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Sensitivity of dengue virus NS-1detection in primary and secondary infections

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We have evaluated a commercial enzyme immunoassay for the rapid detection of dengue NS1 antigen in human sera. The PLATELIA™ Dengue antigen assay was compared with the in-house IgM and TaqMan real-time RT -PCR using a panel of sera from primary acute and convalescent dengue infections, secondary acute and convalescent dengue infections, IgM-positive samples, tissue culture supernatant and other flaviviral infections. Of the 93 acute serum samples 82 were positive for NS1 antigen using the PLATELIA™ Dengue antigen assay. Overall, the NS1 detection rate was much higher in the acute primary dengue (100%) than in the acute secondary dengue (53.3%) serum samples. Both the PLATELIA™ Dengue antigen assay and the TaqMan real-time RT-PCR assay were highly specific (100%). The overall sensitivity of the PLATELIA™ Dengue antigen assay was 93.9% and 55% in the absence and presence of IgM, respectively. The results indicate that the PLATELIA™ Dengue antigen assay is a specific and sensitive assay for the detection of dengue virus infections during the primary acute phase when IgM is not detectable.

Key words: Dengue virus, NS1 antigen, ELISA, rapid diagnostic assay.

INTRODUCTION

Dengue virus is an arthropod-borne Flavivirus that can be found in tropical and sub-tropical regions world-wide. It is the causative agent of dengue which could present as a mild fever, called dengue fever (DF), or a more severe, often fatal disease such as dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) (Gubler and Clark, 1995). The World Health Organization estimates that there may be up to 100 million dengue infections world- wide which could result in up to 250 000 to 500 000 cases of DHF each year (Gibbons and Vaughn, 2002; WHO, 1997).

There are four serotypes of dengue designated dengue 1 through dengue 4 (DEN 1 to DEN 4) that is antigenically related (Monath and Heinz, 1990). Recovery from infection by one serotype can confer life-long protection against that serotype; however, it provides only partial, transient immunity against subsequent infection by the other three dengue serotypes. All four serotypes cause disease, usually asymptomatic or mild dengue fever (Halstead, 1988). Progression from DF to DHF and DSS usually occurs after a second infection with a different serotype, which is due to immune-mediated enhancement of infection, known as antibody-dependent enhancement (ADE) (Halstead, 1988).

Dengue virus is a positive stranded, encapsulated RNA virus. The genome is approximately 11 kb in size and has a single open reading frame (ORF) encoding for a single polypeptide. The polypeptide is further processed into three structural proteins, that is, the capsid (C), membrane (M), and envelope (E) proteins, and seven non-structural (NS1) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Chambers et al., 1990; Deubel et al., 1988). The NS1 protein is a 50-kilodalton glycoprotein that is expressed on the surface of infected cells, and is found in both secreted and non-secreted forms (Falconar and Young, 1990). Although the exact function NS1 is unknown, this glycoprotein is thought to play a vital role in virus viability.

Diagnosis of dengue virus infection in clinical laboratories is achieved through the detection of the virus by virus isolation or viral RNA, and detection of dengue-specific IgM and IgG antibodies. Although virus isolation is the gold standard for detection of dengue virus, this

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Table 1. Category of samples used in the evaluation.

Group	Category	Number of samples	NS1 assay No. of samples (%)	RT-PCR No. of samples (%)
1	Acute dengue sera			
	DEN 1	20	18(90)	20(100)
	DEN 2	20	18(90)	20(100)
	DEN 3	20	20(100)	20(100)
	DEN 4	6	6(100)	6 (100)
2	Primary Dengue Acute	12	12(100)	12(100)
	Primary Dengue Convalescent	12	5(41.7)	ND
	Secondary Dengue Acute	15	8(53.3)	13 (86.7)
	Secondary Dengue Convalescent	15	1(6.7)	ND
3	IgM positives	60	33(55)	60(100)
4	Other Viral infections			
	Japanese Encephalitis	14	0	0
	Chikungunya	6	0	0
	West Nile	6	0	0

