Original Article

Detecting Dengue Virus Nonstructural Protein 1 (NS1) in Urine Samples Using ELISA for the Diagnosis of Dengue Virus Infection

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SUMMARY: Dengue virus (DENV) infection is a serious global health threat. For the surveillance and control of dengue, there is a need for robust diagnostic tools that are relatively easy to use and reliable in various clinical settings. We investigated the applicability of NS1 antigen detection in urine samples for the diagnosis of DENV. About 118 urine samples, obtained from 96 dengue patients at various phases of disease, were used for this study. NS1 antigen was detected by ELISA in the urine samples obtained from patients after 2–17 days of disease onset. Positive detection rates of NS1 antigen ranged between 13–43%. Based on real-time RT-PCR, positive detection rates of viral genome in the urine samples ranged between 20–33% on days 0 to ≥15. On days 11 to ≥15 after the disease onset, NS1 antigen was detected at similar rates in serum and urine samples. Additionally, NS1 antigen was detected in 2 urine samples, but not in the serum samples, on days 7 and 16 after the onset of the disease. The results confirm the applicability of NS1 antigen detection in urine samples using ELISA to diagnose acute DENV infection and suggests that the assay is potentially useful when only limited amounts of serum samples are available and in limited resource settings.

INTRODUCTION

Dengue virus (DENV) belongs to the genus Flavivirus, which consists of 4 serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) (1). DENV infection occurs in tropical and subtropical areas (2), where it is transmitted by vector mosquitoes Aedes aegypti and Aedes albopictus. In recent years, there has been an exponential increase in the number of dengue cases worldwide. It has been speculated that such increase is associated with increased mosquito activity and range expansion, which has been speculated to be associated with increased global warming and urbanization (3–5). About 40% of the world’s population lives in areas where there is risk of DENV infection (5,6). Although DENV has previously not been endemic in Japan, the number of imported cases has increased from 32 cases in 2003 to 249 cases in 2013. A recent autochthonous dengue outbreak in Japan resulted in 160 cases (7,8). It is estimated that annually there are 390 million cases of dengue infection (9). Although most cases of DENV infection are asymptomatic (3), approximately 500,000 patients per year are hospitalized as a result of severe dengue with a mortality rate of 2.5% (6,9). In symptomatic infection, dengue infection causes symptoms that range from mild dengue fever to severe and life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (10). Currently, there are no suitable vaccines available for dengue and also, there is a lack of specific treatment. Therefore, early diagnosis is important for clinical management, both for prevention of severe disease and infection control. Laboratory confirmation of dengue infection includes virus isolation, detection of viral genome, viral antigen, and a 4-fold rise in antibody titers (10–13).

NS1 antigen is a useful marker for the laboratory diagnosis of DENV infection. The NS1 antigen, which is essential for viral replication, is expressed in the intracellular organelles and the cell surface, and also secreted into the extracellular environment during DENV infection (14–16). NS1 is detectable in the sera of dengue patients, although the amount of secreted NS1 varies among different individuals and is speculated to be correlated with the levels of viremia and disease severity (17). NS1 antigen is detected in the serum samples after the onset of the disease up to ≥14 days post-infection and after the disappearance of viral genome or in presence of IgM antibody (13,18).

Serum samples have been proven useful for the laboratory diagnosis of DENV infection. In the field, however, or in DHF patients with severe bleeding tendency and infants, the use of samples such as urine (which can be obtained by non-invasive procedures) is advantageous (19). Previous studies by other investigators have shown the presence of viral genome in the urine samples of dengue patients (12,20). Additionally, NS1 antigen has been detected in urine samples from days 2–10 after the onset of the disease (21). However, determining the applicability of NS1 ELISA at each phase of the disease would confirm the usefulness of NS1 antigen detection in urine samples for DENV diagnosis. In this study, we investigated the use of NS1 Ag

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ELISA (for urine samples) for the laboratory diagnosis of DENV infection. NS1 antigen detection rates were determined using urine samples obtained from 96 dengue fever patients who returned to Japan from overseas travel during 2008-2012. All the samples were sent from clinics and hospitals to the National Institute of Infectious Diseases, Japan, for diagnosis of arbovirus infection. Patient samples were examined for the presence of the viral genome by fluorogenic real-time reverse transcriptase polymerase chain reaction (TaqMan real-time RT-PCR), and ELISA with NS1 antigen and anti-DENV antibody (IgM and IgG antibody) (13,22). For the concentration of urine samples, the urine samples were transferred to Vivaspin 20 (Sartorius Stedim Biotech, Goettingen, Germany) and centrifuged for 30 min at approximately 1,400 × g. The concentrated urine samples were used at concentration of 10-times that in the original. The day of disease onset was defined as day 0. DENV infection was confirmed by the presence of the viral genome and NS1 antigen or a 4-fold rise in antibody titters (10). Ten urine samples from non-dengue patients were used as negative controls. Patients were de-identified prior to the conduction of laboratory tests. The study protocol was approved by the ethics institutional review board of the National Institute of Infectious Diseases.

