

# Serotype 1-Specific Monoclonal Antibody-Based Antigen Capture Immunoassay for Detection of Circulating Nonstructural Protein NS1: Implications for Early Diagnosis and Serotyping of Dengue Virus Infections

Hua Xu,<sup>1†</sup> Biao Di,<sup>2†</sup> Yu-xian Pan,<sup>1</sup> Li-wen Qiu,<sup>1</sup> Ya-di Wang,<sup>1</sup> Wei Hao,<sup>1</sup>  
Li-juan He,<sup>2</sup> Kwok-yung Yuen,<sup>3</sup> and Xiao-yan Che<sup>1\*</sup>

Center of Laboratory, Zhujiang Hospital, Southern Medical University,<sup>1</sup> and Center for Disease Control and Prevention of Guangzhou,<sup>2</sup> Guangzhou, and Department of Microbiology, The University of Hong Kong, Hong Kong Special Administrative Region,<sup>3</sup> People's Republic of China

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**Rapid diagnosis and serotyping of dengue virus (DV) infections are important for timely clinical management and epidemiological control in areas where multiple flaviviruses are endemic. However, the speed and accuracy of diagnosis must be balanced against test cost and availability, especially in developing countries. We developed a specific antigen capture enzyme-linked immunosorbent assay (ELISA) for early detection and serotyping of DV serotype 1 (DV1) by using well-characterized monoclonal antibodies (MAbs) specific to nonstructural protein 1 (NS1) of DV1. With this assay, a total of 462 serum specimens from clinically probable DV1-infected patients during the DV1 epidemic in Guangdong, China, in 2002 and 2003 were analyzed. DV1 NS1 was detectable in blood circulation from the first day up to day 18 after onset of symptoms, with a peak at days 6 to 10. The sensitivity of DV1 NS1 detection in serum specimens with reference to results from reverse transcriptase PCR was 82%, and the specificity was 98.9% with reference to 469 healthy blood donors. No cross-reactions with any of the other three DV serotypes or other closely related members of the genus *Flavivirus* (*Japanese encephalitis virus* and *Yellow fever virus*) were observed when tested with the clinical specimens or virus cultures. These findings suggest that the serotype-specific MAb-based NS1 antigen capture ELISA may be a valuable tool for early diagnosis and serotyping of DV infections, while also providing a standardized assay for the analysis of a great number of clinical samples with convenience and cost-effectiveness.**

Despite improvements in public health, epidemics of emerging and reemerging infectious diseases continue to occur in a manner that makes accurate predictions difficult (5, 18). Mosquito-borne *Flavivirus* diseases, such as dengue fever (DF), are currently considered reemerging infections because of the dramatic increase in recent decades, with an estimated annual occurrence of 100 million new cases in tropical and subtropical regions of the world (7, 8, 10). *Dengue virus* (DV) has four distinct serotypes (DV1, DV2, DV3, and DV4). Infection with any of the four serotypes of DV causes a spectrum of clinical features ranging from asymptomatic infections, undifferentiated fever, and classical DF to life-threatening manifestations such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which are often attributed to reinfection by heterologous serotypes (6). Infection with one serotype provides lifelong immunity against homologous reinfection, but protection against subsequent infection by the other three serotypes is only partial and transient. Therefore, people who live in epidemic areas may be susceptible to four infections in their lifetime. Seroepidemiological studies have shown that

subsequent heterologous infections may increase the risk of development of more-severe manifestations (15). At present, however, there is no protective vaccine or specific treatment available for DV infections. Thus, early clinical management can reduce the morbidity and mortality of DHF or DSS.

Since symptoms of DV infections are insufficiently specific for accurate clinical differentiation from other febrile illnesses and hemorrhagic fever, definitive diagnosis of DV infections relies on laboratory tests. A rapid and accurate dengue diagnosis in the acute phase of illness is important for enrolling patients in clinical trials for novel antiviral treatment or early enhancement of epidemiological control measures in areas with low endemicity. Furthermore, for epidemiological and pathological investigations, it is important to determine the correlations of different DV serotypes with disease severity (23). Currently, laboratory diagnosis of DV infections is based on virus isolation, serology, and RNA detection. Virus isolation is the "gold standard" for diagnosis and serotyping of DV infections, but this method is time consuming and requires a sophisticated laboratory. Viral nucleic acid detection typically provides more sensitive and rapid diagnosis than the traditional virus isolation method does. However, molecular diagnoses, such as reverse transcriptase (RT) PCR, require experienced technicians and specialized laboratory equipment. In the field setting in developing areas, false-positive results due to amplifications that carry over contamination are not uncommon.

\* Corresponding author. Mailing address: Center of Laboratory, Zhujiang Hospital, Southern Medical University, Guangzhou 510282, People's Republic of China. Phone: 86-2061643592. Fax: 86-2061643592. E-mail: linche@pub.guangzhou.gd.cn.

† Hua Xu and Biao Di contributed equally to this work.

mon. Although the detection of antibodies with whole virus antigen-based enzyme-linked immunosorbent assay (ELISA) is most commonly used, the limitations of this assay are cross-reactivity with all serotypes of DV as well as other members of the *Flavivirus* family, especially in cases of secondary DV infection (9, 14). Therefore, early diagnosis and determination of the serotype still remains a problem, as it mainly depends on RT-PCR or virus isolation methods.

