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ORIGINAL ARTICLE

Improving dengue viral antigens detection in dengue patient serum specimens using a low pH glycine buffer treatment

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Abstract *Background/Purposes:* Early diagnosis of dengue virus (DENV) infection to monitor the potential progression to hemorrhagic fever can influence the timely management of dengue-associated severe illness. Nonstructural protein 1 (NS1) antigen detection in acute serum specimens has been widely accepted as an early diagnostic assay for dengue infection; however, lower sensitivity of the NS1 antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA) in secondary dengue viral infection has been reported.
Methods: In this study, we developed two forms of Ag-ELISA capable of detecting E-Ag containing virion and virus-like particles, and secreted NS1 (sNS1) antigens, respectively. The temporal kinetics of viral RNA, sNS1, and E-Ag were evaluated based on the *in vitro* infection experiment. Meanwhile, a panel of 62 DENV-2 infected patients' sera was tested.
Results: The sensitivity was 3.042 ng/mL and 3.840 ng/mL for sNS1 and E, respectively. The temporal kinetics of the appearance of viral RNA, E, NS1, and infectious virus in virus-infected tissue culture media suggested that viral RNAs and NS1 antigens could be detected earlier than E-Ag and infectious virus. Furthermore, a panel of 62 sera from patients infected by DENV Serotype 2 was tested. Treating clinical specimens with the dissociation buffer increased the detectable level of E from 13% to 92% and NS1 antigens from 40% to 85%.

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Conclusion: Inclusion of a low-pH glycine buffer treatment step in the commercially available Ag-ELISA is crucial for clinical diagnosis and E-containing viral particles could be a valuable target for acute DENV diagnosis, similar to NS1 detection.

Introduction

Dengue virus (DENV) serotypes 1—4 (DENV-1, DENV-2, DENV-3, and DENV-4) are the most widespread mosquito-borne viral pathogens worldwide in humans, accounting for >390 million infections/y.[1] Currently, there is no approved human vaccine or antiviral treatment available for DENV infection. Thus, an accurate and early diagnosis of DENV infection is essential for implementing the early clinical management critical to reduce the morbidity and mortality of dengue hemorrhagic fever and dengue shock syndrome.

DENV belongs to the genus *Flavivirus* of the family Flaviviridae. It is an enveloped virus containing single-stranded, positive-sense RNA, which encodes three structural proteins: capsid (C), premembrane (prM), and envelope (E), and seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.[2] Viral RNA synthesis can be detected 3—6 hours after infection, and progeny virions are released from infected mammalian cells starting approximately 10—12 hours postinfection.[3] Maximal extracellular virus titers are not usually observed until approximately 24 hours postinfection. In DENV-infected mosquitos[4] or mammalian cell culture, a soluble hexameric form of NS1 protein is secreted into the cultured supernatant in a glycosylation-dependent fashion and in free-form association with virion particles,[5,6] and the levels of secreted NS1 (sNS1) correlate with infectious virus titers.[7] The circulating sNS1 during the acute phase of the disease is higher in patients' serum specimens experiencing primary rather than secondary infection.[8,9]

Recent studies suggested the lower sensitivity of the NS1 antigen-capture enzyme-linked immunosorbent assay (NS1-ELISA) in secondary infection compared with primary infection is due to the early formation of NS1-antibody complex impending the plate-bound capturing and/or detecting antibodies to access antibody-targeted epitope(s).[10,11] In this study, an optimized NS1- and E-specific murine monoclonal antibody (mAb) based NS1- and E-ELISA, respectively were developed to focus on three specific aims: (1) to detect and quantify the levels of DENV E and sNS1 in virus-infected tissue culture fluids, and utilize this assay to examine an extensive number of well-characterized dengue patient serum samples; (2) to investigate whether E-antibody complexes reduced the sensitivity of E-ELISA similar to the phenomena observed for NS1-ELISA in human serum as suggested in the previous study[12]; and (3) to evaluate if serum specimens pretreated with dissociation buffer improved the sensitivity of NS1- and E-ELISA detection in DENV-infected patient serum specimens.