**TaqMan real-time RT-PCR:** The viral genome in serum and urine samples was detected using real-time RT-PCR (20). Viral RNA was extracted from 200 µl of serum or 200 µl of urine samples using a High Pure Viral RNA kit (Roche, Madison, WI, USA). For real-time RT-PCR, 5 µl of RNA was mixed with serotype-specific primer and probe, to make up a final volume of 20 µl of reaction mixture (TaqMan Fast Virus 1-Step Master Mix kit, Life Technologies, Carlsbad, CA, USA) (20). Real-time RT-PCR was used with the following cycle conditions: (1) Real-time RT-PCR for 5 min at 50°C, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Data was collected and analyzed after 40 cycles.

**Anti-DENV IgM and IgG Antibody:** Dengue virus-specific IgM antibodies in serum and urine samples were determined using IgM capture ELISA (Dengue fever virus IgM capture ELISA, Focus Diagnosis, Cypress, CA, USA) according to manufacturer’s instructions. Dengue IgG ELISA (Dengue IgG Indirect ELISA, Panbio, Queensland, Australia, and Dengue ELISA IgG, Vir-cell, Granada, Spain) was used for the detection of anti-DENV IgG antibody according to manufacturer’s instructions (13).

**DENV NS1 Antigen ELISA:** Dengue NS1 antigen was detected in each sample using a Platelia Dengue NS1 Ag kit (Bio Rad, Marnes La Coquette, France): the assay was performed according to the manufacturer’s instructions. Briefly, 50 µl of sample diluent, negative control, positive control, calibrator, and the serum or urine samples were added to each anti-NS1 monoclonal antibody-coated microwell. One hundred microliters of diluted conjugate (1 × ) was then added to each microwell. The samples were mixed in the wells and incubated for 90 min at 37°C. After washing 6 times in 1 × wash buffer, 160 µl of tetramethylbenzidine was added to the wells and incubated for 30 min at room temperature in the dark. The reaction was terminated with 100 µl stop solution (1N H₂SO₄). Optical density (OD) was measured at a wavelength of 450 nm using a spectrophotometer. All experiments were performed in duplicates. Index or sample ratio was calculated using the following formula: mean OD of urine or serum samples/ mean OD of cut-off controls. Index of <0.5, 0.5-1.0, and >1.0 were classified as negative, equivocal, and positive, respectively. Equivocal results were regarded as negative.

**Statistical analysis:** The tabulation, management, and analysis of data were performed using Microsoft Excel. Positive detection rate (%) was calculated using the following formula: the number of positive samples/ the total number of samples × 100. The results were compared using the chi-squared test and Microsoft Excel.

**RESULTS**

**NS1 antigen detection in the serum and urine samples of DENV patients:** We examined the viral genome, NS1 antigen, IgM, and IgG antibody levels in 175 serum samples and 118 urine samples collected from 96 dengue patients. Of the 96 patients, 19 patients were infected with DENV-1, 17 with DENV-2, 17 with DENV-3, and 10 with DENV-4. DENV serotype could not be determined in 33 patients.

Positive rates of NS1 antigen were compared between the serum and urine samples from days 0 to ≥15 (Fig. 1) after disease onset. Urine samples from non-dengue fever patients were confirmed to be negative (10/10, 100%) for the presence of NS1 by ELISA and were used as the negative controls. The filtration method has previously proven useful in sample concentration and adenovirus detection (23). Therefore, this method was adapted to concentrate urine samples for dengue virus detection in this study. Serum samples from dengue patients were divided into 4 groups according to days after disease onset: 0-5 days, 6-10 days, 11-14 days, and on and after 15 days. The NS1 antigen positive rate in the serum samples was >90% on days 0-10, but decreased thereafter to 43% (6/14) on days 11-14 and 12% (3/26) on and after 15 days. In comparison, the NS1 antigen detection rate in the urine samples was 36% (18/50) on days 0-5, 43% on days 6-10 (19/44), 33% (3/9) on days 11-14, and 13% (2/15) on and after 15 days. The NS1 antigen ELISA index ranged from 2.2 to 14.6 in the urine samples determined as positive. Although the NS1 detection rates were higher in the serum samples than in the urine samples during the early phase of the disease (days 0-10) (Fig. 1), the NS1 antigen was detected in 2 urine samples obtained on day 7 and day 16, but not in the serum samples collected on the same days (Table 1). These results indicate that the NS1 antigen can be detected in urine samples even after NS1 antigen clearance (or decrease to undetectable
Detection of DENV NS1 Antigen in Urine Samples