As an alternative, the detection of viral antigens has been proposed, and more recently attention has been focused on nonstructural protein 1 (NS1) of DV (1, 13, 26). This protein has been identified as a highly conserved glycoprotein expressed in either membrane-associated or secreted forms. It possesses not only group-specific but also type-specific determinants and has been recognized as an important immunogen in DV infections (4, 11, 20). Therefore, the NS1 protein might be used as a serotyping marker and an early diagnostic marker. Recently Shu et al. illustrated that detection of serotype-specific immunoglobulin G (IgG) antibody responses to the NS1 antigen of DV could be a valuable tool in seroepidemiologic study for differentiating different serotypes of DV infections (21). A high circulating level of NS1 was demonstrated in the acute phase of dengue by antigen capture ELISAs (1, 26). Polyclonal antibodies were employed as detector or capturer in these antigen capture assays, which may widen the spectrum of strains recognized and improve the assay sensitivity. Cross-reactive epitopes, however, may in turn reduce the detective specificity, as NS1 amino acid sequence identity among the four serotypes of DV is about 80%. In this study, we developed and characterized a panel of monoclonal antibodies (MAbs) to serotype-specific epitopes of NS1 from DV1, by which a rapid and sensitive serotype-specific assay for the early diagnosis of DV infections was established. This two-site sandwich antigen capture ELISA with high affinity and highly specific MAbs was tested and optimized for detection of DV1 NS1. Clinical applications of this technology in the early diagnosis of DV infections and identification of the serotype of DV1 were also investigated.

#### MATERIALS AND METHODS

**Viruses and cells.** Four serotypes of DV standard strains (DV1, DV2, DV3, and DV4) were kindly provided by Center for Disease Control and Prevention of Guangzhou, China. The viruses were propagated in *Aedes albopictus* clone C6/36 cells in minimum essential medium (MEM) supplemented with 10% fetal calf serum. Virus stocks were used to infect 70% confluent cell monolayers in MEM supplemented with 2% fetal calf serum and were incubated at 33°C until cytopathic effect was observed, and then the viral culture supernatant and cell monolayers were harvested.

**Preparation of recombinant DV1 NS1 protein for immunization.** The sequence encoding the DV1 NS1 was amplified from total RNA of DV1-infected C6/36 cells with a forward primer (5'-CGGGATCCGATTCGGGATGTGTAAC-3') and a reverse primer (5'-CCCAAGCTTTGCAGAGACCATTTGA-3') and then was ligated into the BamHI and HindIII sites of the prokaryotic expression vector pQE30 (QIAGEN, Hilden, Germany) in frame and downstream of the six-His tag coding sequence. Recombinant DV1 NS1 protein was expressed in *Escherichia coli* and purified with Ni-nitrilotriacetic acid affinity chromatography (QIAGEN) according to the manufacturer's instructions. The results of high-level expression and purification of DV1 NS1 protein were shown in Fig. 1. The DV1 NS1 protein was detected by Western blot analysis using convalescent-phase sera at a dilution of 1:100 from serologically documented DV1-infected patients as the primary antibody. A panel of normal human sera was concurrently run while the DV1-immune rabbit serum (prepared in our laboratory) and anti-His monoclonal antibodies were used as positive controls. Horseradish peroxidase (HRP)-labeled goat anti-human/rabbit/mouse IgG was used as the sec-

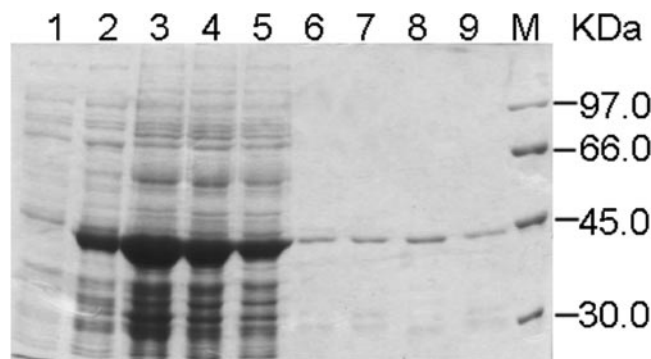


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of expression and purification of recombinant DV1 NS1 protein from *Escherichia coli*. Lane 1, uninduced crude cell lysates; lanes 2 to 5, induced crude cell lysates under different conditions; lanes 6 to 9, recombinant DV1 NS1 protein purified by Ni-nitrilotriacetic acid affinity chromatography; M, markers with their corresponding molecular masses.

ondary antibody, followed by signal detection with aminoethyl carbazole Single Solution (Zymed Laboratories, Inc., South San Francisco, Calif.). The concentration of purified recombinant DV1 NS1 protein was determined by the Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Rockford, Ill.).

**Preparation of serotype-specific MAbs against DV1 NS1.** BALB/c mice were immunized intraperitoneally with inactivated DV1 mixed with Freund's adjuvant (Sigma-Aldrich, St. Louis, Mo.) twice and then given three boosts of recombinant DV1 NS1 protein. Hybridomas were produced by fusion of spleen cells from the immunized mice with myeloma cells, according to our published procedure (3). Hybridoma supernatants were screened for the presence of MAbs against DV1 NS1 by indirect ELISA with recombinant DV1 NS1 and DV1 culture supernatant used as coated antigens. Positive hybridoma cells were cloned by limiting dilution. Serotype-specific MAbs against DV1 NS1 were further validated by immunofluorescence assays (IFA) using C6/36 cells infected with DV1, DV2, DV3, and DV4. The isotypes of the MAbs were determined with the Mouse Monoclonal Antibody Isotyping Kit (Zymed). The MAbs were purified by using protein G column chromatography (Amersham-Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The purified MAbs were labeled with HRP (Sigma-Aldrich) by a periodate method (24). The affinity constants of the MAbs were determined based on the method developed by Beatty et al. (2).

