Original Article

Nonstructural Protein 1 Antibody-Based Epitope-Blocking Enzyme-Linked Immunosorbent Assay to Differentiate Japanese Encephalitis Virus from Dengue Virus Infections in Humans

Eiji Konishi1,2,3* and Mayu Konishi1

1Division of Infectious Diseases, Department of International Health, Kobe University Graduate School of Health Sciences, and
2Division of Vaccinology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe 654-0142, Japan; and
BIKEN Endowed Department of Dengue Vaccine Development, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand**

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SUMMARY: Japanese encephalitis virus (JEV) and the four dengue viruses (DENV1–4) are co-distributed in Southeast and South Asia. Since JEV is antigenically cross-reactive with DENV1–4, the differentiation between these viruses using antibody assays may be difficult. Herein, we describe the development of an epitope-blocking enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody specific for the nonstructural protein 1 (NS1) of JEV (JEV-NS1) to differentiate antibodies against JEV from those against DENV1–4. Hyperimmune mouse sera against JEV-NS1 showed $\geq 60\%$ inhibition, whereas those against NS1 of DENV1–4 showed $<30\%$ inhibition. The present assay could therefore detect antibodies specific for JEV. For testing of human sera, a temporary cutoff value ($30.8\%$) was calculated the average and standard deviation obtained for sera of control humans negative for JEV antibodies. Human sera positive for antibodies to any of DENV1–4 NS1 but negative for antibodies to JEV-NS1 showed a lower percentage inhibition than the cutoff value. On the other hand, sera with JEV-NS1 antibody levels of $\geq 0.400$, as determined by the conventional ELISA (medially/strongly positive for JEV-NS1 antibodies), showed percentage inhibition greater than the cutoff. Although this blocking ELISA afforded false-negative results for most sera that were weakly positive for JEV-NS1 antibodies, it may be useful for investigating the seroepidemiology of JEV antibodies in dengue-endemic areas.

INTRODUCTION

Japanese encephalitis virus (JEV) causes a serious disease with a mortality rate of approximately 20% (1). The virus is a member of the genus *Flavivirus* and the family *Flaviviridae* and is distributed across Asia, where approximately 30,000–50,000 cases of JEV infection occur every year. In Southeast Asia and South Asia, four types of dengue viruses have been recognized; these viruses also belong to the genus *Flavivirus* (2). Since JEV antigenically cross-reacts with dengue viruses, conventional serological methods for antibody testing, such as a conventional enzyme-linked immunosorbent assay (ELISA), may afford false-positive results. This is sometimes the case even with plaque-reduction neutralization tests that are the most specific of the existing serological tests (3). Thus, seroepidemiological surveys of JEV antibodies in dengue endemic areas are often difficult. Therefore, development of an antibody assay method to differentiate JEV from dengue virus infections is required in areas where JEV is co-circulated with dengue viruses.

The epitope-blocking ELISA (hereinafter, blocking ELISA) is an antibody assay used to differentiate between infections with highly cross-reactive viruses. In flaviviruses, blocking ELISA systems have been reported or used to differentiate antibodies against West Nile virus from those against Murray Valley encephalitis virus (4), St. Louis encephalitis virus (5–12), and JEV (13). These four viruses belong to the JEV serocomplex, which is one of the eight serocomplexes established in the genus *Flavivirus* on the basis of antigenic cross-reactivity and genetic homology (14). These blocking ELISAs detect antibodies specific for nonstructural protein 1 (NS1). This is different from conventional antibody assays, such as the neutralization test and hemagglutination-inhibition test, which measure antibodies to the viral surface proteins and, predominantly, the envelope (E) protein. NS1 has been reported to have more virus-specific epitopes than E (15) and, thus, NS1-based assays are more suitable than E-based assays for the detection of specific antibodies and differentiation of flavivirus infections.
Dengue type 1–4 viruses (DENV1–4) constitute the dengue virus serocomplex (1). A previous report described a method for the differentiation between JEV and DENV1–4 antibodies (16), much earlier than the above mentioned reports on blocking ELISAs targeting flavivirus NS1 antibodies. Although the method involved the use of the E protein as an antigen, it was successfully used to differentiate these antibodies. Therefore, inter-serocomplex differentiation may be considered more easily achievable than intra-serocomplex differentiation. However, the blocking ELISA using NS1 antigen is believed to provide better results than assays using E antigen because of the differences in cross-reactivity.

