EVALUATION OF RAPID IMMUNOCHROMATOGRAPHIC NS1 TEST, ANTI-DENGUE IgM TEST, SEMI-NESTED PCR AND IgM ELISA FOR DETECTION OF DENGUE VIRUS

Praewpilai Tontulawat[1, 2], Piyathida Pongsiri [2], Chittima Thongmee[2], Apiradee Theamboonlers[2], Nuantip Kamolvarin[1] and Yong Poovorawan[2]

[1]Department of Biochemistry, [2]Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Abstract. Dengue virus (DENV) causes various clinical symptoms of differing severity based on time of infections. The existing laboratory methods, semi-nested PCR and Dengue IgM ELISA, still have limitations for diagnosis. A commercially available rapid immunochromatographic dengue NS1 antigen and IgM antibody tests in comparison with semi-nested PCR and IgM ELISA for confirmation of DENV infection were evaluated. In total, 237 single acute serum specimens and 50 paired sera of dengue patients were examined using the rapid dengue NS1 antigen test, IgM antibody test, semi-nested PCR and Dengue IgM ELISA. The NS1 and IgM rapid tests showed sensitivity of 70.6%, and 75.6%, respectively, and specificity of 73.4% and 97.1%, respectively. The combination of NS1 and IgM tests enhanced diagnosis. Thus rapid dengue NS1 antigen and IgM antibody tests are highly appropriate for diagnosis of dengue infection as it is rapid, easily applicable, sensitive and highly specific.

Key words: dengue, rapid immunochromatographic test, semi-nested PCR, IgM ELISA

INTRODUCTION

Dengue virus (DENV) infection is one of the most important arthropod-borne viral diseases in tropical and subtropical countries throughout the world (Monath, 1994; Gubler, 2002; Guzman and Kouri, 2002). DENV causes various clinical symptoms, ranging from asymptomatic or undifferentiated fever, known as dengue fever (DF), to fever with plasma leakage, called dengue hemorrhagic fever (DHF). Some cases of DHF become an even more serious form, called dengue shock syndrome (DSS), leading to death, especially among children (Gubler and Meltzer, 1999; WHO, 2000).

DENV is transmitted by the mosquito vector, *Aedes aegypti* (Gubler and Clark, 1995; Gubler, 1998). It has been classified into 4 antigenic serotypes (DENV 1-4) of the genus *Flavivirus* and family Flaviridae. DENV comprises 7 nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (Henchal and Putnak, 1990;

Correspondence: Prof Yong Poovorawan, Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.
Tel: +66 (0) 2256 4909; Fax: +66 (0) 2256 4929.
E-mail: Yong.P@chula.ac.th

Monath and Heinz, 1990). Among the nonstructural proteins, NS1 is a highly conserved glycoprotein, which is essential for virus replication. During the acute phase of DENV infection, NS1 protein is found associated with intracellular organelles and can be transported via cellular secretion pathway to the infected cell surface. NS1 protein also released from infected mammalian cells and may then be found circulating in the sera of patients (Mackenzie *et al*, 1996; Flamand *et al*, 1999; Young *et al*, 2000).

Several methods have been used to diagnose DENV infection. Enzyme-linked immunosorbent assay (ELISA) detects immunoglobulin M (IgM) antibody directed against DENV in sera of patients who have fever for at least 7 days (WHO, 1997; Shu and Huang, 2004). When patients present with an acute phase or prior to day 7 post-infection, polymerase chain reaction (PCR) based on amplifying 3'-UTR (Lanciotti *et al*, 1992; Chutinimitkul *et al*, 2005; Johnson *et al*, 2005) can be performed to arrive at a definite diagnosis, but PCR is not widely performed because the technique requires special equipment and laboratory skill. Thus, a rapid dengue immunochromatography test is developed to detect the NS1 antigen and IgG/IgM antibody against DENV. As the assay can detect both NS1 antigen and IgG/IgM antibody, it should facilitate diagnosis of patients with DENV infection at any time period in their clinical course.

