The development of a novel serotyping-NS1-ELISA to identify serotypes of dengue virus

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Abstract

Background: Dengue virus (DENV), which causes mosquito-borne disease dengue hemorrhagic fever (DHF), consists of four serotypes co-circulating in endemic areas. Currently, DENV serotypes can be identified by laborious virus isolation followed by immunofluorescent assay and sophisticated RT-PCR. **Objective:** To establish a new assay designated as “serotyping-NS1-ELISA” to detect the NS1 protein and to identify DENV serotypes simultaneously.

Study design: The monoclonal antibodies (Mabs) against NS1 of each DENV serotype were produced and characterized for their serotype-specificity. To develop serotyping-NS1-ELISA, the selected serotype-specific anti-NS1 Mabs were applied to detect the NS1 antigen, which was previously captured by a flavivirus cross-reactive anti-NS1 Mab. Serotyping accuracy of the developed assay was validated with NS1 from DENV-infected cell culture supernatants and from well-characterized clinical specimens.

Results: Of 30 anti-NS1 Mabs, 1 serotype-specific anti-NS1 Mab to each DENV serotype was selected based on NS1 capture ELISA results for developing the serotyping-NS1-ELISA. Using DENV-infected cell culture supernatants for validation, the selected antibodies were shown to be capable of differentiating four DENV serotypes. When acute phase plasma from DENV-infected patients was used for validation, 65 out of 85 specimens (76.5% overall sensitivity) were positive to one of the four serotypes developed in our assay. Interestingly, identification of DENV serotypes by our serotyping-NS1-ELISA was 100% accurate for DENV1, 3 and 4 and 82.4% for DENV2 as compared with standard RT-PCR. Assay specificity was 100% (90/90).

Conclusions: The developed serotyping-NS1-ELISA provides an alternative for simultaneous detection of DENV NS1 and identification of its serotype in acute patients’ specimens. The assay would be applicable for dengue diagnosis and epidemiological studies.

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1. Background

An infection by DENV in human causes a range of symptoms from mild dengue fever (DF) to severe DHF or dengue shock syndrome (DSS). DENV can be transmitted by Aedes mosquitoes infected with the virus. Co-circulation of four DENV serotypes has been found in all the endemic areas in the tropics. Serotypes and

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strains of DENV that cause infections could be one of the risk factors contributing to disease severity and major epidemics within populations. Severe DHF has been associated with a secondary response to an infection by heterotypic viruses. Determination of dengue serotypes in current and previous epidemics will be crucial for predicting of the severity of the current epidemics and also in detection of switch in predominant serotype, which could serve as a warning to dengue outbreak.

Conventional methods of identifying DENV serotypes involve isolation of DENV in mosquitoes or cell cultures followed by immuno-staining with serotype-specific anti-DENV Mabs, or detection of viral RNA by sophisticated RT-PCR with serotype-specific oligoprimers. During the past decade, detection of the NS1 antigen has become an alternative assay for early diagnosis of DENV infection. This is due to the findings that NS1 is secreted from infected cells into the blood during the pyrexia phase, and that its level is correlated with disease severity. Most NS1 assays, however, detect NS1 without being able to differentiate its serotype. Recently, NS1 assays specific for serotype-1 and -2 have been established, but none of the remaining serotypes (DENV3 and DENV4) has yet been reported.

2. Objectives

In this study, we aimed to develop a “serotyping-NS1-ELISA” system to detect dengue NS1 and to identify its serotype simultaneously.

3. Study design

3.1. DENV strains

Prototype strains of each DENV serotype (DENV1-Hawaii, DENV2-16681, DENV3-H87 and DENV4-H241) were used in this study.

3.2. Generation of anti-NS1 Mabs to each DENV serotype

The supernatants from DENV-infected PS clone D (a swine fibroblast cell line) containing NS1 protein (for DENV1 and DENV2) or immuno-affinity purified NS1 were used to immunize BALB/c mice separately for each serotype. Fusion of mouse splenocyte and myeloma cells was performed as previously described. Hybridoma clones producing anti-NS1 Mabs to each DENV serotype were screened by indirect NS1 ELISA using NS1 antigen obtained from homologous serotypes.

3.3. Characterization of the generated anti-NS1 Mabs

The types of the epitope recognized by the Mabs were characterized as linear or conformational through Western blot analysis. The DENV-infected cell lysates were treated with SDS-PAGE sample buffer containing reducing agent β-mercaptoethanol to completely denature NS1 antigen, or no reducing agent to maintain disulfide bonds on NS1 protein. The treated lysates were heated at 95°C to generate monomer NS1 (approx. 45 kDa), or non-heated to maintain its dimeric form (approx. 90 kDa) followed by subjecting to Western blot analysis and probed with the Mab to be tested. The Mabs that always react with NS1 prepared by four conditions were identified to recognize a linear epitope type. In contrast, the Mabs that recognize a conformational epitope type selectively reacted with NS1 obtained by only non-reducing conditions.