Methods

Virus, cells, and antibodies

The DENV-2 virus (strain 16681) used in this study was propagated in *Aedes albopictus* clone C6/36 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown at 28°C in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and nonessential amino acids (Gibco BRL, Grand Island, NY, USA). Vero cells for viral infection and plaque assay and COS-1 cells (ATCC CRL 1650) for E production were grown at 37°C with 5% CO_2 in DMEM supplemented with 10% heat-inactivated FBS. Virus stocks were used to infect 80% confluent Vero cell monolayers in DMEM medium supplemented with 2% FBS and incubated at 37°C until the day designated, at which point the supernatants and cell monolayers were harvested.

Flavivirus group-cross reactive, E-specific DB42-3, and NS1-specific DB20-6 monoclonal antibodies (mAbs) were generated and purified as previously described.[13,14] These two mAbs were chosen based upon their strengths of binding to E and NS1 and their cross-reactivity with other flaviviruses. Mouse hyperimmune ascitic fluid (MHIAF) against DENV-2 strain New Guinea C was provided by the US Centers for Disease Control and Prevention (US-CDC). Polyclonal rabbit anti-DENV-2 E and NS1 were generated by intramuscular injection of E or NS1 expressed DNA plasmids as described in the plasmid construction section below and were kindly provided by one of the authors, Dr. GJ Chang's laboratory at the US-CDC.

Plasmid construction, protein expression, and purification

The pcDNA3-based vaccine plasmids encoding the prM/E or NS1 genes of the DENV-2 strain 16681, named pCBD2E and pCBD2NS1, respectively, were constructed as described previously.[15,16] The prM/E containing virus-like particles (VLPs) and NS1 were expressed by COS-1 cells electroporated with pCBD2E and pCBD2NS1 using the protocol described previously.[17,18] Supernatants were harvested and clarified from media of electroporated cells 4—5 days post-transformation. Media containing VLPs were concentrated by polyethylene glycol precipitation (10% PEG), and media containing NS1 were concentrated by ultrafiltration using a 10-kD pore size centricon (Millipore, Billerica, MA, USA); finally, concentrated VLPs and NS1 antigens were further purified using Cellufine Sulfate columns (Chisso, Tokyo, Japan) as previously suggested.[19] Columns were washed with

10 mL adsorption buffer (0.01M sodium phosphate, 0.1M NaCl, pH 7.5) and then 0.4M and 0.6M NaCl in adsorption buffer were used to elute NS1 and VLPs, respectively. The purity of DENV-2 VLPs and NS1 was determined using western blot and ELISA using MHIAF. Protein concentration was measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

E- and NS1-specific ELISA

Microtitration plates (Nunc Maxisorp; Nunclon Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μL/well of the E- or NS1-specific mAb at a concentration of 27 ng/mL in phosphate buffered saline (PBS). Wells were washed with PBS-Tween buffer (PBS, 0.05% Tween-20) and blocked with 200 μL/well of blocking buffer (PBS, 0.05% Tween-20, 3% skim-milk) at room temperature for 2 hours. Samples, VLPs, or NS1 diluted serially were added to wells (50 μL/well) and incubated for 2 hours at room temperature. The wells were washed again and incubated for 1 hour at 37°C with 50 μL/well of anti-E or anti-NS1 rabbit antiserum (diluted 1:500 in blocking buffer). The wells were washed again and incubated for 1 hour at 37°C with 50 μL/well of peroxidase-conjugated goat anti-rabbit antiserum (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:5000 in blocking buffer. After three further washes, each well received 50 μL of a freshly prepared 3,3′,5,5′-tetramethylbenzidine solution (TMB; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The color reaction was stopped after 10 minutes with 50 μL/well of 2N sulfuric acid, and optical density (OD) was measured at 450 nm. Bovine serum albumin (BSA, 1%) and mock infection culture supernatant were included in each plate as negative controls for constructing E- or NS1-specific standard curves and for the *in vitro* reconstitutive assay. Five normal healthy human serum samples obtained from local residents were used as the normal serum to determine the background cutoff ELISA OD value when the assay was used to determine the presence of E or NS1 in dengue patient serum specimens. Absorbance values were corrected by subtracting the mean OD values of the negative controls. A sample is considered positive if its corrected OD value is two times greater than or equal to the average OD of the negative control (P/N ratio $\geq$2).

