# USE OF DENGUE NS1 ANTIGEN FOR EARLY DIAGNOSIS OF DENGUE VIRUS INFECTION

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**Abstract.** Accurate and timely diagnosis of dengue virus is important for early detection of dengue virus infection. In this study, the usefulness of the dengue NS1 antigen test was evaluated as a routine, rapid diagnostic test for dengue virus infection. A total of 208 sera from patients suspected of having dengue virus infection were collected and tested for dengue antibody, dengue genome and dengue NS1 antigen. Dengue antibody test, dengue PCR test and dengue antigen test were able to detect dengue virus infection from Days 1 to 8 in 72.8, 52.8 and 44.0% of samples, respectively. Of the 208 sera tested, 69.2% (144/208) of the acute sera were positive for dengue virus infection based on IgM antibody, IgG antibody, NS1 antigen and PCR tests. Thirty-two point two percent of the samples (67/208) were found positive for dengue NS1 antigen, 38.5% (80/208) were PCR positive, 40.9% (85/208) were IgM positive and 36.1% (75/208) were IgG positive for dengue virus. The results reveal the detection rate of dengue virus infection was similar for PCR and dengue antibody (65.9%) and for NS1 antigen and dengue antibody (62.0%) combinations. Therefore, the dengue NS1 antigen test can be used to complement the current antibody test used in peripheral laboratories. Thus, the combination of the NS1 antigen and antibody tests could increase the diagnostic efficiency for early diagnosis of dengue infection.

**Keywords:** dengue virus, NS1 antigen, rapid diagnostic test

## INTRODUCTION

Dengue has become a major international public health problem due to the human morbidity and mortality it causes. Globally, more than 2.5 billion people live in areas where there are risks of exposure to dengue virus (DENV) infection and the WHO currently estimates that worldwide, 50 million people are infected with dengue virus every year (WHO, 2000). Controlling dengue infections is challenging because it requires not only effective control of vectors responsible for transmitting the virus but also accurate and rapid diagnosis. To date, accurate and timely diagnosis of early detection with DENV remains a problem for management of dengue infected patients in many parts of the world, especially in countries with limited resources.

Several laboratory methods, such as virus isolation, genomic RNA, antigen and antibody detection methods are available to diagnose dengue infections. However, methods such as virus isolation and genomic RNA detection (PCR), need a specialized laboratory, well trained laboratory personnel, which are

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not widely available in hospital settings. In most cases, serologic tests which detect DENV-specific immunoglobulin M (IgM) and immunoglobulin G (IgG) by enzyme-linked immunoassay (ELISA), are the most commonly used methods for diagnosis of dengue infection. During the acute phase, the presence of IgM antibodies alone suggests primary infection and detection of newly formed IgM antibodies occurs after viremia ends or after fever subsides (Halstead, 2007). IgM antibodies are detectable approximately 4 to 6 days after the onset of fever. By Day 5 of symptoms, 80% of cases have detectable IgM antibodies and 93-99% of cases have detectable IgM by Days 6 to 10 (Guzman and Kouri, 2004). Once IgM is detected, the level increases rapidly and appears to peak about 2 weeks after the onset of symptoms, then decreases to undetectable levels over 2 to 3 months. However, in secondary infection, IgG antibodies present at high levels before or simultaneously with the IgM antibody response. IgG antibodies increase to high levels within the first week of infection and decline slowly over 3 to 6 months (WHO, 2000).

Recently, DENV nonstructural 1 (NS1) antigen has gained a lot of interest as a new biomarker for early diagnosis of DENV infection. Dengue NS1 antigen, a highly conserved glycoprotein, produced in both membrane-associated and secretion forms, is abundant in the serum of patients during the early stages of DENV infection (Young *et al,* 2000). Several studies conducted revealed the importance of dengue NS1 antigen as a biomarker; these antigens can be detected before the formation of antibodies (Young *et al,* 2000; Alcon *et al,* 2002; Dussart *et al,* 2006; Ramirez *et al,* 2009). NS1 antigen is detectable in blood from the first day after the onset of fever up to Day 9; once the clinical phase of the disease is over it is still detectable even when viral RNA is negative by RT-PCR and in the presence of IgM antibodies (Alcon *et al,* 2002). Currently, NS1 antigen-capture ELISA and rapid NS1 antigen commercial kits for detection of NS1 antigen have been developed and evaluated. Studies revealed the detection rate of NS1 antigen is higher in acute primary dengue than in acute secondary dengue infection (Kumarasamy *et al,* 2007; Blacksell *et al,* 2008; Zainah *et al,* 2009). Its use has been suggested for early diagnosis of dengue infection after the onset of fever (Chaiyaratana *et al,* 2009; Ramirez *et al,* 2009; McBride, 2009). Detection of dengue NS1 antigen represents a new approach for the diagnosis of acute dengue infection.