**Competition ELISA.** The binding epitopes of the MAbs were analyzed by use of competition ELISA with recombinant DV1 NS1 protein as coated antigen. Microwell plates (Costar Corning Inc., Corning, N.Y.) were coated with 100  $\mu$ l/well of DV1 NS1 at concentration of 1  $\mu$ g/ml in coating buffer. After the blocking steps, a constant concentration of one of the non-HRP-conjugated MAbs (50  $\mu$ l/well) was incubated with various amounts of a different HRP-conjugated MAbs (50  $\mu$ l/well) for 1 h at 37°C. After the plates were washed, the binding of HRP-labeling MAb was determined by incubation with 100  $\mu$ l/well of TMB (tetramethylbenzidine) (Zymed) for 10 min at 37°C. The reaction was stopped with 100  $\mu$ l/well of 1 N sulfuric acid, and absorbance was read at 450 nm in a microplate autoreader (Bio-Tek Instruments). An irrelevant, unlabeled MAb was used as a control. The percentage of inhibition was calculated by the following formula:  $[1 - (\text{OD}_{450} \text{ of the test well} / \text{OD}_{450} \text{ of the control well})] \times 100$ , where  $\text{OD}_{450}$  is the optical density at a wavelength of 450 nm. The results were described as competition if the inhibition was greater than 75%, inhibition between 75% and 25% was described as relative competition, and <25% inhibition was described as noncompetition.

**MAb-based antigen capture ELISA procedure.** The procedure for antigen capture ELISA was carried out as previously described, with modification (3). In brief, microwell plates (Costar) were coated with 100  $\mu$ l/well of capture MAbs overnight at 4°C, and then the wells were incubated with a blocking reagent. After removal of the blocking solution, a series of diluted 100- $\mu$ l/well samples was added and incubated for 1 h at 37°C. After the plates were washed, 100  $\mu$ l/well of diluted HRP-conjugated MAb was added and incubated for 30 min at 37°C. After further washing, 100  $\mu$ l/well of TMB solution was added, and the reaction was stopped after incubation for 10 min with 1 N sulfuric acid. The absorbance was determined as described above.

TABLE 1. Characteristics of MAbs against the NS1 protein of DV1

| Hybridoma cell strain | Isotype | Affinity constant (liters/mol) | Reaction with DV1 NS1 protein |              | IFA result from cells infected by: |     |     |     | Epitope group <sup>b</sup> |
|-----------------------|---------|--------------------------------|-------------------------------|--------------|------------------------------------|-----|-----|-----|----------------------------|
|                       |         |                                | ELISA <sup>a</sup>            | Western blot | DV1                                | DV2 | DV3 | DV4 |                            |
| M1                    | IgG1    | $2.1 \times 10^{10}$           | +++                           | +            | +                                  | -   | -   | -   | I                          |
| M2                    | IgG1    | $6.0 \times 10^6$              | ++                            | +            | +                                  | -   | -   | -   | III                        |
| M3                    | IgG1    | $3.0 \times 10^9$              | +++                           | +            | +                                  | -   | -   | -   | VI                         |
| M4                    | IgG1    | $1.4 \times 10^{10}$           | +++                           | +            | +                                  | -   | -   | -   | II                         |
| M5                    | IgG1    | $7.7 \times 10^9$              | +++                           | +            | +                                  | -   | -   | -   | VI                         |
| M6                    | IgG2a   | $6.1 \times 10^9$              | +++                           | +            | +                                  | +   | +   | +   | IV                         |
| M7                    | IgG1    | $6.0 \times 10^8$              | +++                           | +            | +                                  | -   | -   | -   | VI                         |
| M8                    | IgG1    | $1.0 \times 10^7$              | ++                            | +            | +                                  | -   | -   | -   | VI                         |
| M9                    | IgG1    | $1.0 \times 10^8$              | ++                            | +            | +                                  | -   | -   | -   | V                          |
| M10                   | IgG1    | $1.8 \times 10^8$              | +                             | +            | +                                  | -   | -   | -   | V                          |

<sup>a</sup> The purified recombinant DV1 NS1 protein was used as the coating antigen and was reacted with purified MAbs against the DV1 NS1 protein. The absorbance was measured at 450 nm: +, OD<sub>450</sub> = 0.8 to 1; ++, OD<sub>450</sub> = 1 to 2; +++, OD<sub>450</sub> > 2.

<sup>b</sup> Competition ELISA was performed to analyze the epitope specificity of the different MAbs. MAbs M3, M5, M7, and M8 effectively competed ( $\geq 75\%$  inhibition) with each other, which suggests that these four MAbs recognized the same epitope. Similarly, M9 and M10 are suggested from another group, with  $> 75\%$  inhibition of each other's binding. MAbs M1, M2, M4, and M6 did not interfere with heterologous MAb binding ( $< 25\%$  inhibition), suggesting that these MAbs may recognize discrete epitopes. Therefore, these 10 MAbs were divided into six groups, each group reacting with the same epitope or sterically overlapping epitopes on the NS1 protein.

