

## Horseradish peroxidase-labeled rabbit anti-non-structural protein 1 of dengue virus-2 for the diagnosis of dengue virus infections

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**ABSTRACT**

**BACKGROUND** Early diagnosis of dengue virus (DENV) infection is essential for patient management and disease control. Detection of the antigen non-structural protein 1 (NS1) has been proven to provide early diagnosis of DENV infection. Thus, commercial NS1 antigen detection assays have been increasingly used and are becoming the tool of choice among clinicians to confirm DENV infection in Indonesia.

**METHODS** To obtain anti-NS1 DENV antibody, NS1 protein (90 µg/ml) from the collection of the Department of Microbiology, Faculty of Medicine, Universitas Indonesia was injected into a rabbit. The anti-NS1 antibody from the rabbit was then labeled with horseradish peroxidase (HRP) using the periodate oxidation method. Sera were tested by enzyme-linked immunosorbent assay (ELISA) to detect NS1 from DENV-infected patients.

**RESULTS** Serially diluted antibody labeled with HRP tested using the direct ELISA method showed the highest absorbance value at a 1:100 dilution (Mean [SD] = 1.35 [0.35]); even at a dilution as high as 1:3,200 (0.22 [0.15]), antibody labeled with HRP was able to detect the NS1 protein, although the absorbance value did not differ greatly from that of the negative control (0.13 [0.01]).

**CONCLUSIONS** In an attempt to develop an NS1-based diagnostic test, polyclonal anti-NS1 DENV antibody was successfully produced as a diagnostic assay to determine the presence of DENV NS1 antigen in patients' sera.

**KEYWORDS** dengue virus, horseradish peroxidase, NS-1 antigen, polyclonal antibody

Dengue virus (DENV) infection is an important arthropod-borne viral infection threatening 2.5 billion people who live in endemic areas worldwide. DENV belongs to the Flaviviridae family and is transmitted to humans by infective female mosquitos of the genus *Aedes*, especially *Aedes aegypti* or *Aedes albopictus*.<sup>1,2</sup> There are four related but antigenically distinct serotypes of DENV 1–4. DENV causes two types of infection: primary and secondary. Primary infection results in an acute febrile illness known as dengue fever (DF), which is cleared in approximately 7 days by a complex immune response. Secondary infection is

more severe and results in dengue hemorrhagic fever (DHF) or dengue shock syndrome.<sup>3,4</sup>

Rapid and accurate diagnosis of DENV infection is critical for prompt initiation of appropriate clinical care and has been associated with a significant (50- to 100-fold) decrease in mortality from the disease.<sup>5</sup> Detection of dengue-specific IgM/IgG class antibodies remains the most commonly utilized diagnostic method. However, serologic evaluation has several limitations, including prolonged time to seroconversion (3–7 days), IgM seropersistence following disease resolution, and cross-reactivity with other flaviviruses.<sup>6</sup>

Recently, detection of DENV non-structural protein 1 (NS1) has emerged as a potential alternative to both serologic and molecular-based techniques for the diagnosis of active dengue infection.<sup>7</sup> NS1 is a non-structural glycoprotein essential for replicability and is detectable within 24 hours and up to 9 days after fever onset. Kinetically, NS1 antigenemia overlaps with the DENV viremia phase and is present prior to IgM seroconversion, positioning the NS1 antigen as a ideal biomarker for identification of acute dengue infection.<sup>6,8</sup> The NS1 antigen appears in higher concentrations in the sera of DHF patients as compared with those suffering from DF, which indicates its association with disease severity. On account of its high concentration in serum, it could be used for the early diagnosis of DF or DHF with an immune-chromatographic device or enzyme-linked immunosorbent assay (ELISA).<sup>9,10</sup>

Indonesia is facing outbreaks of DENV, where the diagnosis and management of DENV infection is the main issue due to the complicated, time consuming, and expensive procedures needed to diagnose infection at an early stage. Commercial NS1 antigen detection assays have been used increasingly often and are becoming the tool of choice among clinicians to confirm DENV infection in Indonesia. In this study, an immunoassay using horseradish peroxidase (HRP)-labeled anti-NS1 polyclonal antibody was developed to detect the NS1 antigen in serum, which could ensure rapid, cost-effective, and early diagnosis of DENV infection.