In this study, the potential use of the dengue NS1 antigen test to improve dengue laboratory diagnosis is analyzed and compared to the current antibody and genomic RNA detection methods available in our laboratory.

## MATERIALS AND METHODS

Two health clinics were selected for this study and a total of 208 sera were collected from patients with suspected DENV infection. These patients were selected based on a clinical diagnosis of DENV infection and fulfilled the WHO case definition for dengue fever. The status of dengue infection in the patients was determined by serological detection of dengue IgM and IgG, molecular detection of dengue genomic RNA and detection of dengue NS1 antigen.

### Dengue IgM- and IgG-capture ELISA

All samples were tested using a commercial Dengue IgM- and IgG-capture ELISA (Panbio, Brisbane, Australia) following the manufacturer's instructions.

Table 1
Results of samples (*n*=208) tested by antigen, PCR and antibody tests.

| | NS1 antigen | PCR | IgM-ELISA | IgG-ELISA |
|---|---|---|---|---|
| Positive | 67 (32.2%) | 80 (38.5%) | 85 (40.9%) | 75 (36.1%) |
| Negative | 141 (67.8%) | 128 (61.5%) | 123 (59.1%) | 133 (63.9%) |

Table 2
Results of samples tested by dengue NS1 antigen test compared to PCR.

| | | Dengue PCR | |
|---|---|---|---|
| | | Positive (*n*=80) | Negative (*n*=128) |
| Dengue NS1 antigen | Positive (*n*=67) | 43 | 24 |
| | Negative (*n*=141) | 37 | 104 |

**Detection of dengue genome (PCR)**

Two methods were used to detect dengue genomic RNA: conventional reverse transcriptase-polymerase chain reaction (RT-PCR) (Lanciotti *et al*, 1992) and SYBR Green real-time RT-PCR (rRT-PCR) as described by Chutinimitkul *et al* (2005).

**Dengue NS1 antigen capture ELISA**

The assay was performed in accordance with the manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France). Briefly, 50 l of serum was diluted 1:2 with sample diluent and combined with 100 l horseradish peroxidase-labeled anti-NS1 monoclonal antibody. The mixture was incubated at 37°C for 90 minutes in the microplate of which the wells were coated with anti-NS1 capture antibodies. Antigen-antibody complexes were detected after washing by addition of 160 l of substrate chromogen solution and incubating in the dark at room temperature for 30 minutes. The reaction was stopped by adding 100 l of 1 N sulfuric acid and the optical densities were read at 450/620 nm within 30 minutes. Results were interpreted based on the ratios, >1.0, <0.5, 0.5 to <1.0 for positive, negative and equivocal results, respectively.

## RESULTS

In this study, all serum samples were tested for the presence of dengue NS1 antigen, dengue genomic RNA, IgM antibodies and IgG antibodies. From the 208 samples tested, 69.2% (144/208) of the sera were found to be positive for DENV infection based on the IgM antibody, IgG antibody, NS1 antigen and PCR tests. Of these, 32.2% of the samples (67/208) were positive for dengue NS1 antigen, 38.5% (80/208) were PCR positive, 40.9% (85/208) were IgM positive and 36.1% (75/208) were IgG positive for DENV (Table 1).

**Combination of dengue NS1 antigen and PCR tests**

Based on the combination of dengue NS1 antigen and PCR tests, only 50.0% (104/208) of patients were found positive for DENV infections. Table 2 indicates the performance of the dengue NS1 antigen

Table 3
Results of samples tested with the dengue NS1 antigen test compared to dengue antibody tests.

| | | Dengue antibody (IgM and/or IgG) | |
|---|---|---|---|
| | | Positive (*n*=107) | Negative (*n*=101) |
| Dengue NS1 antigen | Positive (*n*=67) | 45 | 22 |
| | Negative (*n*=141) | 62 | 79 |

Table 4
Results of samples tested by dengue PCR compared to dengue antibody tests.

| | | Dengue antibody (IgM and/or IgG) | |
|---|---|---|---|
| | | Positive (*n*=107) | Negative (*n*=101) |
| Dengue PCR | Positive (*n*=80) | 50 | 30 |
| | Negative (*n*=128) | 57 | 71 |

ELISA kit compared to the results obtained from the PCR test. Dengue NS1 antigen was not detected in 46.3% (37/80) of PCR positive samples but was detected in 18.8% (24/128) of PCR negative samples.

