Evaluation of a Commercial SD Dengue Virus NS1 Antigen Capture Enzyme-Linked Immunosorbent Assay Kit for Early Diagnosis of Dengue Virus Infection

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Early definitive diagnosis of dengue virus infection may help in the timely management of dengue virus infection. We evaluated the Standard Diagnostics (SD, South Korea) dengue virus nonstructural protein NS1 antigen enzyme-linked immunosorbent assay (SD dengue NS1 Ag ELISA) for the detection of dengue virus NS1 antigen in patients’ sera, using a total of 399 serum samples in a comparison with real-time reverse transcription (RT)-PCR, an in-house IgM capture (MAC)-ELISA, and a hemagglutination inhibition (HI) assay. Of the 320 dengue sera, 205 (64%) tested positive for NS1 antigen compared to 300 (93.75%) by either MAC-ELISA or RT-PCR, 161 (50.31%) by RT-PCR, and 226 (70.36%) by MAC-ELISA only. The assay was able to detect NS1 antigen in convalescent-phase sera until day 14 of infection. The NS1 detection rate is inversely proportional while the IgM detection rate is directly proportional to the presence of IgG antibodies. The overall sensitivity and specificity of the SD dengue NS1 Ag ELISA in the detection of “confirmed dengue virus” sera are 76.76% and 98.31%, respectively. This suggests that the SD kit is highly specific and sensitive for the detection of NS1 antigen. However, caution is needed when the kit is used as a single assay, as detection in samples that contained the virus was only about 81.97%. Combining this assay with an IgM and/or IgG assay will increase the sensitivity of detection, especially in areas with a higher prevalence of secondary dengue virus infections.

Dengue virus infection is a major public health problem worldwide, and dengue is among the most important human diseases caused by mosquito-borne viruses. The global prevalence of dengue has grown dramatically in recent decades. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, the Western Pacific, and particularly, in Southeast Asia. The World Health Organization (WHO) estimates that more than 2.5 billion people are at risk of dengue virus infections, with 50 to 100 million cases occurring annually. Among these infections, approximately 500,000 cases are dengue hemorrhagic fever (DHF), with 24,000 deaths which mostly occurred in children (12, 18).

Dengue virus is a mosquito-borne flavivirus belonging to the family Flaviviridae. It comprises four closely related but antigenically distinct serotypes, namely, DENV-1, DENV-2, DENV-3, and DENV-4. All these four serotypes of dengue virus can be distinguished by both serological and molecular methods. The dengue virus genome is infectious and consists of a single-stranded, positive-sense RNA with an approximate size of 11 kb. The RNA genome is 5’ capped but lacks a poly(A) tail and is translated from a single open reading frame to yield a polyprotein consisting of three structural proteins (the core protein C, membrane protein M, and envelope protein E) at the N terminus followed by seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (11, 16).

The NS1 protein is an ~50-kDa (353 or 354 amino acids) glycoprotein which has a high amino acid and nucleotide homology among flaviviruses (2). NS1 does not form part of the virion but is released from the dengue virus-infected cells. Preliminary studies have shown that this nonstructural glycoprotein is involved in viral RNA replication, and it has been found in acute-phase blood samples of patients with primary or secondary dengue virus infections (19). This has suggested a possible major involvement of NS1 in dengue virus pathogenesis and its possible use as a suitable marker for dengue virus infection (19).

For a diagnosis of “confirmed dengue,” dengue virus should be identified by isolation or there should be a 4-fold rise in acute- and convalescent-phase serum antibody titer. Several approaches have been applied for laboratory diagnosis of dengue virus infections. These methods include detection of the virus (by cell culture or immunofluorescence), detection of virus antigen (Ag) (by enzyme-linked immunosorbent assay [ELISA]), detection of anti-dengue virus antibody (by hemagglutination inhibition [HI], complement fixation test [CF], neutralization test, or ELISA), and detection of virus nucleic acids (by reverse transcription [RT]-PCR or real-time RT-PCR). Recently, commercially available kits for the detection of dengue virus NS1 antigen have been developed, and studies have shown that dengue virus NS1 antigen could be useful for the detection of early stages of dengue virus infections (1, 4, 7, 9, 13). In this study, we describe the evaluation of an ELISA kit manufactured by Standard Diagnostics, Inc. (SD, South Korea), that is designed to detect the presence of dengue virus
NS1 antigen in samples from patients with dengue virus infections.