**MAb-based antigen capture ELISA for DV1 NS1 antigen in serum.** The antigen capture ELISA for detection of the DV1 NS1 antigen in serum specimens was performed as described above. Since NS1 protein involved in immune complexes may interfere with antigen detection, a method for preceding antibody-antigen immune complex dissociation was employed as previously described (13). All serum samples were treated with dissociation buffer (1.5 M glycine, pH 2.8) to dissociate the immune complexes, followed by neutralization with neutralization buffer (1.5 M Tris-HCl, pH 9.7).

**DV IgM antibody.** Detection of IgM antibody against DV was performed with a commercial capture ELISA kit (MAC-ELISA; PANBIO, Brisbane, Australia), and absorbance values were determined according to the manufacturer's instructions.

**RT-PCR assay.** Detection of viral RNA in serum specimens was carried out by a conventional RT-PCR with the QIAGEN OneStep RT-PCR kit using consensus primers targeting the C/pre-M genes of DV, followed by a nested PCR with serotype-specific primers for DV1 to DV4, as described previously (16).

**Clinical samples.** A total of 462 serum specimens were collected from 462 clinical dengue patients during the DV1 epidemic in Guangdong, China, in 2002 and 2003 (27). Because documentation of serotype diagnosis by virus isolation or RT-PCR was not available for all of these patients, we defined probable DV1-infected patients on the basis of clinical symptoms and serological criteria according to the World Health Organization definition of DV infections (25). These serum specimens were collected between days 1 and 24 after the onset of symptoms, including 394 acute-phase serum specimens (i.e., days 1 to 7 after onset). Another 20 DV2-positive and 1 DV3-positive acute-phase serum specimens identified by both virus isolation and RT-PCR were also used in this study. In addition, 120 acute-phase serum specimens obtained from patients with other *Flavivirus* or non-*Flavivirus* infections diagnosed by virus isolation and/or RT-PCR or serological diagnosis were used as controls. Of these serum specimens, 13 were collected from patients with Japanese encephalitis virus (JEV) infections, 51 were collected from Hantaan virus infections, and 56 were collected from measles virus infections. And another 20 acute-phase serum specimens from 18 patients with leptospirosis were also included as controls; these were confirmed by demonstration of seroconversion with the microagglutination test. Normal serum specimens obtained from 469 healthy blood donors were used to establish the normal range of the assay. All serum specimens were stored at  $-20^\circ\text{C}$  until tested.

## RESULTS

**Selection and characterization of MAbs for DV1 NS1 antigen capture ELISA.** A total of 10 hybridoma cell lines that produced MAbs against DV1 NS1 were established from six fusions. These MAbs specific to NS1 of serotype DV1 were obtained by immunization with DV1 followed by a boost with

the purified recombinant DV1 NS1 protein, which was confirmed as having strong immunoreactivity by Western blot analysis (data not shown). The high-affinity and -specificity MAbs were selected on the basis of strong positive reactions with both recombinant DV1 NS1 protein and DV1-infected cell lysates in ELISA. The serotype specificity of the MAbs was further evaluated by IFA with four serotypes of DV. The characteristics of these MAbs are shown in Table 1. Experiments with reference viruses indicated that nine MAbs reacted exclusively with serotype DV1, and only M6 cross-reacted with the other three serotypes of DV. The results demonstrated that the MAbs could be useful for developing a two-site sandwich antigen capture assay for serotyping of DV infections. However, the assay sensitivity of the sandwich formation requires a pair of antibodies that are capable of binding to discrete, non-overlapping epitopes on the antigen (19). Thus, selection of MAbs with distinct epitope binding was done with competition experiments. The results showed that 10 MAbs bound to at least six different epitopes on the NS1 protein of DV1. On the basis of the six groups of epitopes among the 10 MAbs listed in Table 1, all combinations of MAbs were evaluated in a sandwich assay. The most effective nine pairs of capturer and detector MAbs that recognized different epitopes were primarily selected based on the sensitivity in detection of both DV1 and recombinant DV1 NS1 protein (Fig. 2). Although the MAb pair of immobilized M5 and HRP-M4 was demonstrated to be the best in detection of DV1 among the nine pairs of MAbs, its strong background signals appeared when a panel of normal human serum specimens were analyzed (data not shown). Thus, MAb M1 as a solid-phase immobilized capture antibody and MAb M4 as a labeled detecting antibody exhibited a higher combination of sensitivity and specificity than other eight pairs of MAbs. Checkerboard analysis of dilution series of capturer and detector antibody reagents was performed to optimize the reaction conditions of the antigen capture assay. The optimal concentrations for MAb M1 and HRP-M4 were determined to be 10  $\mu\text{g/ml}$  and 1:500, respectively.



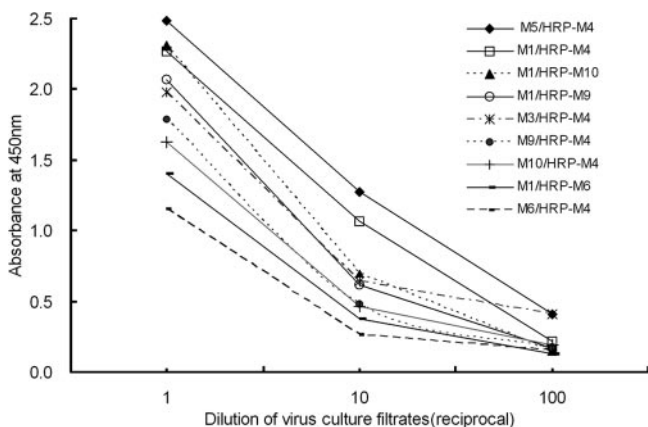


FIG. 2. Sensitivity of sandwich formations in antigen capture ELISA from different pairs of capturer and detector MAbs determined by replicates of several dilutions of DV1 culture filtrates.