**Combination of dengue NS1 antigen and antibody tests**

The results of dengue NS1 antigen detection were also compared to the results of the dengue antibody tests (IgM and/or IgG). A total of 107 samples were positive for IgM and/or IgG, giving the serological tests a detection rate of 51.4% (107/208). Based on the combination of dengue NS1 antigen and antibody tests, a total of 129 patients (62.0%) were positive for DENV infections. This combination of dengue NS1 antigen and antibody tests was 12.0% higher than the combination of dengue NS1 antigen and PCR tests (50.0%). Table 3 shows the dengue NS1 antigen was not detected in 57.9% (62/107) of the IgM and/or IgG positive samples but dengue NS1 antigen was detected in 21.8% (22/101) of dengue antibody negative samples.

**Combination of PCR and antibody tests**

When the PCR and antibody tests were combined, a total of 65.9% of patients (137/208) were positive for DENV infection (Table 4). This test combination was 3.9% and 15.9% higher than the antigen with antibody and antigen with PCR combinations, respectively. Of the 137 positive samples, 50 (36.5%) were positive for both PCR and antibody tests, 30 samples (21.9%) on the PCR only and 57 samples (41.6%) on the antibody tests only. Both tests were found to be negative on the remaining 71 samples (34.1%).

**Combination of dengue NS1 antigen, PCR and antibody tests**

When all three tests were carried out, a total of 69.2% of patients (144/208) were found positive for DENV infection. The combination of these 3 tests were 3.3%, 7.2% and 19.2% higher than the PCR with

Table 5
Results of samples tested by dengue NS1 antigen test compared to PCR and antibody tests.

| | | PCR (+) / antibody (+) (*n*=50) | PCR (+) / antibody (-) (*n*=30) | PCR (-) / antibody (+) (*n*=57) | PCR (-) / antibody (-) (*n*=71) |
|---|---|---|---|---|---|
| Dengue NS1 antigen | Positive (*n*=67) | 28 | 15 | 17 | 7 |
| | Negative (*n*=141) | 22 | 15 | 40 | 64 |

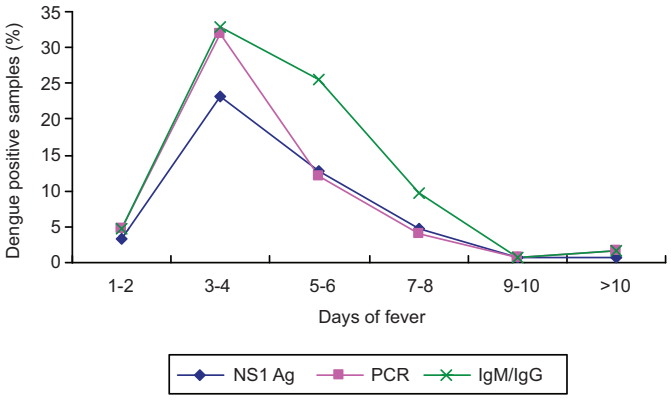


Fig 1–Detection of dengue infection by days of fever.

antibody, antigen with antibody and antigen with PCR combinations, respectively. Of the 144 dengue virus positive samples, 28 samples (19.4%) were positive for all three tests, 7 (4.9%) for dengue NS1 antigen only, 15 (10.4%) for PCR only and 40 (27.8%) for antibody only (Table 5). All three techniques were found to be negative on the remaining 64 samples (30.8%).

**Comparison of detection of DENV infection by days of fever**

Of the 144 dengue positive samples, only 125 samples (86.8%) indicated the day of fever. Data suggest the dengue antibody test, dengue PCR test and dengue antigen test were able to detect DENV infection between Day 1 and Day 8 in 72.8% of samples (91/125), 52.8% of samples (66/125) and 44.0% of samples (55/125), respectively. Fig 1 indicates all tests had the highest detection rate between Day 3 and Day 4. However, detection of antibodies was significantly higher compared to the dengue NS1 antigen and dengue PCR ($z$=2.489; 95% CI) between Day 5 and Day 6 of fever. Detection of DENV infection for all three tests decreased significantly after Day 9, even though data show that 0.8% to 1.6% of samples were found to be positive.