The present study was aimed at developing and evaluating a blocking ELISA for the differentiation of JEV from DENV1–4 antibodies, using the NS1 antigen of JEV (JEV-NS1) and a monoclonal antibody specific for JEV-NS1. In this study, we also analyzed the sensitivity of the blocking ELISA to measure JEV-NS1 antibodies by comparing it with the conventional ELISA, since investigations on the sensitivity of the assay have been only preliminary hitherto, and thorough investigation of the sensitivity is critical for applying this method to seroepidemiology.

**MATERIALS AND METHODS**

**Cells:** Vero cells were cultivated in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum and 60 μg/ml kanamycin (17). CHO cells and C6/36 cells were grown in the same medium as that used for Vero cells, except for the addition of non-essential amino acids (17). All cells were cultivated in a humidified atmosphere containing 5% CO2 at 37°C for Vero and CHO cells and at 28°C for C6/36 cells.

**Viruses:** The strains used in this study were as follows: the Nakayama strain of JEV, Mochizuki strain of DENV1, New Guinea C strain of DENV2, H87 strain of DENV3, and H241 strain of DENV4 (18). Culture fluids harvested from C6/36 cells infected with each of these viruses were used for neutralization tests. Viruses in the form of a 10% homogenate of an infected suckling mouse brain in phosphate-buffered saline (PBS) containing 7.5% bovine serum albumin (BSA) were used for booster immunization of mice to induce hyperimmunity, and serum samples of these mice were collected as hyperimmune sera.

**Plasmids:** The cDNA encoding NS1 and its signal sequence for each of DENV1–4 was produced from viral RNA (purified from infected C6/36 cells) by reverse transcriptase-polymerase chain reaction (RT-PCR) using a Thermo Script RT-PCR System (Invitrogen, San Diego, Calif., USA) and the previously reported method for constructing plasmids expressing NS1 of JEV (19) and West Nile virus (13). Primers used for RT-PCR were designed using nucleotide sequences registered in GenBank (accession no. AB074760.1 for DENV1, AF038403.1 for DENV2, M93130.1 for DENV3, and AY947539.1 for DENV4). Antisense primers for the production of cDNAs included the C-terminal 6–8 codons of NS1, which were adjacent to the termination codon and a restriction enzyme site (Table 1). The sense primers included a BamHI site, an efficient eukaryotic initiation site (ACC; 20) and a start codon, followed by the 6–8 codons of the NS1 signal sequence (Table 1). Amplified cDNA was inserted into the pcDNA3 vector (Invitrogen) between the strong eukaryotic promoter derived from human cytomegalovirus and the polyadenylation signal derived from bovine growth hormone. The constructs for DENV1, DENV2, DENV3, and DENV4 were designated pcD1NS1, pcD2NS1, pcD3NS1, and pcD4NS1, respectively.

**Table 1. Antisense and sense primers used for construction of NS1-expressing plasmids**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Virus</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense</td>
<td>DENV1</td>
<td>CCGTCTAGATTTATGCAGAGACCATTGAC</td>
</tr>
<tr>
<td>DENV2</td>
<td>CCGCTCGAGTTAGGTGAGCAAGAGGATAC</td>
<td></td>
</tr>
<tr>
<td>DENV3</td>
<td>CCGGATATTTATGCTAGGCTAGGATTCAT</td>
<td></td>
</tr>
<tr>
<td>DENV4</td>
<td>CCGCTCGAGTTAGGTGAGCAAGAGGATAC</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>DENV1</td>
<td>GGCCGATACCACATGTCAGCATGCAATTC</td>
</tr>
<tr>
<td>DENV2</td>
<td>GGCCGATACCACATGTCAGCATGCAATTC</td>
<td></td>
</tr>
<tr>
<td>DENV3</td>
<td>GGCCGATACCACATGTCAGCATGCAATTC</td>
<td></td>
</tr>
<tr>
<td>DENV4</td>
<td>GGCCGATACCACATGTCAGCATGCAATTC</td>
<td></td>
</tr>
</tbody>
</table>

1) Sequences corresponding to the restriction enzyme site are underlined and the stop codon is italicized.