Quantification of free and antibody-bound E and NS1

To determine the temporal kinetics of E and NS1 protein expression in virus infected cell cultures, Vero cells were plated in six well plates at a cell density of 6×10^5 cells/well with 2 mL of DMEM growth medium. Cells at 80% confluence were infected with DENV-2 strain 16681 in a given multiplicity of infection (MOI). Cell supernatants from infected and uninfected cells were collected at designated times postinfection, briefly centrifuged, and stored at −70°C prior to analysis. The virus culture supernatants harvested at different time points were diluted in the washing buffer; the human specimens were initially performed at 1:10 dilution, and threefold serial dilutions were carried out and tested. The concentration of E and NS1 present was extrapolated based on the OD_{450} response curve of purified E and NS1 after subtracting the OD_{450} of the noninfected cell supernatants.

The immune complexes formed *in vitro* were performed via incubation of purified VLP and sNS1 with designated dilutions of DENV-2 MHIAF at 4°C overnight. Antibody complexes formed *in vitro* were initially diluted 1:2 in dissociation buffer [1.5M glycine (pH 2.8) in PBS with 1% BSA]. Immune complexes were dissociated for 1 hour at 37°C, and the reaction was stopped by the addition of one volume of neutralization buffer [1.5M Tris-HCl (pH 9.7)]. Finally, 2.5 volumes of PBS were added to achieve an end dilution of 1:10 for the sample. The same protocol was applied to release DENV-2 E and NS1 from immune complexes in dengue patients' plasma samples for detection. All samples were assayed in duplicate (sNS1 and E) in a blinded fashion and were quantified on the linear portion of the standard curves.

Human serum panel

A total of 62 serum samples from DENV-2 infected patients during an epidemic in Taiwan between 2002 and 2003 were used in this study. Diagnosis of DENV infection was based on immunoglobulin (Ig) M antibody-capture ELISA, reverse-transcriptase polymerase chain reaction (RT-PCR), or virus isolation in cell cultures as previously described.[20,21] These serum specimens were collected between Day 1 and Day 13 after onset of fever.

Ethics statement

The use of samples was approved by the National Taiwan University Institutional Review Board (NTUH-REC No. 200903086R), Kaohsiung Veteran General Hospital Review Board (VGHK98-CT8-10), and Kaohsiung Yuang's General Hospital (IRB #20090430B). Written informed consent was obtained from all participants and all samples were coded for anonymity. All of the coauthors and their affiliations in this study followed the protocol stated in Institutional review board (IRB) accepted from the other institutes.

Statistical analysis

The Student *t* test was used for comparisons between normally distributed continuous variables and the Mann-Whitney *U* test was used for comparisons between continuous variables not normally distributed. χ^2 analysis was used for comparisons among proportional data. The Graphpad prism 4.0 software package (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analyses.

Results

Sensitivity of E- and NS1-ELISA

The results of the western blotting of E and NS1 are shown in Figure 1A and B, respectively, and the total proteins of the eluents were further measured as shown in Figure 1C. It was consistent with the result from the western blotting, which suggested that the Cellufine Sulfate columns

successfully purified E and NS1 proteins. To further confirm the results, equal amounts of total proteins from each preparation were applied to E- and NS1-ELISA and the OD_{450} reading in the eluents increased as shown in Figure 1D. To establish the sensitive Ag-ELISA for measuring levels of E and NS1 during dengue viral infection, purified DENV-2 NS1 and E containing VLPs with serial dilutions were used to construct the OD_{450} response curve. As shown in Figure 1E, the minimum concentration of NS1 and E that could generate P/N $\geq$2 by this assay was estimated to be at 3.056 ng/mL and 2.978 ng/mL, respectively.

Temporal kinetics of appearance of viral RNA, E, NS1, and infectious virus in virus-infected tissue culture media

To test whether our NS1- and E-ELISAs can reliably detect sNS1 and secreted E (either in the form of virions or VLPs) from DENV-2 infected mammalian cells, Vero cells were infected with different MOI and harvested culture supernatants at different time points postinfection to assay the presence of NS1, E, viral RNA, and infectious virus. NS1