The objective of this study was to evaluate the commercially available rapid dengue NS1 antigen and IgM antibody immunochromatography test in comparison with existing laboratory methods, such as semi-nested PCR and dengue IgM ELISA for confirmation of DENV infection based on a single specimen during acute phase and paired sera.

MATERIALS AND METHODS

The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The specimens were used with the permission of the Director of King Chulalongkorn Memorial Hospital. The specimens were collected anonymously using coding numbers. All specimens were used exclusively for academic research.

Population study

Specimens were sent to the Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University and stored at -20°C until further examination. They were divided into 2 groups: 1) single sera (n = 237) collected within the first 7 days of fever (acute febrile phase) in accordance with WHO criteria, and 2) paired sera (n = 50) collected twice, once during the acute febrile phase and once during the convalescence phase upon confirmation as being DENV-infected specimens using IgM ELISA as the gold standard.

Inclusion criteria for specimen collection

Sera were collected from suspected DENV patients diagnosed at Ayutthaya, Buri Ram and Khon Kaen Provincial Hospitals in Thailand. Patients were subsequently clinically classified by WHO criteria into DF or DHF. DF was defined as patients presenting within 1-7 days of the onset of fever, headache, rash or myalgia/arthralgia. DHF was defined as patients with symptoms of DF and one or more of the following symptoms: positive tourniquet test, thrombocytopenia (platelets ≤100,000/mm^3) or plasma leakage (WHO, 2000).

Rapid test for NS1 antigen and IgG/IgM

Dengue NS1 antigen (Ag) and IgG/

IgM antibody tests were performed according to the manufacturer's specifications using SD BIOLINE Dengue Duo rapid test (Standard Diagnostics, Kyonggi-do, Korea). In brief, 100 µl of serum were added into the sample well of the test device for NS1 Ag detection. Subsequently, 10 µl aliquot of serum was added into another sample well, and 120 µl aliquot of provided assay diluent was added into the diluent well of the test device for IgM detection and the results were interpreted after 15-20 minutes of incubation. The presence of only a "C" (control) line within the result window indicates a negative result. The presence of both "C" and "T" or "M" lines within the result window indicate a positive result for NS1 Ag or IgG/IgM, respectively. On the other hand, if a "C" line was absent or only a "T" or "M" line were present, the result was considered invalid and the specimen was retested. However, human interpretation may be biased. Subjective bias was reduced by reading and analyzing the results from 3 different readers and insisting on at least two out of three for positive or negative results. Due to the high prevalence of DENV infection in Thailand, IgG results were not included in the data analysis.

Dengue IgM ELISA

SD Dengue IgM Capture ELISA (Standard Diagnostics, Korea) was used for the detection of IgM antibody to dengue virus in sera according to the manufacturer's instructions. In brief, each well of a flat-bottom microplate was coated with mouse monoclonal anti-human IgM antibodies. The patient's sera were diluted at 1:100 with sample diluent and incubated for 1 hour at 37ºC. The microplate was washed 5 times with 350 µl of diluted washing solution, soaked for at least 10 seconds per well and all liquid was dispensed from the wells. Then 100 µl aliquot of diluted Anti-Dengue Horseradish peroxidase (HRP) conjugate solution, a 1:1 combination of the Anti-Dengue HRP conjugate and the diluted Dengue Antigen, was added into each well. The microplate was incubated for 1 hour at 37ºC and washed as described above. Subsequently, 100 µl aliquot of tetramethylbenzidine (TMB) solution, a 1:1 combination of TMB substrate A and B, was added into each well and incubated for 10 minutes at room temperature. Finally, 100 µl aliquot of stopping solution was added and absorbance was measured at a wavelength of 450 nm within 30 minutes.