Detection of the NS1 antigen was compared between 37 paired concentrated urine and non-concentrated urine samples (Table 2). There was no significant difference in the NS1 antigen detection rates between the concentrated urine and non-concentrated urine samples (chi-squared test, $P = 0.64$). Although the NS1 antigen was detected in 3 concentrated urine samples that were negative before concentration, the results suggest that the utility of concentrating urine samples for increasing the sensitivity of NS1 antigen ELISA was limited.

The NS1 antigen detection rates in 1 dengue patient (Patient #14) were determined during the course of the disease. Serum samples obtained on days 4–10 and on day 16 and urine samples obtained on days 5–10 and on day 16 were used (Fig. 2). On days 5–10, all serum and urine samples tested positive for the NS1 antigen. The NS1 antigen index values of serum samples, however, decreased from day 6 onwards (index value = ≥15 on levels) from the serum of some dengue patients.

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days 6–9, index value = 5.9 on day 10). Although the NS1 antigen index values of the urine samples on days 5–10 were lower than those of the serum samples, the NS1 antigen index values of the urine samples were constant on days 6–10 (index value range = 3.2–4.6). Interestingly, on day 16 after disease onset, the NS1 antigen was not detected in the patient’s serum, but was detected in the urine.

Positive detection rates of the viral genome, NS1 antigen, anti-DENV IgM, and anti-DENV IgG: Positive detection rates of the NS1 antigen by NS1 antigen ELISA were compared to those of the viral genome, anti-DENV IgM, and anti-DENV IgG in both the serum and the urine samples (Fig. 1). The positive detection rate for the viral genome in the serum samples was 81% (57/70) on days 0–5, but decreased to 36% (12/33) on days 6–10 (Fig. 1B). In contrast, the viral genome detection rates in the urine samples remained at similar levels throughout the study period—32% (16/50), 30% (13/44), and 33% (3/9) on days 0–5, 6–10, and 11–14, respectively. Viral genome detection rates in the urine samples were higher than those in the serum samples on and after day 11. The NS1 antigen detection rates in the serum samples were constant on days 6–10 at 90% (47/52), but subsequently decreased to 43% (6/14) on days 11–14 (Fig. 1A). In contrast, the NS1 antigen detection rates in the urine samples were constant at 36% (18/50), 43% (19/44), and 33% (3/9) on days 0–5, 6–10, and 11–14, respectively. In the serum samples obtained on days 6–10, the detection rate of IgM was high at 98% (51/52). The detection rate of the viral genome in the serum samples was 36% (12/33) (Fig. 1B). On and after 11 days, the positive rates of IgM antibody detection in the serum samples increased to 100% (14/14), and the positive rates of NS1 antigen detection decreased to 43% (6/14) (Fig. 1A and 1C). The IgM antibody detection rate in the serum samples was 100% (38/38) on and after 11 days (Fig. 1C), but the antibody was detected in only 1 urine sample obtained on day 5 (1%, 1/118). Similarly, the IgG antibody detection rate in serum samples was 95% (38/40) on and after11 days (Fig. 1D), but the IgG antibody was detected in only 1 urine sample obtained on day 12 (1%, 1/118). Although, the viral genome and NS1 antigen were detected in urine samples using real-time RT-PCR and NS1 antigen ELISA (Fig. 1A and 1B), the detection rates in the urine samples were lower than in those in the serum samples (chi-squared test: viral genome \( P < 0.01 \), NS1 antigen \( P < 0.01 \)). Concurrent with the results from previous studies (12), the viral genome was detected in some cases in the urine samples (9/86, 10%), but not in the serum samples. The results indicate that the viral genome and antigen are present in urine samples.

**DISCUSSION**

Dengue, a global public health problem, is also a serious health threat in areas with limited resources and access to diagnostic tools (9,10,24). For dengue disease surveillance and control, there is a need for robust diagnostic tools that are relatively easy to use and reliable in various clinical settings. NS1 antigen ELISA has emerged as a promising diagnostic tool and is relatively easy to use (14). NS1 antigen could be detected from the day of disease onset (disease onset day 0), with IgM levels increasing from day 3 (13–15,25). The sensitivity and specificity of NS1 Ag ELISA were high, in serum samples, which demonstrate the utility of the assay in dengue diagnosis (13–15). In this study, we evaluated the utility of NS1 antigen detection in urine samples for the laboratory diagnosis of dengue. Real-time RT-PCR and IgM/IgG antibodies by ELISA compared detection rates of NS1 ELISA in urine samples to those of serum samples and detection rates of virus genome.