technique is both tedious and time-consuming. In addition, indirect immuno-fluorescence using serotype- specific monoclonal antibodies is required to identify the dengue serotype (Henchal et al., 1983). Thus, more rapid alternatives are generally used. Serology has been the main method employed in the diagnosis of dengue infections. Antibody detection using IgM and IgG capture ELISA assays have been developed and used in diagnostic laboratories (Lam et al., 1987), whereas NS1 antigen detection is becoming increasingly useful, particularly in plasma samples from patients with acute primary and secondary dengue infections (Koraka et al., 2003). Molecular diagnosis of dengue infections has increased significantly over the years (Shu and Huang, 2004). More recently, the real-time RT-PCR assay has been developed with many advantages over conventional methods (Kong et al., 2006).

The objective of this study was to evaluate the PLATELIA^{TM} DENGUE NS1 AG Assay for the diagnosis of dengue using sera/plasma obtained from the Diagnostic Laboratory of University Malaya Medical Center (UMMC) from patients with primary and secondary dengue infections, as well as sera from patients with other flaviviral infections.

MATERIALS AND METHODS

Serum samples

A total of 206 serum samples were used to evaluate the kit. These sera were from patients admitted to University Malaya Medical Center for acute viral infections that included 12 pairs of confirmed primary dengue infections, 15 pairs of confirmed secondary dengue infection, and 60 single serum samples from acute phase of dengue that were IgM-positive and 26 single samples from patients with other viral infections. In addition, 66 serum samples from which dengue virus was isolated were included (Table 1). Supernatants from 8 tissue culture of four dengue virus serotypes were used.

Primary and secondary dengue infections are generally differenttiated based on the titres of haemagglutination inhibition (HI) test. Patients are classified as having secondary dengue virus infections when the HI test titer is greater than or equal to 1:2 560 and a 4-fold rise is observed in the paired sera. Patients are classified as having acute primary dengue infection when the HI test tire is less than 1:1 280 and a 4-fold rise is observed in paired sera that were collected with an interval longer than 7 days (WHO, 1997).

In-house IgM capture ELISA

The in-house IgM capture ELISA was performed as previously described (Lam et al., 1987). A 100 l of a 1:100 dilution of sample was added to human anti-IgM coated 96-well flat- bottomed plates and incubated for 1 h at 37°C. After washing three times with PBS-Tween 20 (0.05%), 100 l of a 1:100 dilution of sucrose acetone-extracted stock dengue antigen, which had an HA titre of 1:2 560, was added and incubated for another 1 h at 37°C. The plates were then washed three times with PBS-Tween 20 (0.05%) and 100 l of a 1:5 000 dilution of mouse monoclonal antibody was added, and incubated at 37°C for 1 h. The plates were washed again with PBS-Tween 20 (0.05%) and 100 l of a 1: 50 000 dilution of goat anti-mouse IgG conjugated with HRP was added and incubated for 1 h at 37°C. After a further three washes, 100 l of OPD (ortho-phenylenediamine.2HCl) was added to all wells and incubated in the dark at 28°C. The reaction was stopped with 50 l of 4N sulphuric acid and the absorbance (OD) of each well was read at a wavelength of 490 nm with a reference filter of 630 nm using an ELISA plate reader. 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 ration of greater or equal to 2.0 is considered positive. A result with a P/N ratio of less than 2.0 is reported as a negative if the sample is collected two weeks after disease onset.

PLATELIA^{TM} DENGUE NS1 AG Assay

This is a one-step sandwich-format microplate enzyme immunoassay for detecting dengue virus NS1 in human serum or plasma. The assay was carried out according to the manufacturer's instructions (Biorad). The presence or absence of NS1 in a sample is based on the comparisons of the OD of sample versus OD of control sera provided. Samples were considered (i) non-reactive for

Table 2. Sensitivity, efficiency and specificity of the NS1 assay in IgM positive and negative samples.