Dengue RNA detection by PCR

RNA was extracted from 200 µl of serum specimens using Viral Nucleic Acid Extraction Kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's instructions. The extracted RNA samples were eluted in 50 µl elution buffer and subsequently reverse transcribed into cDNA using Improm-II™ reverse transcription system (Promega, Madison, WI) following the producer's recommendation. First, 5 µl of the extracted RNA were used as template for reverse transcription (RT) with 1 µl of 0.5 µg/µl random hexamer primers and 5 µl of DepC-treated sterilized water. These mixtures were heated at 70ºC for 5 minutes and immediately put on ice for 5 minutes. Subsequently, 12.5 µl aliquot of reaction mixture (3.5 µl of Improm-II™ reaction buffer, 2.5 µl of 3 mM $MgCl_2$, 2.5 µl of 0.5 mM dNTP, 0.5 µl of 1 µg/µl RNasin® ribonuclease inhibitor, 1 µl of Improm-II™ reverse transcriptase and 2.5 µl of DepC-treated sterilized water) was added and kept at room temperature for 5 minutes. Finally, the reaction mixtures were incubated at 42ºC for 1 hour and the reaction was terminated by heating at 70ºC for 15 minutes.

The 3′-UTR was analyzed as is considered to show maximum homology between the 4 dengue virus serotypes. For the first round of semi-nested PCR, DenF (5′-TTAGAGGAGACCCCTCCC-3′) and $DenR_2$ (5′-GAGACAGCAGGATCTCTGG-3′) were used as forward primer and reverse primer, respectively (Chutinimitkul *et al*, 2005). The reaction mixture consisted of 5 µl of Eppendorf MasterMix (Eppendorf, Hamburg, Germany), 0.5 µl of 25 mM $MgCl_2$, 0.25 µl of 10 µM of each primer, 1 µl of cDNA template and nuclease-free water to make a final volume of 13 µl. PCR was performed in a thermocycler (Eppendorf, Hamburg, Germany) under the following thermal cycling conditions: at 94ºC for 5 minutes; 40 cycles of 30 seconds at 94ºC, 30 seconds at 53ºC and 30 seconds at 72ºC; and a final step at 72ºC for 10 minutes. The second round of semi-nested PCR was performed using the PCR product from the first round as a template, with the same reaction mixture and under identical thermal cycling conditions but using DenR (5′-TCTCCTCTAACCTCTAGTCC-3′) as an inner reverse primer. The final PCR amplicon was analyzed by 2% agarose gel-electrophoresis at 100 volts for 30 minutes and staining with ethidium bromide. The expected band of approximately 100 bp was visualized on a UV trans-illuminator.

The agarose gel slice containing the DNA fragment of interest was excised and purified using HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Taiwan) according to the company's protocol. The purified DNA was sequenced commercially (1st BASE Laboratories, Selangor Darul Ehsan, Malaysia).

Data analysis

Diagnostic accuracy of the DENV commercially available rapid test, IgM ELISA, and semi-nested PCR were evaluated relative to the final patient diagnosis, DENV-positive or negative. The results were calculated using diagnostic tests that considered sensitivity, specificity, positive predictive values (PPVs) and negative predictive values (NPVs) as follows: sensitivity: a/a+c x 100%; specificity: d/d+b x 100%; negative predicted value: d/d+c x 100% and positive predicted value: a/a+b x 100%; where: a = number of true positives, b = number of false positives, c = number of false negatives and d = number of true negatives. The commercially available rapid test was compared with semi-nested PCR and Dengue IgM ELISA, and ELISA was used as the gold standard for dengue diagnosis.

RESULTS

This study diagnosed dengue virus infection using 3 methods; semi-nested PCR, the rapid test, and IgM ELISA for comparison of test results in single sera and paired sera. Samples were analyzed the day after the onset of symptoms and the diagnostic accuracy of the three methods was evaluated.

Comparison of test results in single sera

Of 237 acute phase sera, the percentage of DENV-positive samples by semi-nested PCR, NS1 rapid test, IgM rapid test, NS1/IgM rapid test and IgM ELISA was 46% (109/237), 47% (111/237), 44% (105/237), 67% (159/237) and 57% (135/237), respectively Fig 1 (A). The combination of NS1 and IgM rapid tests increased the percentage of DENV detection when compared to the single NS1 rapid test or single IgM rapid test.