To determine their reactivity to four serotypes of DENV, five immuno-detection methods were performed. The lysates or supernatants prepared by mosquito cells separately infected with each serotype were employed to test with Mabs by dot blot assay and Western blot analysis, and indirect immunofluorescent assay and flow cytometry (FACS), while supernatants derived from DENV-infected PS clone D were tested with Mabs by NS1 capture ELISA. For the latter assay, the secreted NS1 from culture supernatant of each DENV serotype was separately captured by DENV cross-reactive, IgM, anti-NS1 Mab (2E11 or NS1-1F). The anti-NS1 Mab (IgG isotype) to be tested was then added to the captured NS1 antigens of all four serotypes. The antigen–antibody complexes were detected by goat-anti-mouse IgG-HRP, O-phenylenediamine (OPD)-H2O2 substrate and measured at 492 nm using an ELISA reader.

3.4. Optimization of the serotyping-NS1-ELISA

Based on the results of NS1 capture ELISA, four anti-NS1 Mabs that exhibited highest reactivity and serotype specificity were selected. Optimal concentrations of serotype-specific anti-NS1 Mabs were obtained with NS1 of homologous serotypes in culture supernatants of which their initial concentration were previously determined by NS1 capture ELISA with a purified NS1 standard curve. Detection limits of the assay for each serotype were measured as in the previous study. Briefly, OD readings obtained from NS1 capture ELISA using 25 µg/ml of serotype-specific Mabs were plotted against the various concentrations of corresponding NS1. The cut-off value of the assay was obtained by mean OD reading of negative control antigen (mock culture supernatant) +2 standard deviation (SD). Detection limit of the assay was the lowest NS1 concentration which gave the OD reading above the cut-off.

3.5. Validation of the developed serotyping-NS1-ELISA

3.5.1. Supernatant from cell culture

The secreted NS1 in culture supernatant derived from each serotype of DENV- or Japanese encephalitis virus (JEV)-infected cells was separately captured by coated cross-reactive 2E11 Mab. Four serotype-specific anti-NS1 Mabs were added at optimal concentrations to the captured NS1. Antigen–antibody complexes were detected as described above.

3.5.2. Clinical specimens

A total of 85 acute-phase plasma specimens, from the patients who were clinically diagnosed as DF/DHF according to WHO criteria with serological confirmation by anti-DENV IgM/IgG capture ELISA and DENV RNA detection by RT-PCR, were taken following ethical approval and consent from patients admitted in Khon Kaen and Songkhla Hospitals, Thailand. These specimens were collected during pyrexia phase, 3 days before and 1 day after defervescence (day −3; n = 9, day −2; n = 21, day −1; n = 41, day 0; n = 13, day 1; n = 1), and were identified for DENV serotype by serotyping RT-PCR. For non-dengue samples, 40 acute specimens from other febrile illness (OFI) pyrexia cases and 50 specimens from healthy individuals were included.

3.5.3. Evaluation of the serotyping-NS1-ELISA in clinical samples and interpretation of DENV serotype

Each clinical specimen at a dilution of 1:10 in PBS was aliquoted for NS1-capturing with coated cross-reactive anti-NS1 Mab 2E11 and probing with four different serotype-specific Mabs. Each clinical specimen was treated in duplicate. In parallel, a pool of human plasma specimens from healthy individual (PNS) was treated by the same method as a negative control for setting the baseline. The mean OD reading of a test specimen (T) obtained from each serotype-specific Mab was normalized by that of PNS (N) and represented as the T/N ratio for each serotype-specific Mab. With a cut-off T/N ratio at 1.7 (the value that none of the 20 healthy human individuals have exceeded)
were either DENV cross-reactive (reactive to all 4 serotypes), sub-
complex (reactive to 2–3 serotypes) or serotype-specific (reactive
to one serotype) (Fig S1, Table 1). Based on NS1 capture ELISA, four
anti-NS1 Mabs (i.e., D1NS1-84, D2NS1-3D1, D3NS1-46 and D4NS1-
4) were selected to develop the serotyping-NS1-ELISA due to their
serotype-specificity and high reactivity in comparison to others
(Fig 1, Table 1 in bold).

### 4. Results

#### 4.1. Production and characterization of anti-NS1 Mabs

A total of 30 hybridoma clones producing anti-NS1 Mabs (i.e., 2,
13, 10 and 5 clones obtained from mice immunized with NS1 of
DENV1 to DENV4, respectively) were generated (Table 1). The Mabs recognized both linear and conformational epitopes of the
NS1 protein. The reactivity patterns obtained from those Mabs to
NS1 proteins of DENV1 to DENV4 by various immuno-detection
assays were generally consistent. However, a few inconsistent
results were observed, which might reflect the different forms of
NS1 used by each assay, i.e., intracellular NS1 (in fixed cells or
cell lysates) or secreted NS1 (in culture supernatant). The Mabs
were either DENV cross-reactive (reactive to all 4 serotypes), sub-
complex (reactive to 2–3 serotypes) or serotype-specific (reactive
to one serotype) (Fig S1, Table 1).