## METHODS

### Patients' sera

A total of 40 clinical samples from the Community-Based Dengue Study Group Faculty of Medicine, Universitas Indonesia (FMUI), Cipto Mangunkusumo Hospital (Approval number 471/UN2.F1/ETIK/2015) were collected during dengue surveillance in 2010 from a health center in Jakarta. Febrile patients with suspicion of dengue and clinical features suggestive of acute febrile illness, rashes, bleeding tendencies, leucopenia, or thrombocytopenia were evaluated according to the World Health Organization criteria for probable dengue infection and<sup>11</sup> a previous study<sup>12</sup> and confirmed to have DENV infection by NS1 (KOREA, NS1 Ag Combo Dengue Duo Kit, Standard Diagnostic Inc), reverse transcription polymerase chain reaction (RT-

PCR),<sup>13</sup> and the hemagglutination inhibition test. Sera from patients with other viral and bacterial infections were obtained from the collection of the Department of Microbiology, FMUI, Cipto Mangunkusumo Hospital.

### Purification of DENV-2 NS1 protein

DENV-2 NS1 protein belonging to the Department of Microbiology was purified using Sephadex® G-100 (Pharmacia fine chemicals). The NS1 protein was applied to the column, and each fraction was collected in a tube to a total volume of approximately 250 µl. Fractionation results were tested by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stored at -30°C until use.

### Production of anti-NS1 antibody

Immunogenicity analysis was carried out in a rabbit and conducted in compliance with the ethical standards for animal experimentation of the Health Research Ethics Committee of the FMUI (Approval number 149/UN2.F1/ETIK/2015). The rabbit was immunized by a method described previously, with minor modifications.<sup>14</sup> Briefly, one male New Zealand White rabbit aged 4 months was immunized with four subcutaneous injections of 90 µg of purified DENV type 2 NS1 protein suspended in 100 µl of Freund's complete adjuvant (FCA, Sigma Aldrich, Missouri) at 1-week intervals. A week after the first immunization, samples of rabbit serum were drawn from the auricular vein. Then, the rabbit was boosted with a suspension of 90 µg in 100 µl of Freund's Incomplete Adjuvant (Sigma Aldrich, Missouri) after 3 and 4 weeks. One week after the booster, blood was sampled and tested for anti-NS1 antibody by ELISA to detect the NS1 antigen.

### Detection of anti-NS1 antibody in rabbit serum by indirect ELISA

Indirect ELISA was carried out according to the method described by Igarashi in the Technical manual for the study of arboviruses with special emphasis on Japanese encephalitis and dengue viruses.<sup>14</sup> Each well was coated with 100 µl of New Guinea C (NGC) DENV-2 viral culture supernatant ( $2.1 \times 10^5$  FFU) in 1:25 coating buffer ( $\text{Na}_2\text{CO}_3$ ,  $\text{NaHCO}_3$ , pH 9.6) (Disposable Products Pty. Ltd, South Australia), and the plate was incubated overnight at 4°C. After incubation, the wells were rinsed three times with washing buffer (phosphate buffered saline [PBS] with 0.1% Tween 20). After washing, non-specific sites were blocked by addition

of 300 µl blocking buffer (5% skim milk [Tropicana Slim, PT. Nutrifood Indonesia, Jakarta] in PBS, pH 7.3) into each well, and the plate was incubated for 30 min at 37°C. After incubation, the plate was washed three times with rinse buffer. After washing, 100 µl of 1:50 diluted rabbit serum in 1% skim milk was added to each well, and the plate was incubated for 1 hour at 37°C. Excess rabbit serum (primary antibody) was removed by washing the well three times with rinse buffer. After washing, 50 µl of goat anti-rabbit IgG-HRP (Sigma Aldrich, Missouri) diluted 1:5,000 in 1% skim milk was added to each well, and the plate was incubated for 1 hour at 37°C. The excess antibody-enzyme conjugate was removed by washing the well three times with rinse buffer. For detection, 100 µl of the chromogenic substrate H<sub>2</sub>O<sub>2</sub> + TMB (3,3',5,5'-tetramethylbenzidine) (Kirkegaard & Perry Laboratories, Maryland) 1:1 was added to each well, and the plate was incubated for 10 min at room temperature in the dark until the color developed. The reaction was stopped by addition of 100 µl of 3 N sulfuric acid (H<sub>2</sub>SO<sub>4</sub>). The absorbance of the colored solution was read at 450 nm in an automatic microplate ELISA reader (Bio-Rad Model 550, California).