When the sera were divided into 3 groups with regards to duration of fever (Days 1-3, 4-5 and 6-7), the percentage of

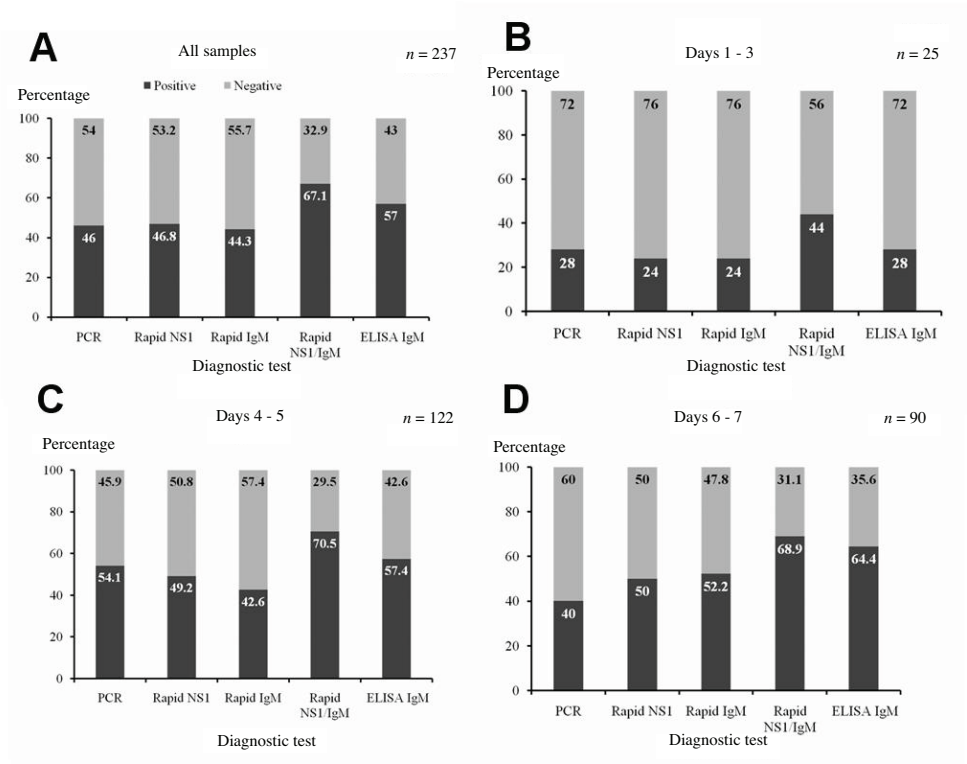

Fig 1–Single sera tested by semi-nested PCR, the rapid test (NS1 Ag/IgM) and IgM ELISA. Experimented protocols are described in Materials and in Methods. Pos, positive and Neg, negative. (A) All samples (n = 237); (B) samples collected on Days 1-3 (n = 25); (C) Days 4-5 (n = 122); (D) Days 6-7 (n = 90) after onset of fever.

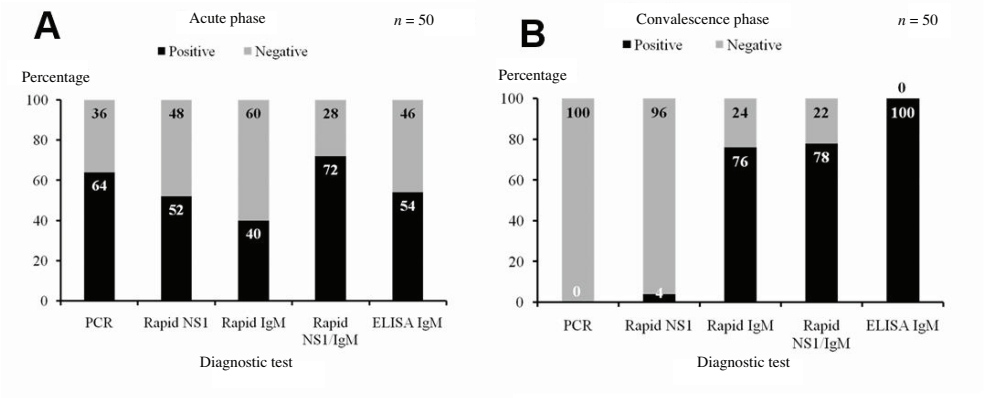

Fig 2–Paired sera during the acute febrile phase (A) and convalescence phase (B) analyzed by semi-nested PCR, the rapid test (NS1 Ag/IgM) and IgM ELISA. Experimented protocols are described in Materials and Methods.