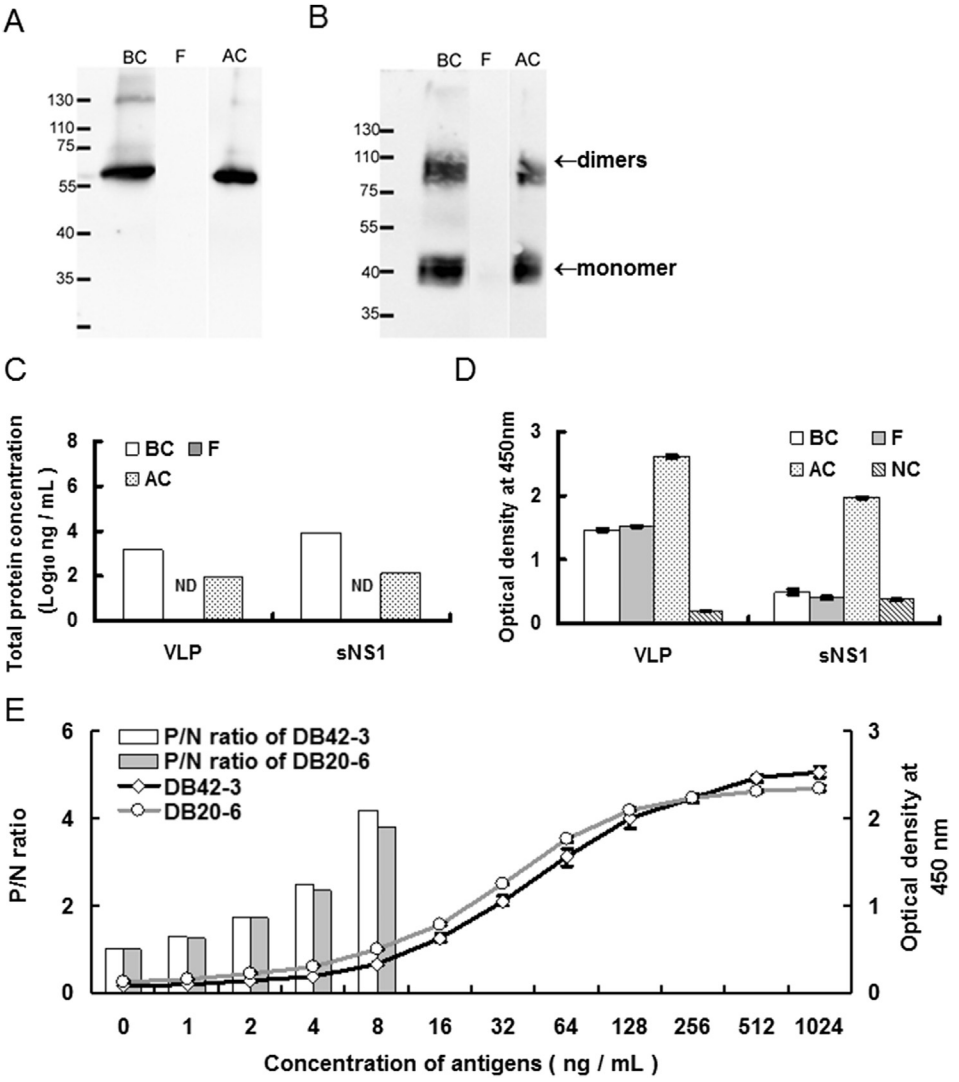

Figure 1. Characterization of native secreted nonstructural protein 1 (sNS1) and virus-like particles (VLP) of dengue virus (DENV)-2 protein standard. Tissue culture medium from cells electroporated with the E- and NS1-encoding plasmids were concentrated and then applied to Cellufine Sulfate columns. Equal volumes (10 μL of each fraction) of VLP and sNS1 concentrates before column purification (BC), flow-through buffer (F), and eluted antigens (AC) were separated using 10% denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels of purified (A) VLP and (B) sNS1 were electroblotted onto nitrocellulose membranes and immunostained with DENV-2 mouse hyperimmune ascitic fluid (MHIAF) as shown in and (B). Optical density (OD) readings were shown from equal volume (50 μL of each) of BC, F, and AC of purified VLP and sNS1 measured using (C) Bradford total protein assay and (D) equal concentrations (100 ng of total protein) carried out with E- or sNS1-Ag capture ELISAs. Wash buffer was used as negative control (NC). Serial dilutions of purified sNS1 and VLP antigens (E) were tested against the reactivity of monoclonal antibodies DB42-3 and DB20-6 (in concentration of 27 ng/mL). The cutoff value for positive samples was decided to be a sample OD reading two times greater than the average OD of the negative control (referred to as P/N ratio $\geq$ 2). Graphs represent the results from two independent experiments and each was performed with duplicate samples.