**Sensitivity, specificity, and reproducibility of the DV1 NS1 antigen capture ELISA.** To evaluate the sensitivity of the antigen capture assay, replicates of serially diluted recombinant DV1 NS1 protein of known concentrations were analyzed. As shown in Fig. 3, a standard curve for the DV1 NS1 protein test was constructed. Bovine serum albumin (BSA) was used to establish the baseline for the assay, and a sample was considered positive if the OD<sub>450</sub> was twice greater than that of BSA. With these criteria, the minimal amount of recombinant DV1 NS1 detection with this assay was approximately 0.5 ng/well. The linear portion of the standard curve ranges from 100 to 2,000 ng/ml and may be used to estimate the NS1 levels in patient sera. Analysis of cross-reaction within the flaviviruses was further performed in the DV1 NS1 antigen capture ELISA. Serial dilutions of the DV-infected cell culture filtrates from the four serotypes of DV were analyzed. Culture filtrates from JEV and yellow fever virus (YFV) were also subjected to the analysis. The results of the assay are presented in Fig. 4. Only the DV1-infected cell culture filtrate gave a positive signal. The sensitivity of the positive assay signal can be detected with an approximately 1:200 dilution of DV1-infected cell culture filtrate. None of the other three serotypes of DV, JEV, or

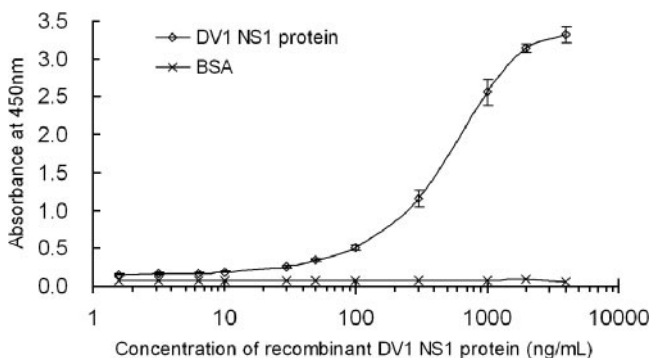


FIG. 3. Standard curve for NS1 determined with purified recombinant DV1 NS1 protein by antigen capture ELISA. Various concentrations of DV1 NS1 protein were analyzed. BSA was used to establish the baseline. Data points represent means ± standard deviations for 10 replicates.

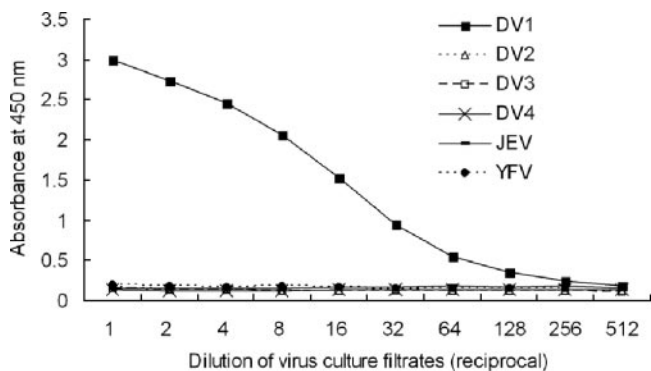


FIG. 4. Evaluation of the sensitivity and specificity of the DV1 NS1 antigen capture ELISA for detection of the DV1 NS1 protein in flaviviruses and nonflavivirus cultures. Serial dilutions of virus culture filtrates that were obtained from the four serotypes of DV, JEV, and YFV were subjected to the DV1 NS1 antigen capture ELISA. The positive OD<sub>450</sub> values are restricted to NS1 of serotype DV1.

YFV had OD<sub>450</sub> values greater than 0.20. The results indicate that the serotype-specific MAb-based antigen capture assay is specific for the detection of serotype DV1 and has no cross-reactivity with the other three serotypes of DV or other closely related members of the *Flavivirus* family (JEV and YFV).

The reproducibility of the assay was evaluated with three samples from DV1-infected cell culture filtrates diluted at 1/10, 1/50, and 1/100; these were run many times within and between assays. The coefficients of variation for 10 replicates tested in the same assay were 3.3%, 8.8%, and 5.2%, respectively, and the test-to-test coefficients of variation, with 10 replicates, were 4.0%, 10.2%, and 7.2%, respectively.

**Detection of NS1 antigen in serum specimens from DV-infected patients.** To establish the baseline of the normal range in the DV1 NS1 antigen capture assay for clinical evaluation, serum specimens from 469 healthy blood donors were analyzed. The mean OD<sub>450</sub> value for these specimens as determined by this assay was 0.129, with a standard deviation of 0.032. Thus, the cutoff OD<sub>450</sub> was set as the average value of the negative control by adding 3 standard deviations: 0.129 + (3 × 0.032) = 0.225. The result was considered positive if a sample yielded an OD<sub>450</sub> value above the cutoff. By these criteria, a total of 5 of 469 sera from healthy blood donors were defined as low-level false positives, with a mean OD<sub>450</sub> value of 0.332. Thus, the detection of DV1 NS1 has a test specificity of 98.9% (5 of 469) (Fig. 5).