DENV positives by semi-nested PCR increased from 28% on Days 1-3 to 54% on Days 4-5 and slightly decreased to 40% on days 6-7 (Fig 1B-D). The percentage of DENV positives by NS1 rapid test, IgM rapid test and IgM ELISA are positively correlated with the duration of fever.

The combined NS1/IgM rapid test yielded a higher detection rate of DENV positives when compared with the other tests at any time point.

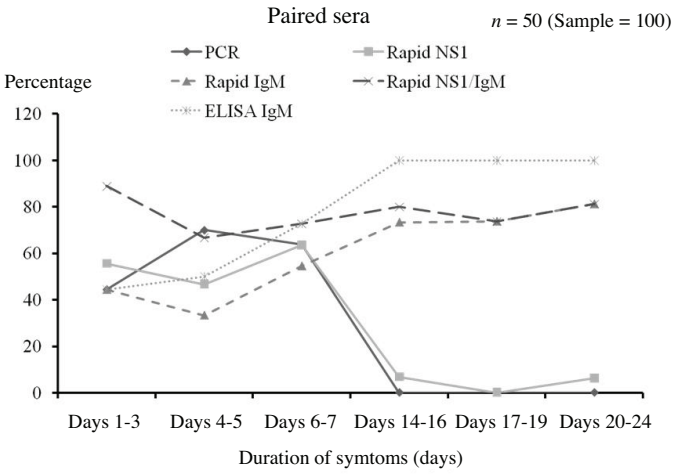

Fig 3–Comparison among semi-nested PCR, NS1 rapid test, IgM rapid test, combined NS1/IgM rapid test and the IgM ELISA in relation to days after onset of fever.

Comparison of test results in paired sera

Paired sera obtained during the acute febrile phase were diagnosed by semi-nested PCR and NS1 rapid test, which showed only 64% (32/50) and 52% (26/50) of positive results respectively. Also, positive results of the IgM rapid test and IgM ELISA amounted to 40% (20/50) and 54% (27/50). Upon combining the results of NS1 and IgM rapid tests, the percentage increased to 72% (36/50) (Fig 2A).

IgM ELISA has been used as the gold standard for DENV infection detection during the convalescence phase and it was used as an inclusion criterion for specimens into this group. Thus, IgM ELISA yielded 100% positive results as opposed to the IgM rapid test with only 76% (38/50). We found that semi-nested PCR exclusively yielded negative results; however, we were able to see positive results from the NS1 rapid test for 4%. Also, the combined results of NS1/IgM rapid test amounted to 78% (Fig 2B).

Sensitivity and specificity of the tests

The NS1 rapid test was compared with semi-nested PCR, with sensitivity of 70.6% and specificity of 73.4%. Furthermore, positive and negative predictive values for the NS1 rapid test was calculated and shown to be 69.4% and 74.6%, respectively. Also, The NS1 rapid test was accurate to 72.2%.

The IgM rapid test was compared with IgM ELISA and showed the sensitivity of 75.6% and specificity of 97.1%. The percentage of positive and negative predictive value of the IgM rapid test was evaluated as 97.1% and 75%, respectively. Thus, accuracy increased to 84.8%.

Comparison of each method on days after onset of fever

During Days 1-3 after onset of fever, semi-nested PCR and NS1 rapid test showed positive result of 44.4% and 55.6%, respectively. Over the next two days, percentage of semi nested PCR increased to 70% while NS1 rapid test decreased to 46.7%. For Days 6-7, semi-nested PCR and NS1 rapid test showed the same percentage of 63.6%. For Days

14-24, which is in the convalescence phase, semi-nested PCR showed no positive results and NS1 rapid test showed less than 7%. When we looked at the combined NS1/IgM test they showed a steady level of positives between 72.7% and 82.9%.