could be detected as early as 16 hours, 14 hours, and 4 hours postinfection at MOI of 0.01, 0.1, and 1, respectively. NS1 drastically accumulated at the first 20 hours of infection and gradually increased until 120 hours postinfection, the last collection point (Figure 2A). The detection of E was delayed and could be detected at 36 hours, 24 hours, and 16 hours postinfection at an MOI of 0.01, 0.1, and 1, respectively (Figure 2B). The pattern of detecting E and detecting viral RNA or infectious viruses was similar. Viral RNAs and NS1 antigens were detected a few hours earlier than E. Viral RNA could be detected at 22 hours, 16 hours, and 6 hours postinfection at an MOI of 0.01, 0.1, and 1, respectively. However, the viral RNA copy numbers peaked at 4 days, 3 days, and 2 days postinfection at an MOI of 0.01, 0.1, and 1, respectively, before beginning to decline (Figure 2C). Consistently, the earliest time points to detect the infectious virus titer were similar to E detection (Figure 2D).

Antibody-antigen complexes influencing E and NS1 detection

Previous studies suggest that decreased detection of NS1 antigens in plasma is correlated with the production of anti-NS1 IgM or IgG antibodies.[12,22] Based on these observations, it was speculated that the presence of anti-E antibodies would similarly decrease the sensitivity of E-ELISA in detecting levels of E-Ag in plasma. The constant concentration (256 ng) of purified VLP and NS1 was premixed with various dilutions of MHIAF to test if preformed E and NS1 antibody-complex reduced the sensitivities of E- and NS1-ELISA. As expected, E-antibody immune complexes markedly reduced the sensitivity of the E-ELISA in a dose-dependent relationship (Figure 3A). Similar results were observed for NS1-antibody complexes (Figure 3B). Then, the E- and NS1-antibody complexes were treated with dissociation buffer or neutral buffer and Ag-ELISA performed after respective treatment. Detection of E was enhanced when immune complexes were treated with low pH dissociation buffer relative to those treated at neutral pH at 37°C for 1 hour as shown by dot blot assay (Figure 3C and D).

Dengue patient sera using Ag-ELISA with or without dissociation buffer treatment

A panel of 62 DENV-2 infected patient serum specimens was examined using our NS1- and E-ELISA. Specimens were assayed for the presence of NS1 and E without pretreating serum with the dissociation buffer. NS1 antigen was detected from Day 1 to Day 10 after onset of fever, peaking during Day 4 with 80% of the samples detected positive (Figure 4A). A significant decline in circulating NS1 was observed after Day 7, although the level of NS1 was still above the detection limit of our NS1-ELISA. Conversely, E was detected in our serum collection only during Days 4—9 and the detectable E was much lower than that of NS1 in

