child Health*, 2012;32(S1):33-8.
5. Halstead. Dengue: Overview and History, In Halstead (Ed). *Dengue*. Imperial College Press, 2008:1-9.
6. Beasley DWC dan Barrett ADT. The Infectious Agent. In Halstead (Ed). *Dengue*. Imperial College Press, 2008:29-57.
7. Henchal EA dan Putnak JB. The Dengue Virus. *Clin Microbiol Rev*, 1990;3(4):376-96.
8. Endy TP, Nisalak A and Vaughn DW. Diagnosis of Dengue Virus Infections. In Halstead (Ed). *Dengue*. Imperial College Press, 2008:327-60.
9. Buchy P, Yoksan S, Peeling RW, Hunsperger E. Laboratory Tests For The Diagnosis Of Dengue Virus Infection. Scientific Working Group, Report on Dengue, 1-5 October 2006, Geneva, Switzerland, 2007.
10. White LA. Susceptibility of Aedes albopictus C6/36 Cells to Viral Infection. J Clin Microbiol, 1987;25(7):1221-24.

11. Kuno G. Cultivation of mosquito cell lines in serum-free media and their effects on dengue virus replication. *In Vitro*, 1983;19(9):707-13.
12. De Paula SO & da Fonseca BAL. Dengue: A Review of the Laboratory Tests a Clinician Must Know to Achieve a Correct Diagnosis *Braz J Infect Dis*, 2004;8(6):390-8.
13. Vaughn DW, Green S, Kalayanarooj S *et al*., 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis* 181:2-9.
14. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, and Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J. Clin Microbiol*, 1992;30:545-51.
15. Harris E T, Roberts G, Smith L, Selle J, Kramer LD, Valle S, Sandoval E, dan Balmased A. Typing of dengue viruses in clinical specimens and mosquitoes by single tube multiplex reverse transcriptase PCR. *J Clin Microbiol*, 1998;36:2634-9.
16. Buckingham L, Flaws M. Molecular diagnostics: fundamentals, methods, and clinical applications. F. A. Davis Company, USA, 2007.
17. Paudel D, Jarman R, Limkittikul K, Klungthong C, Chamnanchanunt S, Nisalak A, Gibbons R dan Chokejindachai W. Comparison of real-time SYBR green dengue assay with real-time taqman RT-PCR dengue assay and the conventional nested PCR for diagnosis of primary and secondary dengue infection, *N Am J Med Sci*, 2011;3(10):478-85.
18. Johnson BW, Russell BJ, Lanciotti RS. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. J Clin Microbiol, 2005 Oct;43(10):4977-83.
19. Hue KD, Tuan TV, Thi HT, Bich CT, Anh HH, Wills BA, Simmons CP. Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma. J Virol Methods, 2011 Nov;177(2):168-73.
20. Pongsiri P, Praianantathavorn K, Theamboonlers A, Payungporn S, Poovorawan Y. Multiplex real-time RT-PCR for detecting chikungunya virus and dengue virus. Asian Pac J Trop Med, 2012;5(5):342-6.
21. Bisordi I, Rocco IM, Suzuki A, Katz G, Silveira VR, Maeda AY, Souza RP, Bassi MG, Del Tedesco EF, Freitas E, Bessa TÁ; Dengue-NS1 Group. Evaluation of dengue NS1 antigen detection for diagnosis in public health laboratories, São Paulo State, 2009. Rev Inst Med Trop Sao Paulo. 2011 Nov-Dec;53(6):315-20.
22. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flammand M. Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in blood during the acute phase of disease in patient experiencing primary or secondary infection. J Clin Microbiol, 2002;40:376-81.
23. Dussart P, Petit L, Labeau B, Bremand L, Leduc A, Moua D, Matheus S dan Baril L. Evaluation of Two New Commercial Tests for the Diagnosis of Acute Dengue Virus Infection Using NS1 Antigen Detection in Human Serum. *PLoS Negl Trop Dis*, 2008;2(8):e280.
24. Hang VT, Nguyet NM, Trung DT, Tricou V, Yoksan S, Dung NM, Van Ngoc T, Hien TT, Farrar J, Wills B, Simmons CP. Diagnostic accuracy of NS1 ELISA and lateral flow rapid

tests for dengue sensitivity, specificity and relation-ship to viraemia and antibody responses PLoS Negl Trop Dis, 2009; 3(1): e360.

25. Anantapreecha S, Anuegoonpipat A, Prakrong S, Chanama S, Sangasang A, Sawanpanyalert P and Kurane I. Dengue Virus Cross-Reactive Hemagglutination Inhibition Antibody Responses in Patients with Primary Dengue Virus Infection. Jpn. J. Infect. Dis, 2007; 60(5): 267-70.
26. Matheus S, Deperis X, Labeau B, Lelarge J, Morvan J, dan Dussart P. Discrimination between Primary and Secondary Dengue Virus Infection by an Immunoglobulin G Avidity Test Using a Single Acute-Phase Serum Sample. *J. Clin Microbiol*, 2005;43(6):2793-7.
27. Russell PK, Nisalak A. Dengue virus identificat-ion by the plaque reduction neutralization test. J Immunol, 1967;99:291-6.
28. Center for Disease Control and Prevention. [cited 2012 Sep]. Avaliable from: URL: http://www.cdc.gov.
29. Thomas SJ, Nisalak A, Anderson KB, Libraty DH, Kalayanarooj S, Vaughn DW, Putnak R, Gibbons RV, Jarman R, Endy TP. Dengue plaque reduction neutralization test (PRNT) in primary and secondary dengue virus infections: How alterations in assay conditions impact performance. *Am J Trop Med Hyg*, 2009;81(5): 825-33.
30. Kuno G, Gomez I, Gubler DJ. An ELISA procedure for the diagnosis of dengue infections. J Virol Methods, 1991;33:101-13.