2) Sequences corresponding to the restriction enzyme site are underlined and the start codon and the Kozak sequence (20) are italicized.

**Table 2. Nucleotide differences between reported sequences and those for the NS1-expressing plasmids**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Position</th>
<th>Alteration in:</th>
<th>Nucleotide</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV2</td>
<td>2544</td>
<td>G to A</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>2735</td>
<td>A to G</td>
<td>Gln to Arg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3130</td>
<td>T to C</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENV3</td>
<td>2398</td>
<td>G to A</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>2537</td>
<td>G to C</td>
<td>Val to Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2660</td>
<td>G to A</td>
<td>Asp to Asn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2770</td>
<td>T to A</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2771</td>
<td>T to A</td>
<td>Leu to Lys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2772</td>
<td>T to A</td>
<td>Leu to Lys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2938</td>
<td>C to T</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENV4</td>
<td>2566</td>
<td>G to A</td>
<td>Glu to Lys</td>
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1) Nucleotide number based on the sequence registered in GenBank (accession no. AF038403.1 for DENV2, M93130.1 for DENV3, and AY947539.1 for DENV4).
pcD2NS1, pcD3NS1, and pcD4NS1, respectively. Proper insertion of the NS1 gene in all constructs was confirmed by sequencing. Although there were 1–7 nucleotide differences from the reported sequences for DENV2–4 that were accompanied mostly by amino acid substitutions (Table 2), the sequences found in the plasmids were identical to those in DENV1–4 that had been passaged through C6/36 cells in our laboratory. All plasmid DNAs were purified using a Qiagen endotoxin-free DNA purification kit (EndoFree Plasmid Mega Kit; Qiagen, Hilden, Germany) and used for the transfection of cells and immunization of mice.

Monoclonal antibody: A mouse monoclonal antibody specific for JEV-NS1 (JE-2D5: 21) was used, along with antibodies against DENV1–4 that included D1-4G2 (flavivirus group-crossreactive, purchased from American Type Culture Collection, Manassas, Va., USA) and D1-I-15C12 (DENV1-reactive, unpublished).

Antigen and immunoaffinity purification: NS1 antigen contained in the culture fluids of JEV-infected Vero cells was affinity-purified with a monoclonal JE-2D5 coupled to Sepharose 4B beads (NHS-activated Sepharose 4B Fast Flow; GE healthcare UK, Buckinghamshire, England), as described previously (21).

Rabbit and mouse serum samples: Hyperimmune rabbit serum (21) was obtained by repeated immunization of a rabbit with an NS1 antigen that was affinity-purified from the culture fluids of JEV-infected Vero cells with the Nakayama strain of JEV. A monoclonal antibody to JEV-NS1, JE-2D5, was used for the affinity purification and blocking ELISA. Normal rabbit serum obtained from the same rabbit prior to immunization. Mouse sera positive for antibodies to JEV-NS1 were obtained by infecting ICR mice (Japan SLC, Shizuoka, Japan) with JEV. To obtain sera positive for antibodies to JEV-NS1, mouse sera were affinity-purified from the culture fluids of Vero cells in our laboratory. All mouse sera were used in the blocking ELISA for differentiating JEV-NS1 from DENV-NS1 antibodies: The protocol established previously for the blocking ELISA used for differentiating West Nile virus from JEV antibodies (13) was modified for differentiating JEV-NS1 from DENV-NS1 antibodies. Microplates (Maxisorp: Nunc A/S, Roskilde, Denmark) were sensitized at 4°C overnight with affinity-purified JEV-NS1 prepared at a concentration of 5 ng/well in 0.1 M sodium carbonate buffer (pH 9.6) and blocked at 37°C for 1 h with ELISA diluent, which was composed of 0.05 M Tris-HCl (pH 8.0) containing 0.2% casein (Sigma-Aldrich Corp., St. Louis, Mo., USA), 0.05% Tween 20, 1 mM EDTA, and 0.15 M NaCl. Plates were incubated serially with test sera at 37°C for 3 h, JE-2D5 at a 1:1,000 dilution at 37°C for 1 h, alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa., USA) at a 1:4,000 dilution at 37°C for 1 h, and p-nitrophenyl phosphate at 1 mg/ml at 37°C for 10 min. Test sera were obtained from rabbits at dilutions of 1:20 to 1:640 and from humans and mice at dilutions of 1:5 to 1:80. Tests were conducted in duplicate. In this system, the test sera were incubated in parallel with the ELISA diluent, and the percent inhibition of monoclonal antibody binding was calculated from absorbances at 415 nm by the following formula: 100–100 × A/B, where A and B are the absorbance obtained at 415 nm by the following formula: 100–100 × A/B, where A and B are the absorbance obtained with the test sera and ELISA diluent, respectively. For testing mouse sera, biotinylated-JE-2D5 was used in place of JE-2D5, followed by incubation with alkaline phosphatase streptavidin conjugate (Vector Laboratories, Burlingame, Calif., USA) at a dilution of 1:500. Biotinylation of JE-2D5 was conducted using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, Ill., USA) according to the manufacturer’s instructions.