MATERIALS AND METHODS

Serum specimens. A total of 399 sera were used in this evaluation. These blood samples were collected from patients admitted to the University of Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia, for acute viral infection. These sera consisted of (i) 30 samples from which dengue virus was isolated, (ii) 50 samples that were dengue virus RT-PCR positive, (iii) 30 samples from which dengue virus NS1 antigen was detected by both the Platelia dengue NS1 antigen kit (Bio-Rad Laboratories, Marnes-la-Coquette, France) and pan-E dengue early ELISA kit (Panbio, Queensland, Australia), (iv) 49 samples that were IgM negative but seroconverted in convalescent-phase sera, (v) 10 pairs of samples from confirmed primary dengue, (vi) 10 pairs of samples from confirmed secondary dengue, (vii) 100 random samples for which IgM to dengue virus was detected, (viii) 20 samples that were diagnosed clinically as dengue but for which laboratory tests were negative, and (ix) 59 samples from healthy donors and patients with infections other than dengue virus. All samples were subjected to virus isolation, in-house IgM capture ELISA (MAC-ELISA), in-house dengue virus real-time RT-PCR, and the hemagglutination inhibition (HI) assay. Sera that tested positive for the presence of virus or NS1 RNA or serum positive for recent acute dengue virus infection were categorized as confirmed dengue, whereas the acute phase of dengue is generally described as fever in the first 7 days of infection. Written informed consent was obtained from the patients. Ethical clearance was obtained from the Scientific and Ethical Committee at the UMMC prior to commencement of studies.

Virus isolation. Inoculation was carried out by inoculating 5 µl of serum into confluent C6/36 mosquito cell lines and incubating at 28°C for 7 to 10 days. Virus was then detected by immunofluorescence with dengue virus-specific monoclonal antibodies obtained from the Centers for Disease Control and Prevention (Fort Collins, CO).

In-house hemagglutination inhibition assay. The hemagglutination inhibition assay was carried out as described by Clarke and Casals (3). Briefly, 25 µl of 0.4% bovine albumin borate saline (BABS) was added to wells 2 to 12 of a 96-well ELISA microtiter plate. Fifty microliters and 25 µl of acetic acid-extracted serum were added to wells 1 and 12, respectively, and the serum was titrated from well 1 to well 12. Twenty-five microliters of antigen was added to the first 11 wells, while BABS was added to the last well (serum control), after which the plate was covered and incubated at 4°C for 18 to 24 h. Freshly prepared goose red blood cells (50 µl) were added to all the wells, and the microtiter plate was further incubated at 37°C for 45 min. The results were then interpreted according to WHO guidelines (17).

In-house IgM capture ELISA (MAC-ELISA). The in-house IgM capture ELISA was carried out as described by Lam et al. (8). Briefly, 100 µl of a 1:100 dilution of sample was added to human anti-IgM-coated 96-well flat-bottom plates and incubated at 37°C for 1 h. After washing thrice with phosphate-buffered saline (PBS)-Tween 20 (0.05%), 100 µl of a 1:100 dilution of dengue virus antigen was added and then incubated at 37°C for another hour. The plates were then washed three times with PBS-Tween 20 (0.05%), followed by the addition of 100 µl of a 1:5,000 dilution of anti-dengue virus mouse monoclonal antibody and further incubation at 37°C for 1 h. The plates were washed again with PBS-Tween 20 (0.05%), and 100 µl of a 1:50,000 dilution of goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was added, prior to incubation for 1 h at 37°C. After three additional washes, 100 µl of OPD (o-phenylenediamine 2HCl) was added to all wells and the mixture incubated in the dark at room temperature. The reaction was stopped with 50 µl 4N sulfuric acid, and the absorbance (optical density [OD]) of each well was read at 490 nm with a reference filter of 630 nm using an ELISA reader. The positive-versus-negative ratio was then calculated. The positive control/sample OD was divided by the mean of the negative OD to obtain a positive/negative ratio (P/N). A P/N ratio equal to or greater than 2.0 was considered positive. A result with a P/N ratio of less than 2.0 was reported as negative if the sample was collected 2 weeks after disease onset.