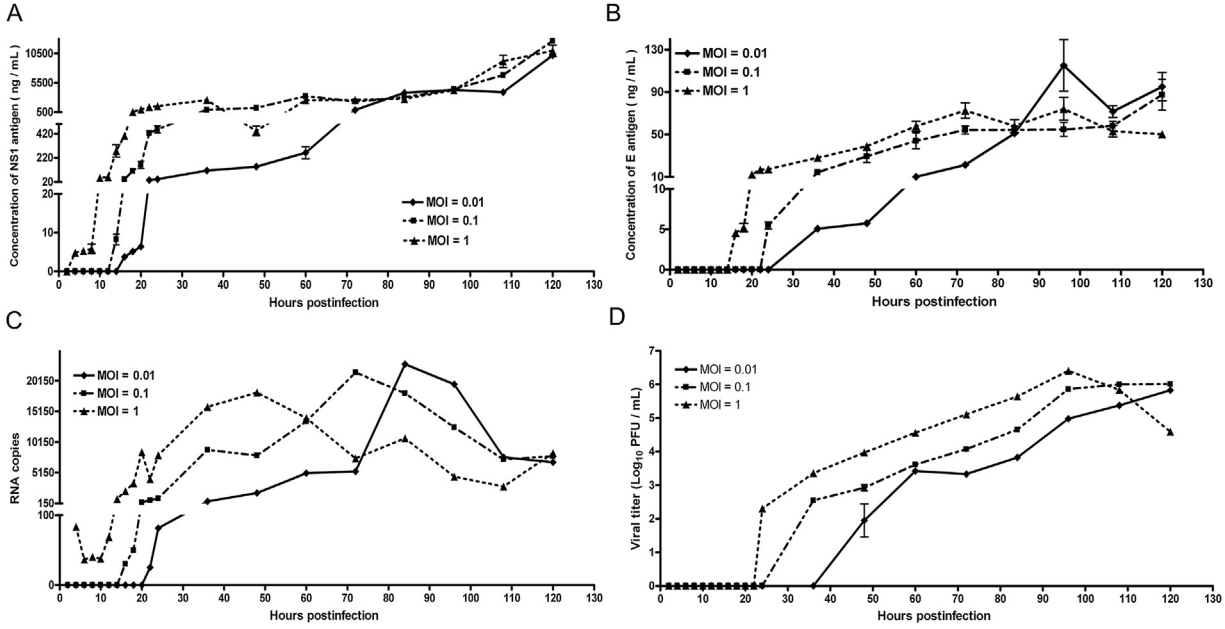

Figure 2. Detection of secreted nonstructural protein 1 (sNS1), E-antigen, RNA copies, and infectious virus from dengue virus (DENV)-2 infected Vero cells. Vero cells were infected with DENV-2 strain 16681 at a multiplicity of infection (MOI) of 0.01, 0.1, and 1. Culture supernatants from infected or uninfected cells were collected at designated times postinfection, and measured using (A) sNS1-antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA) and (B) E-Ag-ELISA. The concentrations were calculated after comparison with a standard curve derived on the same ELISA plate using the purified virus-like particles (VLP) and sNS1 as standard antigens. The graph shows the average results of three independent experiments performed in duplicate. In parallel, the infectious virus in culture fluids was measured using (C) real-time reverse-transcriptase polymer chain reaction (RT-PCR) and (D) the standard plaque assay method. The standard deviations for sNS1/E-Ag concentrations and the infectious virus titers at each time point are indicated.