Conventional ELISA for measuring antibodies to JEV-NS1: The method followed in this study was the same as that described previously (22). Briefly, microplates were sensitized with affinity-purified JEV-NS1 antigens and blocked with the ELISA diluent, followed by serial incubation with the test sera, alkaline phosphatase-conjugated goat anti-human IgG, and p-nitrophenyl phosphate. To eliminate nonspecific reactions, absorbances obtained for the non-sensitized control wells were subtracted from those obtained for the antigen-sensitized wells. To minimize interplate variations, a constant positive control serum sample, prepared from serum samples of JE patients, at a dilution of 1:1,000 was included in every plate, and absorbances obtained with the test samples were adjusted with the
value for the positive control as 1.0. The adjusted absorbances were expressed as ELISA values. ELISA values of 0.185 or higher were determined as positive for NS1 antibodies.

**Sandwich ELISA for measuring JEV-NS1 antigens:**
The method followed has been described previously (17). Briefly, microplates sensitized with rabbit anti-JEV-NS1 hyperimmune serum were incubated serially with test samples, a monoclonal JE-2D5, alkaline phosphatase-conjugated goat anti-mouse IgG, and p-nitrophenyl phosphate. Antigen levels were calculated from absorbances obtained with the sample and a reference standard and expressed as the amount of NS1 in nanogram per milliliter. The reference standard was prepared with JEV-NS1 affinity-purified from JEV-infected Vero cells. The amount of purified JEV-NS1 was estimated by comparing with BSA samples in silver-stained gels.

**Immunostaining method for measuring DENV-NS1 antibodies:** The immunological staining method established previously for measuring JEV-NS1 antibodies in human sera (19) was used with some modification for measuring antibodies to the NS1 of DENV1–4. To obtain the antigen used in this method, CHO cells were transfected with 1 µg of pcD1NS1, pcD2NS1, pcD3NS1, or pcD4NS1, incubated for 24 h, and fixed. Before testing, cells were blocked with normal horse serum prepared in the ELISA diluent (see above). The cells were incubated with test specimens at a dilution of 1:50 for human sera or at serial 2-fold dilutions starting from 1:20 for mouse sera. Antigen-antibody reactions were detected by incubation with biotinylated anti-human or anti-mouse IgG (heavy and light chain-specific: Vector), the avidin-biotinylated peroxidase complex (ABC: Vector) reagent, and then the VIP substrate (Vector). Incubations with the former two were performed at room temperature for 30 min, while that with VIP was performed for 10 min. The reaction was determined to be positive when microscopic examination revealed differences in the stain intensity between cells expressing and not expressing NS1: immunostaining showed that under the transfection conditions of this study, approximately 40–60% of the cells expressed antigens. For mouse sera, the NS1 antibody titer was expressed as the maximum serum dilution yielding a positive reaction.

**Neutralization tests:** Neutralizing antibodies in sera were titrated using plaque-reduction assays, as described previously (18). Briefly, serum samples were incubated with each of the DENV1–4 in the presence of the complement. The antibody-virus mixture was titrated on Vero cells. Plaques were visualized by immunostaining using D1-I-15C12 or D1-4G2 monoclonal antibodies to detect DENV1 or DENV2–4 antigens, respectively. The neutralization titer was expressed as the maximum serum dilution yielding a 90% reduction in plaque number. Serum samples showing neutralizing antibody titers of 1:10 or higher were determined to be positive.