Real-time RT-PCR. The one-step SYBR green I real-time RT-PCR of Kong et al. (6) was carried out in an iCycler (Bio-Rad Laboratories, Marnes-la-Coquette, France) thermocycler using an iScript one-step RT-PCR kit with SYBR green I (Bio-Rad Laboratories, Marnes-la-Coquette, France). Briefly, 5 µl of the extracted sample RNA was added to 25 µl reaction mixture containing 1× SYBR green I, 75 nM each primer, and 3 mM MgCl2. The thermal cycling conditions consisted of a 30-min reverse transcription at 50°C and 15 min of Taq polymerase activation at 95°C, followed by 45 cycles of PCR with denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. Following amplification, the melting curves were analyzed. This is to verify the specificity of the PCR product by looking at its melting temperature (Tm). Melting curve analysis consisted of a denaturation step at 95°C for 1 min and 55°C for 30 s, followed by 80 cycles of incubation in which the temperature was increased to 95°C at a rate of 0.5°C/10 s/cycle. The Tm of each specific PCR product was analyzed using iCycler iQ optical system software version 3.0a (Bio-Rad Laboratories, Marnes-la-Coquette, France). The Tm for each sample was used to identify the dengue virus serotype, and the samples sharing the same Tm were interpreted as belonging to the same serotype.

SD dengue NS1 Ag ELISA. The SD dengue NS1 Ag ELISA kit produced by Standard Diagnostics, Inc., South Korea, is an enzyme-linked immunosorbent assay for the qualitative detection of dengue virus NS1 antigen in human serum. Detection for the presence of NS1 antigen was performed according to the manufacturer's recommendations. Briefly, 50 µl each of a patient's serum and sample diluent were added to each well. The microplate was mixed using a vibrating mixer and incubated at 37°C for 60 min. The plate was then washed five times with diluted washing solution, followed by the addition of 100 µl diluted enzyme conjugate. After 1 h of incubation at 37°C, the plate was washed five times with washing solution and 100 µl of TMB (tetramethylbenzidine) substrate solution was added. The plate was further incubated at room temperature for 10 min, during which time a blue color developed for a positive reaction. Following this, 100 µl of stopping solution (1.6 N sulfuric acid) was added to each well to stop the reaction, and the absorbance was read at 450 nm with reference 620 nm using a bichromatic spectrometer. The results were then interpreted in accordance with the manufacturer's instructions.

Data analysis. Tabulation, management and analysis of raw data were carried out using Microsoft Excel (Microsoft, Inc., United States). Statistical analysis was performed with Statistica version 18 (StatSoft, Inc., United States). The sensitivity, specificity, efficiency, positive predictive value (PPV), and negative predictive value (NPV) for the assays were calculated based on confirmed dengue samples (virus isolation/PCR positive, seronegative acute-phase sera, acute primary, or acute secondary) using the following formulas, where a is the number of true positives, b is the number of false positives, c is the number of false negatives, and d is the number of true negatives: % sensitivity = a / (a + c) × 100; % specificity = b / (b + d) × 100; efficiency = (a + d) / (a + b + c + d) × 100; % PPV = a / (a + b) × 100, and % NPV = d / (c + d) × 100.

RESULTS

A total of 399 samples collected from 2007 to 2009 were selected for the evaluation of the SD dengue NS1 Ag ELISA kit in this study. The assay was evaluated against a panel of samples, including “confirmed positive sera” (validated by virus isolation and real-time PCR), IgM-positive sera, NS1 antigen-positive sera, and sera from healthy donors and other disease agents. The results are summarized in Table 1. Of the 320 dengue sera, 205 (64.06%) tested positive for NS1 antigen (with the SD dengue NS1 Ag ELISA kit), compared to 161 (50.31%) by real-time RT-PCR, 226 (70.36%) by MAC-ELISA, and 300 (93.75%) by either real-time RT-PCR or MAC-ELISA. Among the 30 samples that were positive by virus isolation, the SD dengue NS1 Ag ELISA detected 20 (66.7%) as positive. One sample from the category that was clinically dengue but negative by laboratory tests, as well as another sample from the negative-control panel, also tested positive by the SD dengue NS1 Ag ELISA.

The dengue virus NS1 detection rate by the SD dengue NS1 Ag ELISA was compared in the presence and absence of dengue virus-specific antibodies. The detection rate by the SD dengue NS1 Ag ELISA in the absence of IgM was 82.42% (75/91), compared to 56.76% (130/229) in the presence of IgM. As the levels of IgM antibodies increased, the NS1 detection rate decreased. Further analysis of the data indicated that the NS1 detection rate is inversely proportional to the presence of IgG antibodies as depicted by the HI value (Fig. 1). At lower levels of antibodies (HI ≤ 320), the SD dengue NS1 Ag ELISA
gave better detection rates than at high levels of antibodies (≥640 HI ≤10,240). This suggests that the SD dengue NS1 Ag ELISA is more sensitive for diagnosis in the absence of dengue virus-specific antibodies.