IgM rapid test and IgM ELISA yielded 44.4% positive for the first three days. Semi-nested PCR yielded 57.2%, the NS1 rapid test 48.7%, the IgM rapid test 40.8% and the IgM ELISA 55.9%. IgM ELISA showed a slight increase at Days 4-5 of 50%, while IgM rapid test decrease to 33.3%. For Days 6-7, IgM rapid test and ELISA showed an increasing trend, 54.5% and 72.7%, respectively. During Days 14 to 24, both methods showed a consistent increasing trend, between 73.3% and 81.2% for IgM rapid test and 100% for ELISA.

DISCUSSION

Acute febrile illness is listed among the symptoms related to fever such as malaria, influenza, leptospirosis, scrub typhus, typhoid fever and dengue infection. Some symptoms necessitate specific treatment. For instance, antibiotics will be required to treat for bacterial diseases. Knowing which kind of infection the patient has is necessary for proper treatment and helps in avoiding unnecessary administration of antibiotics. In this study, we evaluated rapid DENV diagnosis of DENV infection in acute febrile illness in order to provide information for appropriate management.

Previous studies have shown that the NS1 antigen can be detected between Days 0 to 9 (Falconar, 1997; Shu *et al*, 2002; Dussart *et al*, 2006) and peaks at Days 6 to 10 (Xu *et al*, 2006). In our study, when we compared the NS1 rapid test with semi-nested PCR, the former test showed the highest percentage of detectable NS1 antigen on Days 6-7 of the acute phase in single sera and the highest percentage in paired sera on Days 6-7. During the convalescence phase (after Day 14), NS1 antigen was detected by the rapid test in only 4% of the specimens. Semi-nested PCR amplification yielded the highest percentage of positive results in single sera obtained on Days 4-5, and also in paired sera obtained on Days 6-7 of the acute phase, consistent with the NS1 rapid test. During the convalescence phase, the viral genome could not be amplified by semi-nested PCR.

Sensitivity and specificity tests are essential for accurate laboratory diagnosis of DENV infected patients. Sensitivity of the NS1 rapid test amounted to 70.6%, which appears to be the most sensitive method because of its high sensitivity to the NS1 antigen of DENV. In previous diagnoses of dengue, NS1 rapid test has proven more sensitive than semi-nested PCR due to the fact that the NS1 antigen is detectable for a longer time than viral RNA (Huhtamoa *et al*, 2010). The NS1 rapid test was highly specific to DENV (73%). In addition, the IgM rapid test showed 75.6% sensitivity and 97.1% specificity. It has been reported that a combination of NS1 and IgM rapid tests enhances diagnosis (Huhtamoa *et al*, 2010) and our study has shown a detection rate amounting to approximately 70% upon combining NS1 test with IgM detection.

As the combined test uses both the NS1 as well as the IgM markers, as well as displaying a consistently high rate of diagnosis, it presents as the ideal method of testing. With convalescence sera, there was a higher percentage of IgM expression than during the acute phase, consistent with other previous studies reporting an increase in IgM directly proportional to the number of days after infection (Shu

and Huang, 2004), which indicates that during the viremia phase, antibody response is either non-existent or at a very low level. One particular challenge in this study has been the uncertainty as to the exact day the patient(s) have been infected and due to this limitation, the analysis might vary.

In conclusion, evaluation of rapid dengue NS1 antigen and IgM antibody tests showed that this test is highly appropriate for diagnosis of dengue infection as it has proven to be a rapid, easily applicable, sensitive and specific method. NS1 antigen detection has potential value for screening patient samples during the early acute phase. IgM antibody detection, on the other hand, can help diagnose severe disease due to the fact that IgM antibodies appear in the phase of dengue hemorrhagic fever or in dengue shock syndrome.

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