**RESULTS**

**Preliminary evaluation and optimization of the blocking ELISA using rabbit sera:** To determine whether the JE-2D5 antibody would be suitable for the blocking ELISA, we compared the inhibition percentages in hyperimmune rabbit sera against JEV and normal rabbit sera (Fig. 1A). Nearly 80% inhibition was observed at a 1:20 dilution of hyperimmune serum, and this percentage decreased gradually with an increase in the dilution factor. However, normal serum did not show more than 20% inhibition for dilutions in the range 1:20–1:640. This result indicated that the blocking ELISA using JE-2D5 can recognize JEV-specific antibodies in rabbit hyperimmune serum.

Optimal conditions for the blocking ELISA were determined using hyperimmune rabbit serum. To determine whether the JE-2D5 antibody would be suitable for the blocking ELISA, we compared the inhibition percentages in hyperimmune rabbit sera against JEV and normal rabbit sera (Fig. 1A). Nearly 80% inhibition was observed at a 1:20 dilution of hyperimmune serum, and this percentage decreased gradually with an increase in the dilution factor. However, normal serum did not show more than 20% inhibition for dilutions in the range 1:20–1:640. This result indicated that the blocking ELISA using JE-2D5 can recognize JEV-specific antibodies in rabbit hyperimmune serum.

Optimal conditions for the blocking ELISA were determined using hyperimmune rabbit serum. To determine the NS1 antigen concentration used for sensitization, varying concentrations from 12.5 to 100 ng/ml were tested without changing the other assay conditions described in Materials and Methods (Fig. 1B). Incubation with the ELISA diluent instead of hyperimmune

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**Fig. 1.** Preliminary evaluation and optimization of the blocking ELISA used to differentiate antibodies to JEV from those to DENV. (A) Dose-dependent percentage inhibition curves obtained with hyperimmune rabbit serum against NS1 of JEV (closed circle) and normal rabbit serum (open circle). Assay conditions were the same as described in Materials and Methods. Each datum represents an average obtained in two separate assays with SDs (indicated by bars). (B) Effect of antigen concentrations used for sensitization on absorbances obtained by the blocking ELISA. Absorbances were obtained using hyperimmune rabbit serum at a 1:40 dilution (open circle) or ELISA diluent (closed circle) in the incubation step for test sera with the other assay protocols the same as described in Materials and Methods.
rabbit serum represents the direct reactivity of a monoclonal JE-2D5 with the antigen. Absorbances increased with an increase in the antigen concentration and leveled off at 50 ng/ml in both conditions; thus, this concentration was determined as the optimal value for sensitization. The other optimal conditions were determined in a similar way (data not shown) to establish the assay protocol described in Materials and Methods.

**Evaluation of the blocking ELISA for differentiating the JEV antibody from DENV1–4 antibodies using mouse sera:** To assess the effects of DENV-NS1 antibody cross-reactivity on the results of the blocking ELISA for the specific detection of JEV antibodies, the percentage inhibition was measured using sera from groups of six mice hyperimmune to the NS1 of each of the DENV1–4 and compared with those obtained with four sera of mice hyperimmune to JEV-NS1 and three sera of non-immune mice (Fig. 2A). Sera were used at a 1:5 dilution for all groups of mice. While sera of mice hyperimmune to JEV-NS1 showed more than 60% inhibition, those of mice hyperimmune to NS1 of any of the DENV1–4 showed only less than 30% inhibition. Inhibition obtained with sera of non-immune mice was lower than 5%.

To further investigate the ability of the blocking ELISA to differentiate the JEV antibody from DENV1–4 antibodies, sera with the highest percentage inhibition were selected from each group and examined for dose-dependent percentage inhibition (Fig. 2B). Approximately 80% inhibition was observed using a 1:5 dilution of sera of JEV-hyperimmune mice, and this value decreased gradually to nearly 30% for dilutions in the range of 1:10–1:80. A similar tendency of a decrease in the percentage inhibition with an increase in the dilution factor was also noted in the case of sera from other groups. These results indicated that the blocking ELISA was not affected significantly by the cross-reactivity of antibodies to DENV-NS1 and, therefore, did not appear to produce false-positive results.