The frequency of detection of NS1 through day 7 after the onset of fever was compared (Fig. 2). For the first seven days of fever, 69.57% (192/276) of samples tested positive for NS1 antigen, compared to 56.52% (156/276) by RT-PCR and 67.03% (185/276) by MAC-ELISA, whereas for fever that lasted from day 8 through day 15, 29.55% (13/44) of samples tested positive for NS1 antigen, compared to 13.64% (6/44) by RT-PCR and 100% (44/44) by MAC-ELISA.

We further analyzed the data with regard to day of onset of disease symptoms. As seen in Fig. 2, NS1 antigen could be detected up to day 14 from the onset of fever, while RT-PCR was only able to detect viral RNA up to day 8 postinfection. The kit was also used to detect dengue virus-positive samples comprising all four serotypes of dengue viruses (n = 162), and the results are presented in Table 2. No significant difference was observed between the 4 serotypes with a P value of ≥0.05. The overall sensitivity and specificity of the kit were 76.76% (142/185; 95% confidence interval [CI], 70.61 to 82.90) and 98.31% (58/59; 95% CI, 94.91 to 100), respectively, with an efficiency of 81.97% (142 + 58/244; 95% CI, 77.11 to 86.83), based on confirmed dengue sera (Table 3).

### DISCUSSION

Diagnosis of dengue virus infection based on clinical syndromes is not reliable and should be confirmed by laboratory studies. For a diagnosis of confirmed dengue, dengue virus should be identified by isolation or there should be a 4-fold rise in antibody titer. Isolation of viruses can take from 7 to 10 days, and serological tests depend on the demonstration of the presence of IgM antibody or a rise in IgG antibody titer in paired acute- and convalescent-phase sera. Serological tests are generally the tests of choice to diagnose acute flavivirus infections, with most utilizing IgM capture ELISA formats. More than 90% of patients are IgM positive by the 4th day of illness, but the IgM antibody may be due to infection up to 3 months earlier. Commercial kits for the measurement of antibodies include the ELISA kits, a dipstick, and a rapid dot blot assay. These kits do not require specialized training but their sensitivity and specificity is very variable. The choice of a test, therefore depends on the availability of facilities and human resources and, also, the time of sampling.

With the escalating incidence of dengue infections and the absence of vaccines for the prevention of this disease, early diagnostic confirmation of dengue virus infections in patients is
needed, as it allows for timely clinical intervention, etiologic investigations, and disease control. Hence, diagnosis of dengue disease during the acute phase should be a priority for patients and for public health reasons. In-house IgM capture ELISAs that have been the mainstay of dengue diagnosis in many laboratories throughout the world have several limitations. First, IgM is detected only after day 3 of the onset of symptoms, making early diagnosis impossible since more than half of the patients present early to the clinician. Second, because flaviviruses share common group epitopes, particularly on the envelope (E) protein, a high degree of cross-reactivity is frequently observed. After the onset of illness, the virus is found in serum, plasma, and circulating blood cells, as well as other tissues, for 4 to 7 days. As such, during the early stages of the disease, virus isolation or nucleic acid or antigen detection can be used to diagnose the infection. At the end of the acute phase of infection, serology is the method of choice for diagnosis.

The use of NS1 detection in the diagnosis of dengue virus infections has been evaluated in many laboratories (5, 7, 10, 13). Both the PanBio dengue NS1 antigen capture ELISA and the Platelia dengue NS1 antigen assay were shown to be able to detect NS1 antigen in acute-phase sera from both primary and secondary infections (1, 4, 14, 15). Their sensitivity also decreased with increasing concentrations of antibody. In this study, we evaluated the use of the SD dengue NS1 Ag ELISA for the diagnosis of dengue virus infections. The analysis of all serum samples in the groups revealed that the detection of NS1 antigen was lowest in the RT-PCR-positive group (60%) and in the virus isolation-positive group (66.7%) (Table 1). The sensitivity was highest in the primary acute dengue serum group (100%, 10/10), while with the secondary acute dengue serum group, a lower sensitivity of 30% (3/10) was observed. In both groups, the detection of NS1 in the convalescent phase dropped drastically. Although the analysis was being done on 10 pairs each of primary and secondary dengue, preliminary findings have shown the detection of NS1 antigen in acute patient sera, with the assay being more efficient at the acute phase of primary infection than for secondary infection. This concurs with the findings of Dustart et al. (5) and Kumarasamy et al. (7).