Sample	Sensitivity of NS1 assay	Efficiency NS1 assay	Specificity of NS1 assay
IgM ELISA negative	93.9%	67.4%	100%
IgM ELISA positive	55.0%	38.4%	100%

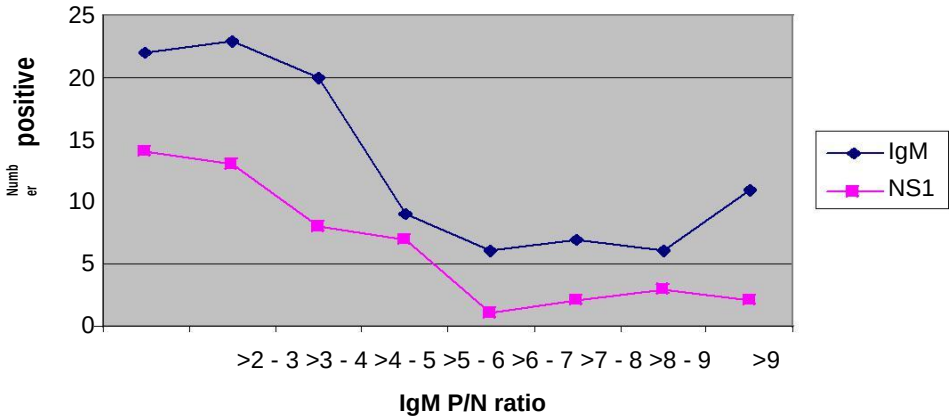

Figure 1. Detection of NS1 with increasing IgM P/N ratios.

dengue virus NS1 if the ratio is less than 0.5, (ii) equivocal for dengue virus NS1 antigen if the ratio is between 0.5 and 1.0, and (iii) reactive for dengue virus NS1 antigen if the ratio is more than 1.0.

TaqMan real-time RT-PCR

The one-step TaqMan real-time RT- PCR was carried out in an iCycler (BioRad) thermocycler using the method of Kong et al. (2006) where 5 µl of viral RNA was added to a 20 µl reaction containing 0.5 µM of each primer, four TaqMan probes (0.25 µM each), and 5.0 nM of $MgCl_2$. The thermal cycling profile of this assay consisted of a 30 min RT step, which was performed at 50°C, 15 min of *Taq* polymerase activation at 95°C of denaturing for 30 s and 60°C of annealing/extension for 1 min.

Calculations of sensitivity and specificity and efficiency

Percent Sensitivity = a/a+c × 100%
Percent Specificity = b/b+d × 100%
Efficiency = a+b/a+b+c+d × 100%

Where a = number of true positives b = number of false positives c = number of false negatives d = number of true negatives

RESULTS

Table 1 summarizes the evaluation of the PLATELIA™ Dengue antigen assay against the in-house IgM and TaqMan real-time RT -PCR. NS1 antigen was detectable in all 8 tissue culture supernatants containing four serotypes of dengue. Of the 66 samples from which dengue virus was isolated and tested positive for dengue virus by RT-PCR, 62 (94%) tested positive with the PLATELIA™ Dengue antigen assay (Table 1). Two sera, one each of DEN1 and DEN2 serotypes, were considered equivocal with the PLATELIA™ Dengue antigen assay. NS1 antigen was also detected in all the primary acute phase sera and in 8 (61.5%) of the 13 secondary acute phase sera. RT-PCR detected viral RNA in the acute sera of all primary dengue sera and in 86.7% of the secondary sera. However, in the convalescent samples the RT-PCR assay detected viral RNA in 5 of the 12 primary convalescent samples and in only 1 of the 15 secondary convalescent sample (Table 1). In the presence of IgM, however, NS1 was detected in 33 (55%) of the 60 samples tested positive by RT -PCR (Table 1). NS1 antigen was not detected in any of the sera from patients with other flaviviral infections, namely JE and Chikugunya. Thus, this assay was equally specific as the TaqMan real-time RT-PCR assay (100%). The sensitivity of the PLATELIA™ Dengue NS1 antigen assay was much higher in the absence of IgM (93.9%) compared with IgM (55%) with an overall efficiency of 67.4 and 38.4%, respectively (Table 2).