A total of 462 serum specimens collected from probable DV1-infected patients during the DV1 epidemic in Guangdong in 2002 and 2003 were examined in the DV1 NS1 antigen capture ELISA. As shown in Fig. 5, high levels of DV1 NS1 were detected in serum specimens obtained during the acute phase of DV infections. DV1 NS1 could be detected as early as day 1 and until day 18 after the onset of symptoms. With the same panel of serum specimens, IgM antibody against DV was also measured by MAC-ELISA, which is currently commercially available for routine dengue diagnosis. Figure 6 shows a profile of DV1 NS1 detection in the 462 serum specimens in comparison with the appearance of IgM antibody. The percentage of DV1 NS1-positive samples was 52.8% on days 1 to 2, peaked at 83.8% on days 6 to 10, and decreased to 50% on

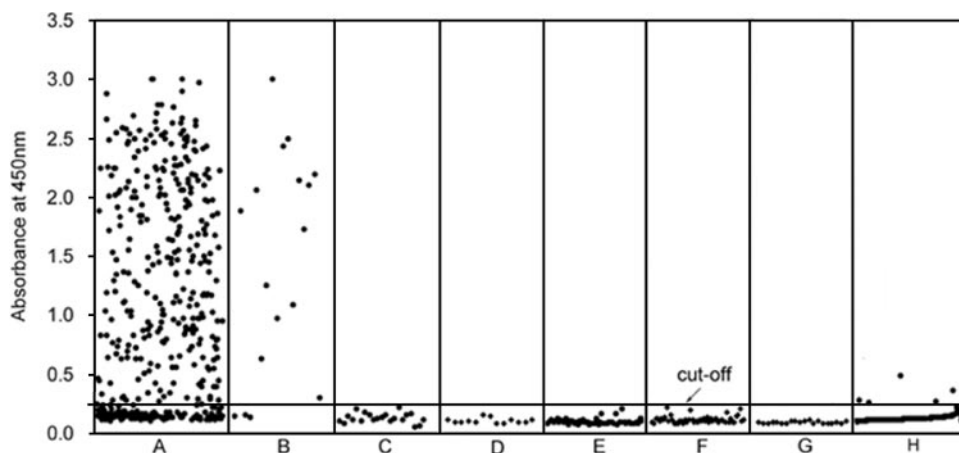


FIG. 5. Evaluation of the sensitivity and specificity of the DV1 NS1 antigen capture ELISA for detection of the DV1 NS1 protein in different serum samples. Data represent the  $OD_{450}$  of sera tested at a dilution of 1/3. Serum samples were taken from the following sources: A, clinically probable DV1-infected patients during the DV1 epidemic in Guangdong in 2002 and 2003 ( $n = 462$ ); B, DV1-infected patients identified by serotyping RT-PCR ( $n = 17$ ); C, 20 documented DV2-infected patients and one DV3-infected patient; D, JEV-infected patients ( $n = 13$ ); E, Hantaan virus-infected patients ( $n = 51$ ); F, measles virus-infected patients ( $n = 56$ ); G, patients with leptospirosis ( $n = 20$ ); H, healthy blood donors ( $n = 469$ ).

days 11 to 15 after the onset of symptoms. However, the positive rate of IgM antibody against DV was only 17.4% on days 1 to 2 and increased from 75% at 6 to 10 days to 100% at 21 to 25 days after onset (Table 2). Obviously, NS1 antigen and IgM antibody were detected concomitantly during the acute phase, but at earlier times, especially from day 1 to day 3, NS1 antigen showed a more sensitive detection. In these 462 serum specimens, there were 75 discordant samples, as evidenced by being NS1 antigen positive while IgM antibody negative. These were almost always collected during the acute phase of illness before day 9 after the onset of symptoms (74/75, i.e., 98.7%) and mainly collected from day 1 to day 3 (64/75, i.e., 85.3%), whereas only 10 IgM antibody-positive serum specimens collected from day 1 to day 3 were negative for NS1 detection. These findings demonstrate that NS1 may be more suitable for early detection of DV infections than is IgM antibody.

The sensitivity of DV1 NS1 detection was also compared to traditional RT-PCR. Of the 17 serum samples for which RNA

of serotype DV1 tested positive by RT-PCR with serotype-specific primers, 14 tested positive for DV1 NS1. The sensitivity of the antigen capture assay for the detection of DV1-NS1 in patient serum samples with reference to the results of RT-PCR was 82%.

**Serotype-specific DV1 NS1 antigen capture ELISA.** The DV1 NS1 antigen capture ELISA was expected to be serotype specific for DV1, as the assay had no cross-reactivity with the other serotypes of DV cultures, as described above. The assay for serotype-specific NS1 of DV1 was further demonstrated in clinical serum samples (Fig. 5). A limited number of acute-phase serum specimens from 20 DV2-infected patients and 1 DV3-infected patient confirmed by both virus isolation assay and RT-PCR were analyzed. None of these serum specimens were detected in the DV1 NS1 antigen capture ELISA, indicating that the antigen capture ELISA is highly specific for DV1 NS1 detection. In addition, DV1 NS1 test results with 13 serum specimens from patients with JEV infections, 51 specimens from patients with Hantaan virus infections, 56 specimens from patients with measles, and 20 specimens from patients with leptospirosis were all negative, indicating that the assay is