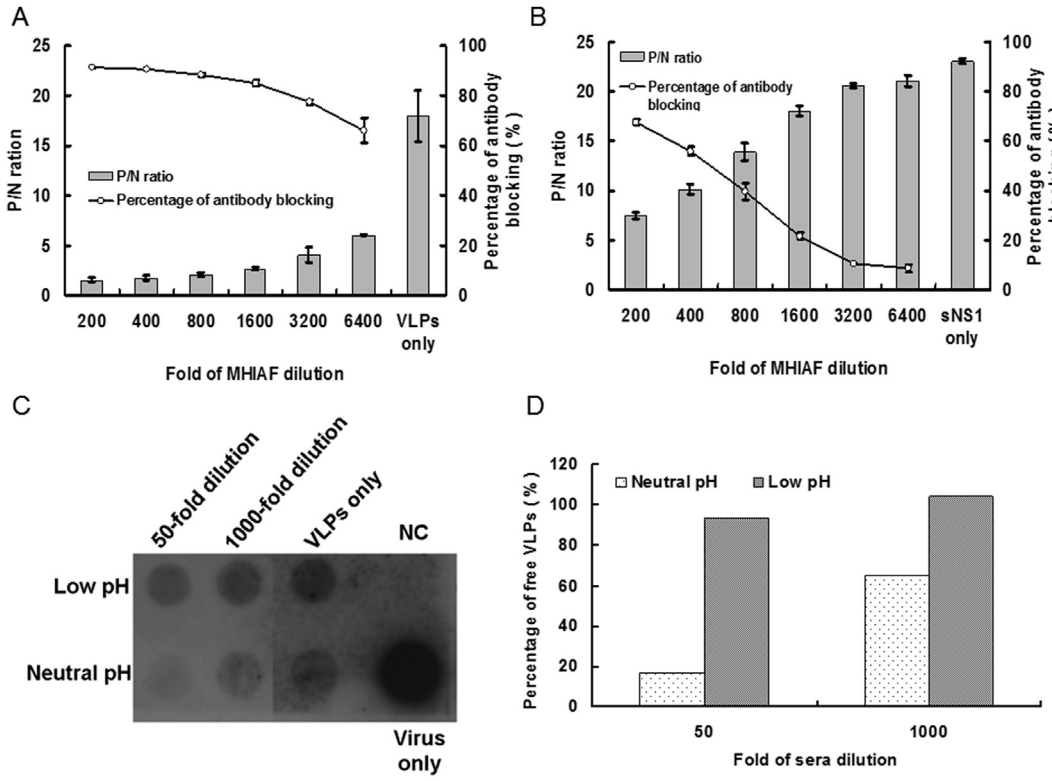

Figure 3. Formation and dissociation of antigen-antibody immune complexes. The immune complexes were formed via incubation of purified virus-like particles (VLP) and secreted nonstructural protein 1 (sNS1) with designated dilutions of dengue virus (DENV)-2 mouse hyperimmune ascitic fluid (MHIAF) at 4°C overnight and detected using (A) E-antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA) or (B) sNS1-Ag-ELISA. Antibody complexes formed *in vitro* were initially diluted 1:2 in dissociation buffer [1.5M glycine (pH 2.8) in PBS with 1% bovine serum albumin (BSA)]. Immune complexes were dissociated for 1 hour at 37°C, and the reaction was stopped by the addition of 1 volume of neutralization buffer [1.5M Tris-HCl (pH 9.7)]. Finally, 2.5 volumes of PBS were added to achieve an end dilution of 1:10 for the sample. Equal amounts were added to nitrocellulose membrane and detected using (C) dot blot assay, in which DB42-3 was used for detection as the immune complexes were formed using anti-envelope rabbit serum. The densities of the antigens detected were quantified using Bio-1D software (SIM International Group, Newark, Delaware, USA) and the concentrations of each density were derived from the density based on using the VLP only (256 ng) as standard. (D) The percentages of E-Ag detected by DB42-3 were calculated using the following formula: 100*(ng of immune complexes/ng of VLP only). Error bars indicate standard deviations. Except NC and PC, each blot was 256 ng/mL. NC = negative control, blocking buffer; PC = positive control, DENV-2 virus.

circulation. The free (antibody unbound) E was never detected beyond Day 9 from our serum collection.

Next, the entire serum collection was pretreated with dissociation buffer and the post-treated specimens were subjected to E- and NS1-ELISA. Treating specimens with the dissociation buffer increased the detectable level of E in serum samples collected between Day 1 and Day 13 (Figure 4B), and the antigen levels detected also increased approximately threefold. Likewise, the level of detectable NS1 increased significantly, and the range of detection was extended to Day 13. The detection rate of NS1 antigens increased from 40% to 85% before and after dissociation buffer treatment, respectively. Similarly, E-Ag detection rate also increased from 13% to 92%.

Discussion

E- and NS1-specific Ag-ELISAs were developed using flavivirus group-cross reactive mAbs as the detectors, respectively, in this study. Using the positive OD_{450}/negative $OD_{450} \geq 2$ as the cutoff P/N ratio, as low as 2.978 ng/mL and 3.056 ng/mL of the purified E containing VLPs and NS1 proteins, respectively, were detected. This E-ELISA could detect E from dengue-infected tissue culture supernatants and from acute dengue patient serum specimens, similar to NS1-ELISA. In addition to NS1 and viral RNA detection, the correlation between E, NS1, and viral RNA in dengue patient serum specimens should provide further insights for the improvement of dengue diagnosis in the future. Using NS1- and E-specific mAbs to recognize flavivirus group cross-reactive epitopes, this assay may not only be suitable for DENV-2 detection but could potentially be expanded for detection of the other three serotypes of DENV.

In this study, the *ex vivo* tissue culture investigation into the kinetics related to viral replication suggests that NS1 viral protein is synthesized and released first, followed by RNA replication, and virion assembly and release. These findings contradict those of the previous study of West Nile virus, which indicated that the release of NS1 into the