Further analysis showed the ability of the PLATELIA™ Dengue NS1 antigen assay to detect dengue NS1 antigen in the presence and absence of dengue virus -specific IgM and is depicted in Table 2 and Figures 1 - 3. The level of IgM, as depicted by P/N ratios, indicated that in the presence of IgM NS1 detection was reduced (Figure 1). Similarly, as seen in Figure 2, that as IgM levels rose,

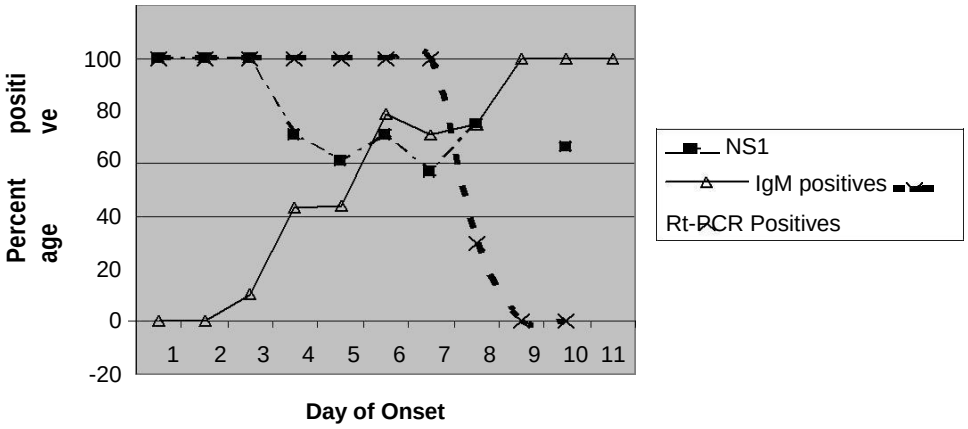

Figure 2. Comparison of IgM, RT-PCR and NS1 antigen detection based on date of onset.

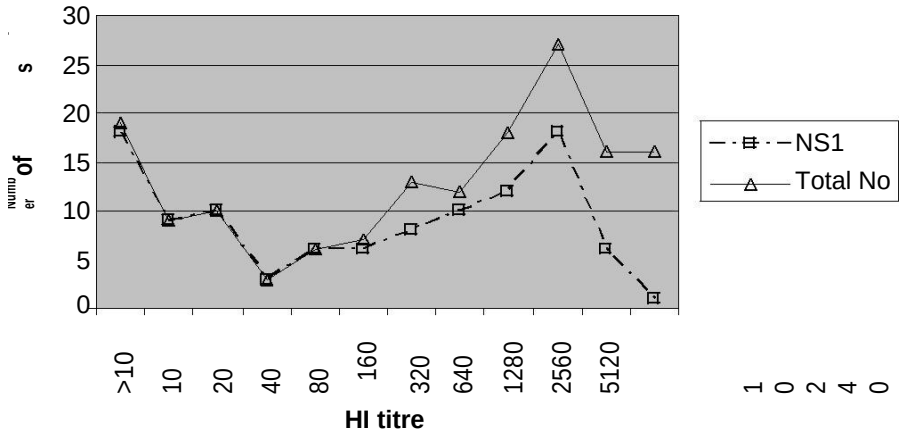

Figure 3. Detection of NS1 in the presence of IgG antibodies.

a drop in NS1 detection was noted. The assay was also evaluated with regard to the date of onset of fever where it was seen that it is possible to detect the presence of NS1 antigen at least up to day 10 from the onset of fever with the detection rate decreasing with an increase in anti- dengue IgM detection. On the other hand, RT-PCR was able to detect viral RNA up to day 8 from the onset of fever. In the presence of IgG antibodies, as measured by the HAI assay, it was also observed that NS1 detec-tion decreased with increasing IgG titers as seen in Fi-gure 3.