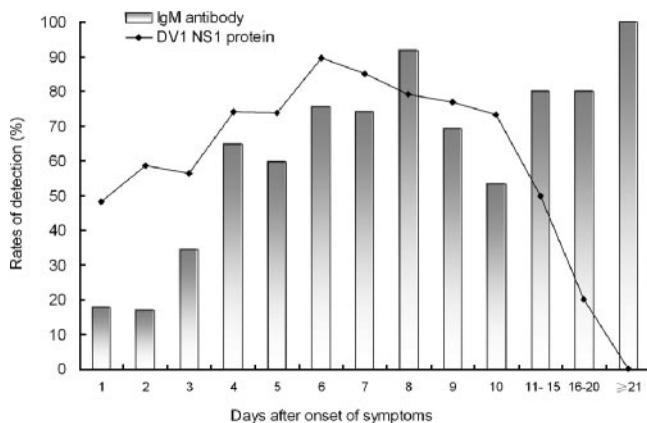


FIG. 6. Profile of DV1 NS1 detection in blood circulation and IgM antibody response to DV from the onset of symptoms to the convalescence phase.

TABLE 2. Comparison of detection of the DV1 NS1 protein by antigen capture ELISA and detection of IgM antibody against DV by MAC-ELISA

| Days after symptom onset | No. of samples | No. of positive samples (%) |              |
|--------------------------|----------------|-----------------------------|--------------|
|                          |                | DV1 NS1                     | IgM antibody |
| 1-2                      | 144            | 76 (52.8)                   | 25 (17.4)    |
| 3-5                      | 166            | 113 (68.1)                  | 88 (53)      |
| 6-10                     | 136            | 114 (83.8)                  | 102 (75)     |
| 11-15                    | 10             | 5 (50)                      | 8 (80)       |
| 16-20                    | 5              | 1 (20)                      | 4 (80)       |
| 21-25                    | 1              | 0 (0)                       | 1 (100)      |
| Total                    | 462            | 309 (66.9)                  | 228 (49.4)   |

highly specific for the DV1, with no cross-reactivity with either other flaviviruses or nonflavivirus infections.

## DISCUSSION

In this study, we successfully developed a DV1 NS1 antigen capture ELISA with serotype-specific MAbs as the capturer and detector antibodies for the detection of DV1 NS1 in serum. These MAbs specifically recognized at least six different epitopes on the NS1 protein of DV1, permitting the selection of an optimal pair of MAbs without overlapping epitopes. Thus, MAbs can be used in pairs for developing a sensitive sandwich formation of antigen capture ELISA. With optimization of assay conditions, the sensitivity of a matched pair of capturer and detector antibodies was determined with the purified recombinant DV1 NS1 protein to be as low as 0.5 ng/well. Using this antigen capture ELISA, we found that DV1 NS1 was detectable in serum specimens of patients even during the early stages of DV infection. The positive detection rates of DV1 NS1 increased from 53% at 1 to 2 days to 84% at 6 to 10 days after onset of symptoms. As a control for the effectiveness of the DV1 NS1 antigen capture ELISA, the 462 serum samples were also analyzed by MAC-ELISA, the routinely used method for the diagnosis of DV infections by detection of IgM antibody against DV. Apart from day 8, the positive detection rates of NS1 antigen always exceeded those of IgM antibody from days 1 to 10, especially in the first 3 days, though both of them were checked concomitantly during this period. Since IgM antibody develops rapidly and is detectable on days 3 to 5 after illness during primary DV infections, MAC-ELISA has been useful for clinical surveillance of dengue, providing faster results with a large number of clinical samples simultaneously and at lower cost (22). However, serological diagnosis of DV infections is often confounded by the existence of cross-reactive determinants of antigens among the four serotypes and some other flaviviruses (9, 14). Moreover, the IgM antibody production varies among patients due to variations in the strength and time of onset of the IgM antibody response. Some patients have detectable IgM antibody by day 2 to day 4, while others do not develop detectable IgM antibody until the eighth day after the onset of symptoms; sometimes IgM antibody is even absent in secondary infections (22). Therefore, NS1 antigen capture ELISA can be a useful adjunct for early diagnosis of DV infections. Furthermore, this strategy for detecting specific circulating antigen eliminates the problem of cross-reactivity between antibodies of homologous and heterologous flavivirus antigens.

The presence of NS1 in acute-phase sera of DV-infected patients has been previously reported (1, 26). However, the antigen capture ELISA used in these tests was based on polyclonal antibodies, which may vary from batch to batch, making it unsuitable for routine large-scale production. Moreover, these assays lack specificity and pose difficulties in standardization compared with those using MAbs (1). The DV1 NS1 antigen capture ELISA described here is established by well-characterized MAbs with high affinity and high specificity to NS1 of DV1. There was no evidence of cross-reaction when tested with cell cultures from the other three serotypes of DV and closely related members of the *Flavivirus* group, such as JEV and YFV. The serotype specificity of the DV1 NS1 anti-

gen capture ELISA was further demonstrated by testing clinical serum samples from other serotypes of DV-infected patients (20 with DV2 infection and 1 with DV3 infection) and serum specimens from patients infected by JEV, Hantaan virus, measles virus, and *Leptospira interrogans*, all exhibiting negative results. These findings suggest that a serotype-specific MAb-based DV1 NS1 antigen capture assay could be reliably used for early diagnosis and serotyping of DV infections.