**Cutoff value for differentiating positive from negative results in human sera:** Sera from 20 healthy American volunteers without any detectable neutralizing antibodies against JEV were used to measure the percentage inhibition of the blocking ELISA (Fig. 3). The mean value was 9.8% with a standard deviation (SD) of 7.0%. The cutoff for differentiating positive from negative results was established tentatively at 30.8% and was calculated as mean + 3 × SD. Serum samples from two JE patients were used as the positive serum samples of this assay, with values for percentage inhibition higher than the cutoff value; these samples were used at a dilution of 1:25 (which is 5-fold higher than that in the protocol) (Fig. 3).

**No detection of cross-reactivity with antibodies to NS1 of DENV1–4 in human sera:** To evaluate the cross-reactivity of the blocking ELISA against antibodies to NS1 of DENV1–4 in humans, we used serum samples of
Fig. 4. Comparison of the blocking and conventional ELISAs using 69 human sera. Sera were used at a dilution of 1:5 for the blocking ELISA and 1:100 for the conventional ELISA. The horizontal and vertical broken lines indicate cutoff values differentiating positive from negative samples in the blocking ELISA (30.8%) and conventional ELISA (0.185), respectively. The dotted line indicates an ELISA value of 0.400 in the conventional ELISA. Asterisks with numerals indicate serum samples used for the experiment shown in Fig. 5.

16 Filipinos who tested positive for DENV1–4 NS1 antibodies but negative for JEV-NS1 antibodies. These samples were selected from 204 samples screened initially for neutralizing antibodies and then for NS1 antibodies to DENV1–4 by the immunostaining method. Serum samples containing relatively high levels of DENV1–4 NS1 antibodies were selected and used with a serum dilution factor of 1:50 (data not shown). The 1:50 dilution factor was 5-fold higher than the cutoff value (1:10) used for differentiating positive from negative results in the immunostaining method established previously for detecting JEV-NS1 (19). The absence of antibodies to JEV-NS1 was confirmed by the conventional ELISA (data not shown).

The results of the blocking ELISA using these 16 serum samples are shown in Fig. 3. These sera were tested at a dilution of 1:5. All sera showed percentage inhibition lower than the cutoff value (30.8%), indicating that the blocking ELISA failed to react with the NS1 antibodies of the DENV1–4 in human sera.

Sensitivity of the blocking ELISA: To evaluate the sensitivity of the blocking ELISA, percentage inhibition values for the blocking ELISA were compared with the NS1 antibody levels obtained using the conventional ELISA (Fig. 4). For this comparison, 69 human sera were selected from those collected during national JE surveillance program (25), such that two-thirds of the population was positive in the conventional ELISA. Of these samples, 26 (38%) were positive using the blocking ELISA when evaluated with the cutoff value of 30.8%; however, 46 (67%) of the samples were positive in the conventional ELISA when evaluated with the cutoff value of 0.185. A quantitative comparison indicated a correlation coefficient of 0.761 (P < 0.001), while a qualitative comparison indicated an agreement of 71% with the cutoff value of 30.8% inhibition for the blocking ELISA and 0.185 in the conventional ELISA. Of the 32 samples with NS1 antibody levels of 0.185–0.400, 20 (62.5%) were negative and 12 (37.5%) were positive using the blocking ELISA. However, 23 samples testing negative using the conventional ELISA also tested negative in the blocking ELISA, while 14 samples with NS1 antibody levels of ≥0.400 tested positive in the blocking ELISA. These results indicated that although the blocking ELISA was less sensitive than the conventional ELISA, samples with NS1 antibody levels of ≥0.400 were consistently positive when tested using the blocking ELISA.

Dose-dependent antibody-response curves were obtained in the case of four sera that showed the highest percentage inhibition in the experiment shown in Fig. 4. As shown in Fig. 5, percentage inhibition decreased with increase in the serum dilution factor. Although one serum sample maintained approximately 80% inhibition even at a 1:80 dilution, three of the four sera showed a rapid decrease within a dilution range of 1:10–1:40. At a 1:80 dilution, all the three sera showed <40% inhibition. This result indicated that a serum dilution of 1:5 may provide the highest sensitivity when testing for the dilution range of 1:5–1:80 for the detection of JEV-specific antibody using the blocking ELISA.