#### Conjugation of anti-NS1 antibody with HRP

Before labeling with HRP, the anti-NS1 antibody was purified with Sephadex® G-100 (Pharmacia fine chemicals). The anti-NS1 antibody was conjugated to HRP as described by Ramesh Kumar et al.<sup>15</sup> First, the HRP was activated by dissolving 2 mg of HRP (Sigma Aldrich, Missouri) in 0.5 ml of distilled water, and 0.2 ml sodium periodate (NaIO<sub>4</sub>) 0.1 M was added. The solution was gently inverted for 2 hours at room temperature until the color changed from orange to green. Immediately after incubation, activated HRP was added to 1 ml of antibody solution and incubated for 20 min at room temperature. After that, 0.2 M Na<sub>2</sub>CO<sub>3</sub> was added, and the mixture was stirred for 2 hours at room temperature. After 2 hours, 100 µl of reductant solution (NaBH<sub>4</sub>) was added, and the solution was mixed for 10 min at room temperature. Then, the solution was dialyzed in PBS at 4°C overnight. The dialysis result was tested by ELISA and stored at -30°C until use.

#### Binding of HRP-conjugated anti-NS1 antibody to the NS1 antigen

Binding of anti-NS1 HRP complexes to the NS1

antigen was tested by direct ELISA. First, each well was coated with 100 µl of NGC DENV-2 viral culture supernatant in coating buffer (Disposable Products Pty. Ltd, South Australia). The anti-NS1-HRP conjugate was diluted 1:100; 1:200; 1:400; 1:800; 1:1,600; 1:3,200; 1:6,400; and 1:12,800 and 100 µl added to each reaction. After incubation for 1 hour at 37°C, the wells were washed, and the substrate was added. The absorbance of the resulting colored solution was read at 450 nm in an automatic microplate ELISA reader (Bio-Rad Model 550, California).

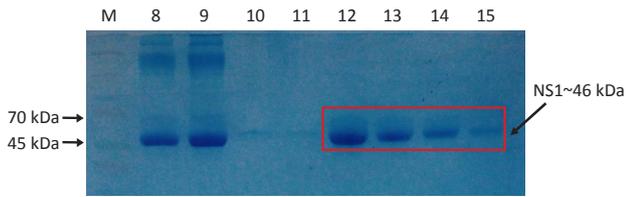
#### Detection of the NS1 antigen in patients' sera

Antibody sandwich ELISA was performed using in-house HRP-labeled rabbit antibody to determine the presence of the NS1 DENV antigen in patients' sera. Briefly, each well was coated with 100 µl of unlabeled serum from an immunized rabbit in coating buffer (Disposable Products Pty. Ltd, South Australia), and the plate was incubated overnight at 4°C. One hundred microliters of patients' sera was added to each well at a dilution of 1:100 and incubated for 1 hour at 37°C. After washing, 50 µl of 1:100 diluted HRP-labeled anti-NS1 antibody was added to each well, and the plate was incubated for 1 hour at 37°C. Excess antibody-enzyme conjugate was removed by washing the well three times with washing buffer. Then, 100 µl of the chromogenic substrate (tetramethyl benzidine/hydrogen peroxide) was added to each well, and the plate was incubated for 10 min at room temperature until the color developed. The reaction was stopped with 1 N sulfuric acid, and the absorbance of the colored solution was read at 450 nm in an automatic microplate ELISA reader (Bio-Rad Model 550, California).

## RESULTS

#### Purification of DENV-2 NS1 protein

In this study, DENV-2 NS1 protein with a molecular weight of 46 kDa was used from the collection of the Department of Microbiology. The presence of purified protein was confirmed using SDS-PAGE 8%. SDS-PAGE showed a single band corresponding to a molecular weight of ~46 kDa in fractions 12–15, which was identified as the NS1 protein. Fractions 10 and 11 also contained NS1 protein, but the band was very thin. In fractions 8 and 9 there were protein impurities despite the