The presence of antibodies, however, does affect the detection of NS1. As seen in Fig. 1 and 2, the presence of both IgG and IgM antibodies affected the detection of NS1. However, NS1 was detectable up to day 14 after the onset of illness. The specificity for NS1 antigen was shown to be 98.31% (with only one sample detected with NS1 antigen). However, the types of samples used to determine specificity are limited, and more samples, especially from other flaviviral pathogens, such as West Nile virus, St. Louis encephalitis virus, Rocio virus, and related viruses in this group, need to be included to ensure adequate coverage of possible cross-reactions. Obtaining such sera that are dengue virus antibody negative is sometimes not possible due to the high endemicity in the tropical belt and also to effects of globalization. Another reason is that more individuals have been exposed to multiple flaviviruses as they travel to various regions as tourists.

The results obtained in this evaluation are consistent with the reports of many others (1, 4, 7, 13) who have shown that NS1 antigen detection is most useful in the acute phase of dengue virus infection. However, the test cannot replace existing assays, as its sensitivity drops with increasing concentrations of antibody. Another factor to consider is that samples that were isolation or RT-PCR positive had a lower percentage of positive detection of NS1. This has several implications, since the monoclonal antibodies utilized may not be detecting the local strains or the samples may not contain NS1 since the blood collection may have been done at an early or later stage of replication. Another point to note is that the kinetics of NS1 antigen detection differ from the kinetics of IgM and IgG detection, and hence, this implies an effect on the inverse correlation between the presence of antibody and the detection of NS1. Comparing the sensitivity of the SD dengue NS1 Ag ELISA with the sensitivities of the other commercial NS1 kits is not justified, as all three kits utilized different amounts of serum or plasma (Platelia dengue NS1 Ag, 50 μl; pan-E dengue early ELISA, 15 μl; and SD dengue NS1 Ag ELISA, 10 μl) for detection. Hence, this is another factor to consider as to how much the sample should be diluted for maximal sensitivity.

In conclusion, the current evaluation of the SD dengue NS1 Ag ELISA shows that this assay has a sensitivity of 76.76% (95% CI, 70.61 to 82.90) and a specificity of 98.31% (95% CI, 94.91 to 100) and is useful, sensitive, and specific for the diagnosis of dengue virus infection. However, caution is needed in using it as a single assay in any laboratory, as detection in samples that contained the virus was only about 81.97%. Hence, the use of more than one test would be necessary in such instances. Combining this assay with IgM and or IgG

### Table 2. Comparative performance of SD dengue NS1 Ag ELISA in detecting dengue virus serotypes

<table>
<thead>
<tr>
<th>Serotype</th>
<th>No. of samples with positive result/total no. of samples (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV-1</td>
<td>78/101 (77.23), 68.91–85.55</td>
<td></td>
</tr>
<tr>
<td>DENV-2</td>
<td>14/21 (66.67), 44.69–88.65</td>
<td></td>
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<tr>
<td>DENV-3</td>
<td>21/23 (91.30), 78.84–100</td>
<td></td>
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<tr>
<td>DENV-4</td>
<td>14/17 (82.35), 62.15–100</td>
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</tbody>
</table>

P value = 0.24

Chi-square test was used for statistical analysis (StatSoft, Inc., United States). A P value of >0.05 indicates that the result was not significant.

### Table 3. Performance of the SD dengue NS1 Ag ELISA in the diagnosis of dengue

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of samples with indicated result/total no. of samples (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>142/185 (76.76), 70.62–82.90</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>58/59 (98.31), 94.92–100</td>
<td></td>
</tr>
<tr>
<td>Efficiency</td>
<td>142 + 58/244 (81.97), 77.11–86.83</td>
<td></td>
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<tr>
<td>Positive predictive</td>
<td>142/143 (99.30), 97.92–100</td>
<td></td>
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<tr>
<td>Negative predictive</td>
<td>58/101 (57.43), 47.62–67.24</td>
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</table>

a Confirmed positive. Samples were derived from the following categories of samples: virus isolation positive (n = 30), dengue RT-PCR (n = 50), dengue NS1 antigen-positive sera which are also positive for dengue RT-PCR (n = 35), seronegative acute-phase sera but convalescent serumoconverted (n = 50), acute primary samples (n = 10), and acute secondary samples (n = 10).

b Confirmed negative. Nondengue sera, which comprise patients with other infectious agents and healthy donors.

Values were calculated as described in Materials and Methods.
detection may therefore be necessary for confidence in diagnosing dengue virus infection.

ACKNOWLEDGMENTS

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REFERENCES