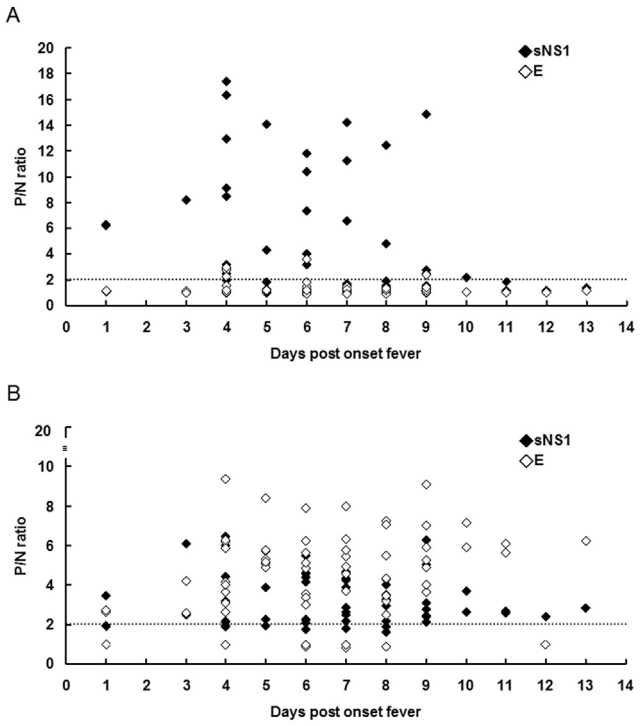

Figure 4. Comparison of detection of secreted nonstructural protein 1 (sNS1) and E-antigen levels in dengue virus (DENV)-2 patient plasma samples with and without dissociation of immune complexes. A panel of 62 serum samples collected from DENV-2-infected patients at different time points after fever onset during an epidemic in Taiwan between 2002 and 2003 was tested for the presence of E and sNS1 antigen via our antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA). A set of 18 serum samples from influenza-infected and normal patients was used as a control panel to determine the background optical density (OD) value for determination of a cutoff. Each diamond represents a sample from one independent patient serum. The detection of sNS1 (closed diamond) and virus-like particle (VLP) (open diamond) were measured using (A) Ag-ELISA without dissociation and then (B) with dissociation by treatment with low pH buffer. All sera samples were plotted against the line indicating the detection limit by P/N ratio of 2 based on the average of OD readings from the 18 control sera. The graph shows the average results of three independent experiments performed in duplicate.

extracellular compartment is delayed compared with the release of mature virion.[22,23] The possible discrepancy could be due to the sensitivity of antibodies performing NS1-ELISA or the nature of different flaviviruses. The level of the sensitivity of the NS1-ELISA in the study by Chung and Diamond[22] was similar to the level we determined in our current study; therefore, the different abilities of antibodies in capturing NS1 antigen may not be the reason as the results were affected in both the study of Chung and Diamond[22] and in this study. Furthermore, the detection of virion released from the infected cells was confirmed by both real-time RT-PCR and E-ELISA for parallel comparison. The pattern of detecting E was similar to that of detecting viral RNA and infectious viruses from our *ex vivo* tissue culture experiments as shown in Figure 2. Using our Ag-ELISA, we demonstrated the presence of both free-circulating NS1 and E in the serum samples collected from Day 1 to Day 10, which covers the entire clinical phase of the disease. Levels of NS1 and E peaked in sera from Day 4 to Day 6, approximately the critical defervescence period of illness (Day 4—7 post onset of fever). The current study provides collaborative evidence relating the temporal kinetics of viral particle secretion and the detectable level of E during infection, which has traditionally been measured by the detection of viral RNA.[24—27]

The recent commercial utility of a rapid dipstick NS1 test for dengue diagnosis is hindered by low sensitivity, particularly in patients with secondary infection, requiring dengue-specific IgM/IgG antibody detection to increase the accuracy of diagnosis.[28—30] Several studies published previously suggested that using different dissociation buffers could efficiently dislodge the antigen from the antigen-antibody immune complex, thereby increasing detection sensitivity by up to 35%.[12,31] In this study, dissociation of DENV immune complexes proved essential for the detection of both DENV E and NS1 in serum samples, particularly among samples from secondary DENV infections, as evidenced by dramatically increased assay detection rates (85% for NS1-ELISA and 92% for E-ELISA) following dissociation. The enhanced detection of E might also be the result of dissociation of immune complexes and hence increased the accessibility of mAb-specific epitopes. However, the reduced P/N ratio for the sNS1 after treating with the low-pH dissociation buffer was noted here (Figure 4) and the reasons could be the labile NS1 to the acid treatment and the selection of mAb that recognized conformationally sensitive epitopes as suggested by a previous study.[22] The results from the current study suggest that the dissociation of immune complexes may render E-containing viral particles a valuable target for acute DENV diagnosis, similar to NS1 detection. Selection of a nonionic detergent for immune complex dissociation to improve the antigen detection should be the focus for future study.

The limitation of the current study was the small sample size and relatively long duration of storage of clinical specimens used. However, these samples were stored in −75°C and only the aliquots without previously freeze—thawed cycles were used. In a planned future study, the intention is to increase the sample size of DENV-2 and expand this protocol to test DENV-1, DENV-3, and DENV-4 patient serum specimens, pretreated specimens with dissociation buffer, and compare our E- as well as NS1-ELISA with the available commercial DENV NS1 detection kits.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

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