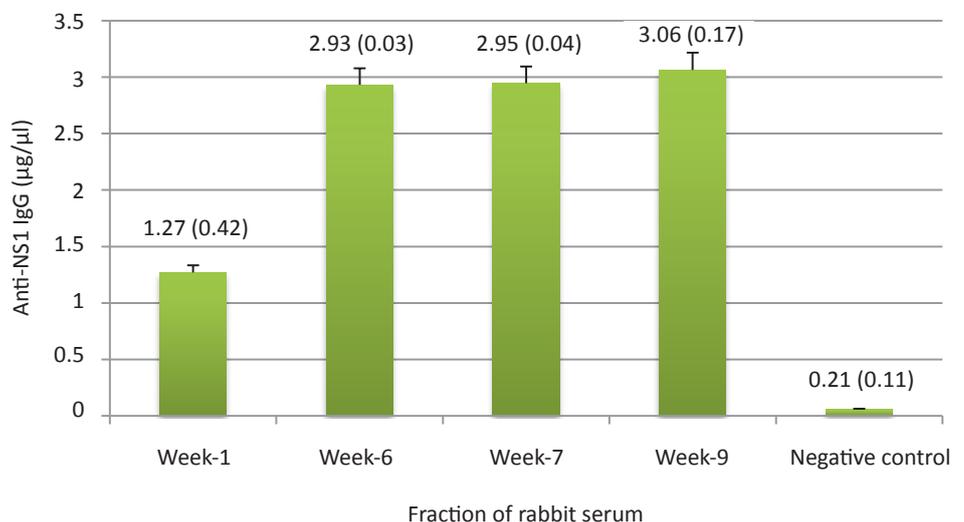
**Figure 1.** SDS-PAGE results for the NS1 protein with a single band of ~46 kDa in fraction 12–15. M=protein marker; 8–15=protein fractions after purification with Sephadex G-100

presence of protein bands corresponding to NS1 (Figure 1). Therefore, the NS1 protein used to immunize the rabbit was from fractions 12, 13, 14, and 15. The presence of NS1 protein was also confirmed using the Dengue NS1 Ag + Antibody Combo Dengue Duo Kit, Standard Diagnostic Inc (immunochromatography strip) and showed a positive result (data not shown).

**Production of the anti-NS1 antibody**

The NS1 protein was injected into the rabbit at a concentration of 90 µg/ml as a single initial injection and three booster injections. After immunization, blood sampling was performed four times. The first blood collection was carried out 1 week after immunization (Code w-1). The next blood samples were collected in the sixth, seventh, and ninth weeks after the last booster injection (Code w-6, w-7, and w-9, respectively). All of the sera were tested by indirect ELISA using virus supernatant as the antigen. This assay was performed to determine the induction of anti-NS1 antibodies in rabbit serum. The results of ELISA showed that the visible absorbance value of the preimmunization serum negative control was low (0.21 [0.11]). The result was obtained with the negative control (0.20 [0.12]).

**Figure 2.** Comparison of the rabbit anti-NS1 antibody fraction determined by indirect ELISA. After immunization, blood sampling was performed four times. The first blood collection was carried out 1 week after immunization. Subsequent blood samples were collected in the sixth, seventh, and ninth weeks after booster injection. At week 1, the mean (SD) of absorbance value begin to increase and continued to increase to at week 9. Negative control=pre-immune rabbit serum