Serum samples have been routinely used for the laboratory diagnosis of DENV infection. Blood collection, however, can be a challenge in areas with limited resources and for patients with severe bleeding tendency and infants. In such cases, urine sample collection is more practical (19), as it is non-invasive, rapid, and can be performed without laboratory instruments such as a centrifuge. In this study, we determined the utility of NS1 antigen ELISA using urine samples from dengue patients. The NS1 antigen was detected in the urine samples of dengue patients on days 2–17 after disease onset.

In a previous study, the NS1 antigen was detected in urine samples collected on days 2–10 after disease onset, positive rates of NS1 antigen using NS1 antigen ELISA were 68.4% and 63.9% in 19 DF and 36 DHF samples, respectively (21). However, the utility of NS1 antigen ELISA was not determined at each disease phase. In the present study, we determined the detection rates of NS1 antigen in urine samples at days 0 to 15 after disease onset and compared the detection rate of NS1 antigen ELISA with that of viral genome (real-time RT-PCR) and IgM/IgG antibodies. IgM and IgG antibodies were detected in 1 urine sample each (1/118, 1%). The results suggest that the detection of IgM and IgG antibody in urine samples is not a feasible method for dengue diagnosis. In contrast, NS1 antigen positive rates in urine samples were 36% (18/50) on days 0–5. The rates were the highest (43%) on days 6–10 (19/44), and they decreased to 33% (3/9) on days 11–14 and to 13% (2/15) on \( \equiv \) day 15. Viral genome detection rates in urine samples were similar to those of NS1 antigen ELISA: 32% (16/50) on days 0–5, 30% (13/44) on days 6–10, 33% on days 11–14 (3/9), and 20% (3/15) on \( \equiv \) day 15. We found that NS1 antigen-positive rates were constant (approximately 30%) in urine samples, although these were lower than those of serum samples on days 0–10. Of note, in the present study, NS1 antigen was also detected in urine samples at days >10 and after the viral genome became undetectable in urine samples. Concurrent with the results of previous investigations, DENV genome was detected at a higher rate in urine samples than in serum samples at days 11 to 15 after disease onset (12,26). Although the NS1 antigen detection rates in serum samples were higher than those of urine samples during the early phase of the disease, during the late phase of the disease (beyond day 11), NS1 antigen was constantly detected at similar rates in urine and serum samples. The results suggest that detection of NS1 antigen in urine samples, using NS1 ELISA, is particularly useful at the late phase of the disease. Interestingly, on days 11 to 15 after disease onset, NS1 antigen detection rates in urine and serum samples were at similar levels. It has been hypothesized that DENV infection deposition of virus-antibody immune-complexes in vas-
cular and glomerular tissue (27–29). There remains a possibility that the prolonged detection of virus genome and NS1 antigen in urine samples occurs due to differences in secretion patterns between serum and urine. Overall, the results suggest the utility of urine samples obtained up to day 17 after disease onset for laboratory diagnosis for DENV infection by NS1 antigen ELISA.

The filtration method has been previously applied to concentrate virus (23). In the present study, there was no significant difference between detection rates in concentrated and non-concentrated urine samples (chi-square test, \(P = 0.64\)). Interestingly, NS1 antigen was detected in serum samples of DENV-4 patients, but not in urine samples. Using the present ELISA method, other investigators have demonstrated that the NS1 antigen-positive rate of serum samples from DENV-4 patients (19%) was lower than that of other DENV serotypes (DENV1, 67%; DENV-2, 68%; DENV-3, 69%) (30). Further studies using a higher number of urine samples obtained from DENV-4 patients are expected to address the discrepancies in NS1 antigen detection rates between serotypes. Overall, the results suggest that where serum sample collections are not practical, urine samples may be a feasible alternative.

The utility of urine samples for laboratory diagnosis of DENV infection using NS1 antigen ELISA was determined in this study. The NS1 antigen detection rates in serum samples were higher than those of urine samples during the early phase of the disease. However, during the late phase of illness (days 11 to 15), NS1 antigen was detected in some urine samples but not in their corresponding serum samples. Interestingly, during the later phase of disease, NS1 antigen-positive rate of urine and serum samples was similar. These findings indicate that detection of the NS1 antigen in urine samples is a useful laboratory diagnostic method for DENV infection, particularly in combination with RT-PCR.

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Conflict of interest  None to declare.

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