#### 4.2. Optimization of the serotyping-NS1-ELISA

Various concentrations of serotype-specific Mabs and dilutions
of NS1 antigen of homologous serotypes were utilized in NS1 cap-
ture ELISA in a checkerboard manner. Similar reactivity patterns
of each serotype-specific Mab at different concentrations to NS1
antigens were obtained (Fig. 2). The reactivity of anti-NS1 Pab at
25 μg/ml, as a positive control antibody, was also equivalent to
most of the serotype-specific Mabs, except D2NS1-3D1, for which
the Pab displayed a superior reactivity to DENV2 NS1. The serotype-
specific Mab at 25 μg/ml was finally selected for each serotype
to establish a serotyping-NS1-ELISA. The detection limits of this assay
to determine soluble NS1 of each DENV serotype were 4.4, 15.8, 2.9
and 1.7 ng/ml of culture fluid for DENV1 to DENV4, respectively.

#### 4.3. Evaluation of the serotyping-NS1-ELISA

The established serotyping-NS1-ELISA was tested against the
supernatant from DENV-infected PS clone D cells. The results
clearly demonstrated that the selected anti-NS1 Mabs can differen-
tiate DENV serotypes and did not cross-react to NS1 of JEV (Fig 3).
Serotyping via this assay was also accurate for NS1 in the super-
natant derived from the other two DENV-infected cell types, i.e.,
mosquito cells C6/36 and human 293T (data not shown).

To evaluate the established serotyping-NS1-ELISA in clinical
specimens, acute plasma from 85 dengue patients and 90 non-
dengue patients (40 from OFI and 50 from healthy individuals)
were tested. The results of all specimens (175 samples) tested by serotyping-NS1-ELISA were analyzed according to our serotype-interpretation criteria, and concluded in Table 2. Of 85 acute DENV-infected specimens, 65 showed positive reactivity by one of the four serotype-specific Mabs (76.5% overall sensitivity). Sensitivities to each serotype were in the range of 64–80%, in which anti-DENV4 NS1 Mab (D4NS1-4) had the highest sensitivity (80%), whereas anti-DENV2 NS1 Mab (D2NS1-3D1) had the lowest sensitivity (63.6%). Interestingly, of those serotyping-NS1-ELISA positive samples (n = 65), their serotypes were identical with those iden-

Fig. 1. Determination of serotype specificity of anti-NS1 Mabs by NS1 capture ELISA. Each anti-NS1 Mab were tested with NS1 antigens from culture supernatants of PS cells infected with DENV1 to DENV4, which were captured by cross-reactive, IgM, anti-NS1 Mab attached on ELISA wells. The representative Mabs belonged to four different groups, i.e., D1NS1, D2NS1, D3NS1 and D4NS1 (A–D) were indicated on the X-axis. Cross-reactive anti-NS1 Pab was included as a positive control antibody. NS1 antigens from each DENV serotype, including mock antigen, were shown in different colored bars as indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 2. Optimization of selected serotype-specific anti-NS1 Mabs and NS1 antigens of each corresponding serotype in NS1 capture ELISA. The dilutions of DENV NS1 antigen in cell culture supernatants are shown on the X-axis. Various concentrations of Mabs in μg/ml [5] to [75] or anti-NS1 Pab at 25 μg/ml [p25] are represented by different dot colors on the right of each panel. (A–D) panels are DENV1 to DENV4 NS1-ELISA, respectively. Each data point represents the mean A492 ± SD of 3–6 replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
The detection of NS1 protein has recently become a promising diagnostic assay for the early stage of DENV infection. Most of the developed NS1 assays detect NS1 in DENV infected patients across four serotypes, but lack serotype specificity. In this study, panels of anti-NS1 Mabs to four individual DENV serotypes were generated and characterized for their serotype-specificity by various methods. Most of the assays used NS1 antigens from DENV-infected mosquito C6/36 cells, as this cell type is the most common and susceptible for DENV infection. However, we finally selected serotype-specific Mabs to develop a new serotyping-NS1-ELISA based on the results of the NS1 capture ELISA, as the selected NS1 in supernatant derived from DENV-infected mammalian PS cells which was used in the assay is assumed to be the most similar form as found in patient plasma.