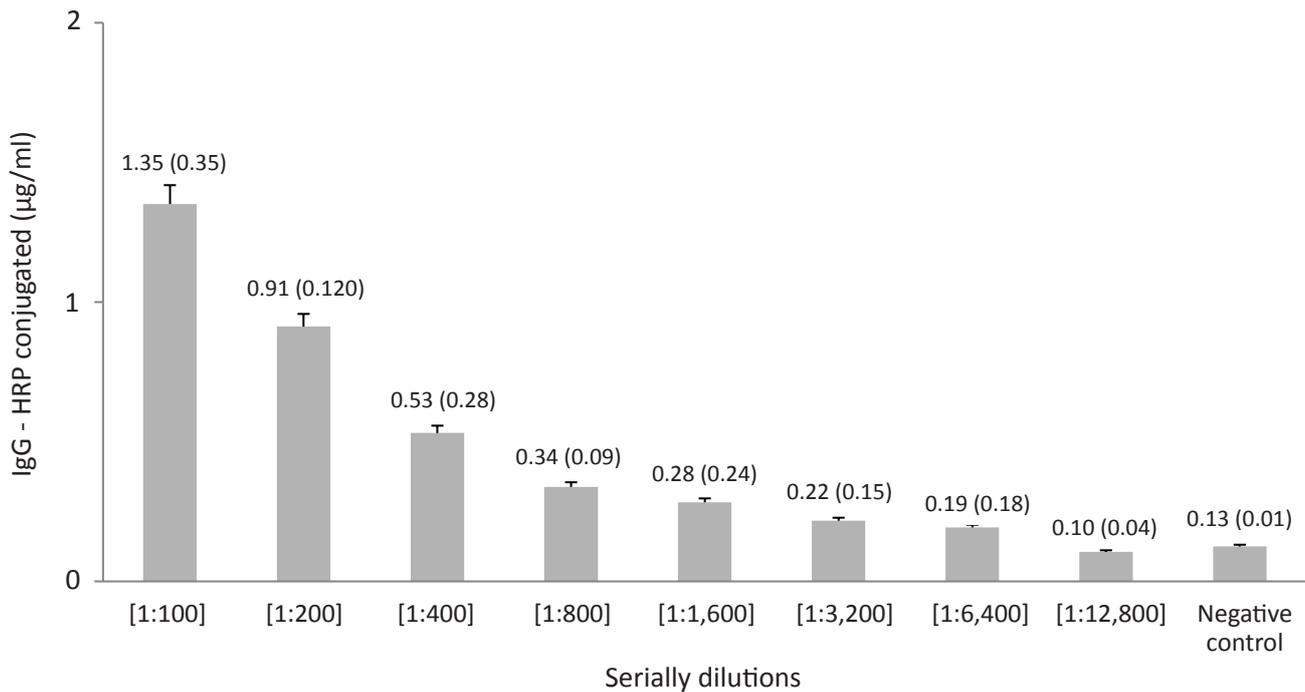
The absorbance value began to increase in w-1 (1.27 [0.42]) and continued to increase to 3.06 (0.17) in w-9. Therefore, only w-9, with the highest OD value, was further purified and labeled with HRP. Sephadex G-100 was used to obtain pure IgG antibodies (Figure 2).

**Conjugation of anti-NS1 antibody with HRP**

Testing of the serially diluted HRP-labeled antibody using the direct ELISA method showed the highest absorbance value in the 1:100 dilution (1.35 [0.35]); even when diluted to 1:3200 (0.22 [0.15]), the HRP-labeled antibody could detect the NS1 protein, although the value did not differ greatly from that of the negative control (0.13 [0.01]) (Figure 3). The absorbance of the labeled antibody was half that of the unlabeled rabbit antibody, presumably due to the dilution effect. This may have been largely due to attachment of HRP to the fragment of antigen binding, which disrupted the binding of HRP-labeled antibody to the NS1 antigen.

**Detection of NS1 antigen in patient’s sera**

The w-9 antibody labeled with HRP was also used to detect NS1 antigen in the sera of patients infected with DENV, DENV-negative patients, and healthy people, by using sandwich ELISA. The test results indicated that the HRP-labeled antibody was able to detect the NS1 antigen, including DENV-1 (10 samples), DENV-2 (10 samples), DENV-3 (10 samples), and DENV-4 (10 samples), in all patients’ sera. To determine whether w-9 antibody labeled with HRP cross-reacted with other antigens, patients’ sera infected with cytomegalovirus and Epstein-Barr virus was used and found no cross-reaction with antigens of other viruses.



**Figure 3.** Direct ELISA to confirm the interaction of the NS1 antigen and HRP-conjugated anti-NS1. The mean (SD) of results were obtained using an HRP-conjugate (2 µg/ml) prepared through periodate conjugation methods as the secondary antibody

## DISCUSSION

Rapid and early diagnosis of DENV infection can lead to early therapeutic intervention and have a significant effect on patient recovery.<sup>16</sup> Detection of the dengue NS1 antigen is suggested as a helpful tool for the early diagnosis of dengue infection after the onset of fever in primary and secondary infection. The NS1 antigen has been found circulating from the first day after the onset of fever up to day 9, once the clinical phase of the disease is over.<sup>17</sup> The NS1 protein is a highly conserved glycoprotein and is believed to be responsible for the pathogenicity of DENV. Therefore, it can be a good candidate antigen for the diagnosis of dengue infection.<sup>18</sup> NS1 is an ideal diagnostic marker due to its presence at high levels in the blood of infected individuals very early in infection, typically at or before symptom onset.<sup>19</sup>