DISCUSSION

The blocking ELISA is an antibody assay based on the binding of a monoclonal antibody specific for an antigen, which is inhibited by prior incubation with a test specimen containing the same epitope-specific antibody induced in infected hosts. Because of the high antigenic similarities among flaviviruses, cross-reactive antibodies, in addition to specific ones, are induced in infected hosts. Thus, the cross-reactivity of flaviviruses complicates the serodiagnosis of the viruses in endemic areas where two or more flaviviruses are co-circulated and sequential infections with different flaviviruses are frequent. Therefore, the identification of the infective flavivirus is difficult using most of the existing serological tests (3).

Conventional ELISA provides quantitative results by measuring antibody molecules bound to the antigen sensitized on the solid phase. However, this method measures both specific and cross-reactive antibodies and is,
therefore, inappropriate for differentiating flavivirus infections. In contrast, the blocking ELISA, although not suited for quantitative measurement of antibody levels, detects only specific antibodies within the test specimens. This is, however, dependent on the specific epitope being located far enough from the cross-reactive epitopes on the antigen molecule so that the binding of the specific monoclonal antibody to the antigen is not hampered. In the present study, the binding of a monoclonal JE-2D5 to the JEV-NS1 antigen was not inhibited by the presence of antibodies to the NS1 of any of the DENV-1–4, in both mice and humans.

The present study indicated that an NS1-based blocking ELISA can differentiate JEV from DENV-1–4 infections. Sera containing antibodies to JEV exhibited high percentage of inhibition, in contrast to the low level of inhibition obtained in mouse and human sera containing antibodies to any of DENV-1–4. In particular, mice demonstrating no detectable cross-reactivity of antibodies to the NS1 of DENV-1–4 were hyperimmune to each of the NS1 of the DENV-1–4 and afforded NS1 antibody titers of >1:320 using immunostaining. Thus, the present blocking ELISA can detect JEV-specific antibodies without any detectable effect of antibodies to DENV-1–4.

In addition to the presence of more virus-specific epitopes on NS1 than E (15), the use of NS1 for blocking ELISAs has another advantage. Since inactivated purified JE vaccine does not contain NS1, the blocking ELISA targeting NS1 antibodies should, theoretically, not be affected by vaccination history. Currently, the inactivated vaccine has been introduced partially in some Southeast Asian countries. The differentiation of JEV from DENV-1–4 antibodies in these areas can be achieved by targeting NS1 and not E.

Although the present blocking ELISA was shown to be specific, some improvements in sensitivity would be needed: a better monoclonal antibody can be identified by a larger population of the panel. However, the blocking ELISA seems to fundamentally, have a lower sensitivity than the conventional ELISA. Since the specific antibody detection in the blocking ELISA is based on the difference between absorbances obtained with and without test specimens, experimental variations in both absorbance values can reduce the assay sensitivity. Specifically, the cutoff value established to differentiate positive from negative samples should be high enough to avoid false-positive results produced by experimental variation. This may result in an increased number of false negatives when the sample contains a low level of specific antibodies. In the present study, 62.5% of the samples with NS1 antibody levels between 0.185 and 0.400 (weakly positive) in the conventional ELISA were determined to be negative by the blocking ELISA, which suggests that the sensitivity of this assay is lower than that of the conventional one. For seroepidemiological surveys, the actual positivity may be approximately 2-fold higher than that obtained using the blocking ELISA, as estimated by the blocking ELISA (38%) and the conventional ELISA (67%) in the present study.

In conclusion, a blocking ELISA for differentiating JEV from DENV-1–4 infections was developed. Although this assay provides false-negative results for samples weakly positive for antibodies to JEV-NS1, it can detect JEV-specific antibodies without false-positives due to cross-reaction with antibodies to DENV-1–4. This assay may, therefore, be useful in epidemiological surveys of JEV antibodies in areas where DENV-1–4 coexist with JEV.

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Conflict of interest None to declare.

REFERENCES