The serotyping-NS1-ELISA is a modification of our previously established cross-reactive NS1 capture ELISA. The same flavivirus-reactive Mab 2E11 was used as a capture antibody, but the second cross-reactive detection antibody was replaced with four individual serotype-specific Mabs. The detection limit of serotyping-NS1-ELISA for each serotype can be as low as 1–15 ng/ml, compared with 50–160 ng/ml in our cross-reactive NS1 ELISA. This might be due to a higher avidity of serotype-specific Mabs than that of the cross-reactive one. In addition, the well-exposed binding epitope of serotype-specific Mabs may be located distinct from that of the cross-reactive one, which is present in all serotypes, thus minimizing any competitive binding to NS1 protein between both capture and detection Mabs in the developed assay. Additionally, no cross-reactivity to JEV NS1 by our serotyping-NS1-ELISA was detected.

Although the serotyping-NS1-ELISA have been shown to differentiate DENV serotypes with supernatant from DENV-infected cell culture, cross reactivity to other serotypes by some serotype-specific Mabs was observed when patients’ plasma was employed to evaluate the assay. This is not surprising, since the assay was developed based on NS1 antigens from DENV prototype strains. However, the serotyping-NS1-ELISA has been found compared to primary cases. This is not surprising, since the assay was developed based on NS1 antigens from DENV prototype strains. Despite the limitation and increase sensitivity of the assay. In addition, the majority of cases enrolled in this study suffered from a secondary infection (83 out of 85 cases), for which lower sensitivity of NS1 assay has been found compared to primary cases.

Focusing on those 65 positive serotyping-NS1-ELISA cases, serotype identification of DENV1, DENV3 and DENV4 was identical to those obtained by standard RT-PCR, and 82.4% was correct for DENV2. These results indicated the accuracy of the serotyping-NS1-ELISA for discriminating NS1 of different serotypes simultaneously. The DENV2 serotype-specific Mab should be modified to improve overall serotype-accuracy of the assay.

Table 2

<table>
<thead>
<tr>
<th>RT-PCR</th>
<th>Serotype accuracy (%)</th>
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<tbody>
<tr>
<td>DENV1</td>
<td>DENV2</td>
</tr>
<tr>
<td>DENV1</td>
<td>17</td>
</tr>
<tr>
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<td>3</td>
</tr>
<tr>
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</tr>
<tr>
<td>Neg</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
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% Sensitivity (overall) = [(17 + 17 + 15 + 16)/(23 + 22 + 20 + 20)] × 100 = (65/85) × 100 = 76.5%.
% Specificity = (90/90) × 100 = 100%.

5. Discussion

The detection of NS1 protein has recently become a promising diagnostic assay for the early stage of DENV infection. Most of the developed NS1 assays detect NS1 in DENV infected patients across four serotypes, but lack serotype specificity. In this study, panels of anti-NS1 Mabs to four individual DENV serotypes were generated and characterized for their serotype-specificity by various methods. Most of the assays used NS1 antigens from DENV-infected mosquito C6/36 cells, as this cell type is the most common and susceptible for DENV infection. However, we finally selected serotype-specific Mabs to develop a new serotyping-NS1-ELISA based on the results of the NS1 capture ELISA, as the selected NS1 in supernatant derived from DENV-infected mammalian PS cells which was used in the assay is assumed to be the most similar form as found in patient plasma.

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Although the serotyping-NS1-ELISA have been shown to differentiate DENV serotypes with supernatant from DENV-infected cell culture, cross reactivity to other serotypes by some serotype-specific Mabs was observed when patients’ plasma was employed to evaluate the assay. This is not surprising, since the assay was developed based on NS1 antigens from DENV prototype strains. Variation of DENV strains in infected humans may cause conformational differences of secreted NS1 in the bloodstream, which will alter the accessibility of those serotype-specific antibodies. Interpretation of DENV serotype of each tested specimen in this study thus relied on the serotype-specific Mabs that provide predominant reactivity to that specimen (determined by the highest T/N ratio obtained among four serotype-specific Mabs).

Evaluation of serotyping-NS1-ELISA in DENV acute phase and non-DENV plasmas indicated an overall sensitivity of 76.5% (65/85) and 100% specificity (90/90). This moderate sensitivity was expected for the NS1 ELISA, as the sensitivity of available commercial kits is in the range of 60–87%. Several possibilities could explain the NS1 negative samples, for example, (i) those samples were collected on the day that NS1 levels were low or none; as was supported by previous studies; (ii) NS1 negative samples, for example, (i) those samples were collected on the day that NS1 levels were low or none; as was supported by previous studies.

In conclusion, panels of anti-NS1 Mabs to four serotypes of DENV were generated and characterized in this study. The serotype-specific Mabs were selected to develop a serotyping-NS1-ELISA, the first dengue NS1 antigen assay that is able to identify four DENV serotypes simultaneously in acute patient plasmas. This assay could be an alternative choice for dengue diagnosis and massive epidemiological studies.
Conflict of interest
None.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2011.01.001.

References