DISCUSSION

With the escalating incidence of dengue and the absence of vaccines for the prevention of this disease, the early diagnostic confirmation of dengue infections in patients is at present one of the ways to efficiently combat the severity of this disease. Furthermore, the clinical diagnosis of dengue is not reliable due to the fact that this infection could result in asymptomatic or mild, undifferentiated fever (Burke et al., 1988; Endy et al., 2002). There have been considerable advances in the development of both serological and molecular methods in the diagnosis of dengue virus infections (Shu and Huang, 2004). Of these, three methods that are used extensively in laboratories world-wide for the diagnosis of dengue infections are virus isolation, detection of anti-dengue IgM via ELISA and detection of viral RNA via RT-PCR. Virus isolation and RT-PCR require specialized facilities, skill and are expensive. Hence, most laboratories use either in-house ELISAs or commercial kits to detect IgM and IgG antibodies. These assays, however, are positive only after the third day of infection. The nonstructural glycoprotein NS1 is an extremely useful diagnostic marker as it has been found to circulate in the plasma of patients infected with dengue virus (Alcon et al., 2002; Alcon-LePoder et al., 2006). Several studies have shown that high concentrations of the NS1 antigen forms immune complexes with NS1-specific IgG antibodies that can be detected in acute phase sera in both primary and secondary dengue virus infections (Koraka et al., 2003; Young et al., 2000).

Recent studies have also shown that this antigen can be detected up to day 18 after onset of symptoms (Alcon et al., 2002; Dussart et al., 2006; Xu et al., 2006).

This study evaluated the PLATELIA™ Dengue NS1 antigen assay and showed that it is specific and sensitive in the detection of dengue NS1 antigen in acute patients' sera with the assay being more efficient with acute phase sera of primary infection as compared to secondary dengue infection. This is concurrent with the findings of Dussart et al. (2006) and Kumarasamy et al. (2007). The current study has also shown that the PLATELIA™ Dengue NS1 antigen assay is able to detect dengue NS1 antigen in some of the patients' sera up to day 10 from onset of fever, but detection levels decreased considerably from day 4 onwards due to the emergence of antibodies, both IgM and IgG, that form immune complexes thus diminishing the amount of free NS1 in the serum. Of importance, however, is the fact that in the acute phase of the illness, especially prior to the emergence of antibodies, nearly all samples had detectable levels of NS1 antigen. Hence, in the first 3 days of illness this assay reached a sensitivity of close to a 100%, making it a suitable test for the diagnosis of dengue infection during the acute stage of the disease. As a large number of patients are clinically diagnosed with dengue during this phase but not serologically confirmed (as IgM is not detectable), the NS1 antigen assay would be useful especially in instances when second samples are not or cannot be obtained. Therefore, the use of both the PLATELIA™ Dengue NS1 antigen assay and the IgM ELISA in the diagnosis of dengue could increase the diagnosis of dengue infections in clinical diagnostic laboratories.

This assay is simple to perform, rapid and easily implementable, especially in laboratories that are already performing ELISAs and thus have the basic facilities for this assay. Within a few hours, physicians in out- patient clinics can make a diagnosis in both acute and early convalescent phases. Early detection of dengue will enable not only better in-patient management but also rapid notification of appropriate public health authorities, especially vector control units. However, these assays are unable to distinguish the serotype of dengue virus causing the infection. PCR is fast becoming the method of choice for the rapid detection of dengue viruses, especially in reference and research laboratories. With the recent advances in real- time PCR, several methods have been developed using this versatile tool that enables rapid detection, serotype identification, as well as viral RNA quantitation (Kong et al., 2006; Laue et al., 1999) . The only drawback in these methods is the need for specialized equipment and the cost incurred using this method. Thus, the choice of an assay that is to be used in the diagnosis of dengue infections depends on several factors, including the laboratory infrastructure, preference and availability of equipment. The current evaluation also showed that this assay was 100% specific when tested with sera positive for other related infections. However, more more such samples need to be tested to ensure minimal or no cross-reactivity especially with other flaviviruses.

In conclusion the current evaluation of the PLATELIA™ Dengue NS1 antigen assay shows that this assay is useful, sensitive and specific for the diagnosis of dengue infection, especially during the acute phase when antibodies are not detectable.

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REFERENCES

Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M (2002). Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural glycoprotein reveals circulation of the antigen in blood during acute phase of disease in patients experiencing primary or secondary infections. J. Clin. Microbiol. 40: 376-381.