The NS1 protein could be detected even when the sample tested negative for viral RNA in RT-PCR or in the presence of immunoglobulin M antibodies. The levels of circulating NS1 in acute-phase serum samples are within the range of 10 µg/ml to 50 µg/ml and do not differ significantly between primary and secondary infection.<sup>14</sup> The commercial development and application of NS1 detection as a diagnostic tool has revolutionized dengue diagnosis and now provides

a simple and relatively low-cost assay with high sensitivity and specificity. More recent studies have shown that NS1 detection may also be applicable to the diagnosis of other flavivirus infections.<sup>20</sup> Recently, commercial diagnostic NS1 kits have become available in Indonesia and show variable specificities and sensitivities. The sensitivity of some NS1 antigen assays ranged from 29–88%, and the specificity ranged from 89–100%.<sup>17</sup> The reasoning behind the disparate sensitivities between kits, serotypes, and geographical sites requires further study. The difference may reflect different levels of avidity of the test monoclonal antibody for the relevant epitope(s) in NS1 from different serotypes and potentially different lineages from the same serotype, as well as differences in the viral burden between serotypes.<sup>1</sup> The other limitation of the NS1 diagnostic kit is its inability to distinguish one dengue serotype from another. Qiu et al<sup>21</sup> developed an NS1 antigen assay using a monoclonal antibody that successfully differentiated DENV-2 from other serotypes with 83.3% sensitivity and 100% specificity. Overall, the results of these studies suggest that the currently available NS1 antigen detection kits require further improvement, mainly with respect to their sensitivity.

In the case of polyclonal antibodies, animals are given injections of antigen or antigen/adjuvant

mixtures to induce an effective antibody response, and it is usually necessary to collect blood to monitor the antibody response during the experiment and to obtain antibodies. When the antigen to which antibodies are to be raised is poorly immunogenic, the immune system requires a stimulus to induce an effective immune response. Adjuvants can be used for this purpose and can induce cellular or humoral responses. FCA is used frequently for the production of polyclonal antibodies because high antibody titers are induced to almost all types of antigens.<sup>22</sup>

Conjugation of enzymes to antibodies involves the formation of a stable, covalent linkage between an enzyme, such as HRP, and an antibody. This enzyme is inexpensive and can be attached to the immune reagent by a variety of methods. Moreover, many chromogenic substrates for it are also available. An ideal conjugation reaction would be the labeling of each IgG with one molecule of HRP without loss of immunologic or enzymatic activities. Some aspects of this ideal reaction may be compromised, but others cannot be without loss of sensitivity of the method. For example, during the reaction, HRP should not lose its enzymatic activity, because IgG labeled with enzymatically inactive HRP will act as unlabeled IgG and compete for the antigenic sites. The conjugation of HRP to an antibody depends on the generation of aldehyde groups by periodate oxidation of the carbohydrate moieties on HRP. Combination of these active aldehydes with amino groups on the antibody forms a Schiff base upon reduction by sodium borohydride, thus stabilizing the conjugate.<sup>15,23</sup>

In this study, the w-9 antibody labeled with HRP was also used to detect NS1 antigen in the serum of patients infected DENV, DENV-negative patients, and healthy people, by using sandwich ELISA. The test results indicate that the HRP-labeled antibody was able to detect the NS1 antigen, including DENV-1, DENV-2, DENV-3, and DENV-4, in all patients' sera. This was due to similarities in the DENV-2 NS1 epitope among serotypes. Based on the results of sequencing the NS1 epitope of DENV-2, B-cell epitopes at amino acids 25–33, 112–116, and 299–303, have an amino acid that is highly conserved among all four serotypes of DENV.<sup>24,25,26</sup> Therefore, the antibody labeled with HRP could be used to develop a diagnostic assay to determine the presence of the DENV NS1 antigen in patients' sera, because early diagnosis of DENV infection is beneficial to patient management and disease control.

A limitation of this study was the unavailability of control sera from patients infected with Flaviviridae or Togaviridae. Therefore, sera from patients infected with other viruses were used. Moreover, further research is needed regarding the optimization of the labeling process to produce an HRP-labeled rabbit anti-NS1 DENV that can detect the NS1 protein at even lowest dilutions.

## Conclusions

In summary, at present the data shows that kit candidate can potentially used for diagnosis of dengue infection. Accuracy and rapidity should be explored in larger study.

## Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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