## DISCUSSION

The development of a commercial dengue NS1 antigen ELISA test kit has allowed early detection of DENV detection. This is possible due to the presence of antigens in the early phase of infection. Viremia in DENV infection generally lasts from 4 to 5 days and DENV antigens remain detectable in the blood up to 5 days after onset of symptoms, and rapidly disappear following the appearance of specific antibodies (Kao *et al*, 2005). Data obtained in this study show DENV antigens were detected from as early as Day 2 (3.2% of samples) up to Day 9 (0.8% samples) of fever. These findings are comparable to a study by Alcon *et al* (2006) who recovered

NS1 antigen until Day 9 of symptoms. Antigen detection was highest between Days 3 and 4 with a detection rate ranging from 10.4% to 12.8%. Similar finding were observed for the PCR test with the percentage of positive samples varying from 15.2% to 16.8%. Both antigen and PCR tests showed a decreased detection rate to 8.8% and 7.2%, respectively on Day 5 of fever. Data revealed the dengue antibodies were prominent from Day 3 onwards and remained at a high level; ranging from 11.2% to 21.6%, before decreasing on Day 8 of fever. Since Malaysia is endemic for dengue, with the majority of the population having been previously infected with DENV, antibodies are expected to present early and at high levels upon reinfection. A study conducted by Koraka *et al* (2003) showed the detection of dengue NS1 antigen in patient samples from areas where DEN is endemic was low due to the presence of immune complexes formed following a secondary dengue infection. Thus, low detection rates with NS1 antigen observed in this study may be linked to immune complexes found in samples collected. Another possible factor influencing the low detection rate of NS1 antigen was inappropriate temperature storage of samples during transportation, because more than 95% of samples were sent at ambient temperature. It is essential to ensure samples are collected, stored and transported properly to maintain the stability and quality of the samples tested.

It is important to establish early detection of DENV infection during the first few days of symptoms in order to provide accurate and timely information for the management of patients as well as for early and effective public health control of dengue outbreaks. Various studies have confirmed the detection of dengue NS1 antigen is useful for rapid and early diagnosis of dengue infections (Chaiyaratana *et al*, 2009; McBride, 2009; Ramirez *et al*, 2009; Zainah *et al*, 2009). Of the 208 patients tested, 32.2% of the patients (67/208) were found positive on dengue NS1 antigen, 38.5% (80/208) were PCR positive, 40.9% (85/208) were IgM positive and 36.1% (75/208) were IgG positive for DENV. Serological testing has always been the method of choice in most laboratory settings. In this study, the results reveal detection of IgM antibodies was the greatest (40.9%) compared to antigen, PCR and IgG antibody tests. The detection rate of DENV antibodies, IgM and/or IgG antibody tests, was found in 51.4% (107/208), an increase in detection of 10.5%. Combinations of the antibody test with other test methods were also analyzed to determine the performance in detecting DENV infections. When the antibody and PCR tests were combined, 65.9% (137/208) of patients tested positive. When antibody and antigen tests were combined, 62.0% (129/208) of patients tested positive for DENV infection. Compared to using the antibody test only, a significant increase in detection of 14.5% ($z$=2.889; 95% CI) and 10.6% ($z$=2.079; 95% CI) was observed with combinations of antibody with PCR and antibody with antigen, respectively. Therefore, application any of these test combinations in the laboratory may increase test efficiency.

Thirty-two point two percent of patients (67/208) were positive for dengue NS1 antigen. However, 104 patients (50.0%) were found to be dengue positive with the combination of antigen with PCR. Although there was an increase of 17.8% in the detection rate, the combination of antigen with antibody tests gave a better yield. The antigen with antibody tests had a detection rate of 62.0% com-

pared to 32.2% with the antigen test alone. The combination of antigen with antibody tests had a significant increase in detection of 29.8% ($z$=5.992; 95% CI) for DENV infection.

The results obtained from this study allow the laboratory to consider a combination of tests that are suitable to increase test sensitivity. The detection rate of DENV infection was similar between the PCR with antibody (65.9%) and NS1 antigen with antibody (62.0%) combinations ($z$=0.716; 95% CI). Since most laboratories have limited funds to set up PCR tests, the NS1 antigen ELISA should be considered as an alternative method for early detection of DENV. However, dengue NS1 antigen ELISA should only be used to complement, not replace, the current antibody test used in the laboratory. This combination of tests (NS1 antigen with antibody) may increase the diagnostic efficiency for early diagnosis of DENV infection. This is in accordance with the findings of some studies showing a commercial NS1 antigen ELISA when used together with IgM capture ELISA is sufficiently sensitive and specific to be clinically informative in the endemic setting. (Sekaran *et al*, 2007; Blacksell *et al*, 2008).

The availability of commercial dengue NS1 antigen test kits has provided an additional laboratory diagnostic tool for early detection of DENV. Such tests may be used in laboratories that have limited resources, lack viral culture or RT-PCR facilities.

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