Alcon-LePoder S, Sivard P, Drouet MT, Talarmin A, Rice C, Flamand M (2006). Secretion of flaviviral non-structural protein NS1: from diagnosis to pathogenesis. In New Treatment strategies for dengue and other flaviviral diseases. G. Bock and J Goode (eds.). Novartis Foundation Symposium 277, Wiley Chichester, pp. 233-250.

Burke DS, Nisalak A, Johnson DE, Scott RM (1988). A prospective study of dengue infections in Bangkok. Am. J. Trop. Med. Hyg. 38: 172-180.

Chambers TJ, Hahn CS, Galler R, Rice CM (1990). Flavivirus genome organization, expression, and replication. Ann. Rev. Microbiol. 44:649-688.

Deubel V, Kinney RM, Trent DW (1988). Nucleotide sequence and deduced amino acid sequence of the nonstructural protein genes of dengue type 2 virus, Jamaica genotype: comparative analysis of the full length genome. Virology 165: 234-244

Dussart P, Labeau B, Lagathu G, Louis P, Nunes MR, Rodrigues SG, Storck-Herrmann C, Cesaire R, Morvan J, Flamand M, Baril L (2006). Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin Vaccine Immunol. 13: 1185-1189 Kumarasamy V, Wahab AH, Chua SK, Hassan Z, Chem YK, Mohamad M, Chua KB (2007). Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. J Virol Methods. 140: 75-79.

Endy TP, Chunsuttiwat S, Nisalak A, Libraty DH, Green S, Rothman AL, Vaughn DW, Ennis FA (2002). Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J Epidemiol. 156: 40-51

Falconar AKI, Young PR (1990). Immunoaffinity purification of the native dimer forms of the flavivirus non-structural glycoprotein, NS1. J. Virol. Methods. 30: 323-332.

Gibbons RV, Vaughn DW (2002). Dengue an escalating problem. BMJ. 324: 1563-1566.

Gubler DJ, Clark GG (1995). Dengue /dengue haemmorhagic fever: the emergence of a global health problem. Emerg. Infect. Dis. 1:55-7.

Halstead SB (1988). Pathogenesis of dengue: challenges to molecular biology. Science 239: 476-81.

Henchal EA, McCown JM, Sequin MC, Gentry MK, Brandt WE (1983). Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay. Am. J. Trop. Med. Hyg. 32: 164-169

Kong YK, Chong HT, Tan CT, Devi S (2006). Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR. J Virol Methods. 138: 123-130

Koraka P, Burghoorn-Maas CP, Flaconar A, Setiati TE, Djamiatun K, Groen J, Osterhaus ADME (2003). Detection of immune-complex-dissociated nonstructural-1 antigen in patients with acute dengue virus infections. J. Clin. Microbiol. 41: 4145-4159.

Lam SK, Devi S, Pang T (1987). Detection of specific IgM in dengue infection. Southeast Asian J. Trop. Med Pub Health. 18: 532-538

Laue T (1999). Detection of dengue virus RNA in patients after primary or secondary dengue infection by using TaqMan automated amplification system. J. Clin. Microbiol. 37: 2543-2547.

Monath TP, Heinz FX (1990). Flaviviruses. In Fields, BW, Knipe DM, Knipe PM, et al. (eds.). Field's Virology, Vol. 1, third ed. Lippincott – Raven Press, New York, pp. 961-1043.

Shu PY, Huang JH (2004). Current advances in dengue diagnosis. Clin Diagn Lab Immunol. 11: 643-50.

World Health Organization (1997). Dengue haemorrhagic fever diagnosis, treatment, prevention and control. WHO, Geneva, Switzerland.

Xu H, Di B, Pan YX, Qiu LW, Wang YD, Hao W, He LJ, Yuen KY, Che XY (2006). Serotype 1-specific monoclonal antibody-based antigen capture immunoassay for detection of circulating nonstructural protein NS1: implications for early diagnosis and serotyping of dengue virus infections. J. Clin. Microbiol. 44: 2872-2878.

Young PR, Hilditch PA, Bletchy C, Halloran W (2000). An antigen capture enzyme-linked immunosorbent assay reveals high levels of dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol. 38: 1053-1057.