Traditionally, laboratory identification of DV infections has relied mainly on the isolation of infectious virus from blood. PCR is more sensitive and rapid than virus isolation and has gradually come to be considered the gold standard for serotyping of DV infections. However, detection of viral RNA is somewhat hampered by its disadvantages, including the risk of amplicon carryover, its high cost, and the expertise needed (12). Although detection of DV1 NS1 by antigen capture assay was less sensitive than detection of viral RNA by RT-PCR in this study, the assay could be performed with a very simple laboratory setup which can handle hundreds of samples with little cost in time and labor. In addition, the NS1 antigen capture assay has the advantage of being a quantitative assay and is considerably more stable and less affected by variations in physical conditions than is quantification of viral RNA. As recent studies have demonstrated that the level of NS1 of DV was significantly higher in patients with DHF and/or DSS than in patients with DF (17), quantification of NS1 in blood samples may contribute not only to the early diagnosis of DV infections but also to an understanding of the pathogenesis of DV by monitoring the progress of clinical manifestations.

This paper describes the development and validation of a DV1-specific MAb-based ELISA for the detection of DV1 NS1 that could be used for early diagnosis and serotyping of DV infections in areas in which multiple flaviviruses are endemic and where laboratory facilities are limited. This is a proof of concept that three other panels of MAbs recognizing distinct epitopes on each DV serotype can be developed as individual tests for the identification of all four serotypes of DV. The development of the antigen capture ELISA for NS1 of the other three serotypes of DV is in progress in our laboratory and may provide a full, rapid serotyping assay for DV1 to DV4 infections.

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## REFERENCES

- Alcon, S., A. Talarmin, M. Debruyne, A. Falconar, V. Deubel, and M. Flamand. 2002. Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. *J. Clin. Microbiol.* **40**:376–381.
- Beatty, J. D., B. G. Beatty, and W. G. Wlanos. 1987. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. *J. Immunol. Methods* **100**:173–179.
- Che, X. Y., L. W. Qiu, Y. X. Pan, K. Wen, W. Hao, L. Y. Zhang, Y. D. Wang, Z. Y. Liao, X. Hua, V. C. Cheng, and K. Y. Yuen. 2004. Sensitive and specific monoclonal antibody-based capture enzyme immunoassay for detection of nucleocapsid antigen in sera from patients with severe acute respiratory syndrome. *J. Clin. Microbiol.* **42**:2629–2635.

4. **Falconar, A. K., and P. R. Young.** 1990. Immunoaffinity purification of native dimer forms of the flavivirus non-structural glycoprotein, NS1. *J. Virol. Methods* **30**:323–332.
5. **Fauci, A. S., N. A. Touchette, and G. K. Folkers.** 2005. Emerging infectious diseases: a 10-year perspective from the National Institute of Allergy and Infectious Diseases. *Emerg. Infect. Dis.* **11**:519–525.
6. **George, R., and L. Lum.** 1997. Clinical spectrum of dengue infection, p. 89–113. *In* D. J. Gubler and G. Kuno (ed.), *Dengue and dengue hemorrhagic fever*. CAB International, New York, N.Y.
7. **Gibbons, R. V., and D. W. Vaughn.** 2002. Dengue: an escalating problem. *BMJ* **324**:1563–1566.
8. **Gubler, D. J.** 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol.* **10**:100–103.
9. **Gubler, D. J.** 1996. Serological diagnosis of dengue haemorrhagic fever. *WHO Dengue Bull.* **20**:20–23.
10. **Gubler, D. J.** 2002. The global emergence/resurgence of arboviral diseases as public health problems. *Arch. Med. Res.* **33**:330–342.
11. **Henchal, E. A., L. S. Henchal, and B. K. Thaisomboonsuk.** 1987. Topological mapping of unique epitopes on the dengue-2 virus NS1 protein using monoclonal antibodies. *J. Gen. Virol.* **68**:845–851.
12. **Kao, C. L., C. C. King, D. Y. Chao, H. L. Wu, and G. J. Chang.** 2005. Laboratory diagnosis of dengue virus infection: current and future perspectives in clinical diagnosis and public health. *J. Microbiol. Immunol. Infect.* **38**:5–16.
13. **Koraka, P., C. P. Burghoorn-Maas, A. Falconar, T. E. Setiati, K. Djamiatum, J. Groen, and A. D. Osterhaus.** 2003. Detection of immune-complex-dissociated nonstructural-1 antigen in patients with acute dengue virus infections. *J. Clin. Microbiol.* **41**:4154–4159.
14. **Koraka, P., H. Zeller, M. Niedrig, A. D. Osterhaus, and J. Groen.** 2002. Reactivity of serum samples from patients with a flavivirus infections measured by immunofluorescence assay and ELISA. *Microbes. Infect.* **4**:1209–1215.
15. **Kurane, I., and F. E. Ennis.** 1992. Immunity and immunopathology in dengue virus infections. *Semin. Immunol.* **4**:121–127.
16. **Lancioti, R. S., C. H. Calisher, D. J. Gubler, G. J. Chang, and A. V. Vorndam.** 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase polymerase chain reaction. *J. Clin. Microbiol.* **30**:545–551.
17. **Libraty, D. H., P. R. Young, D. Pickering, T. P. Endy, S. Kalayanaroop, S. Green, D. W. Vaughn, A. Nisalak, F. A. Ennis, and A. L. Rothman.** 2002. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. *J. Infect. Dis.* **186**:1165–1168.
18. **Morens, D. M., G. K. Folkers, and A. S. Fauci.** 2004. The challenge of emerging and re-emerging infectious diseases. *Nature* **430**:242–249.
19. **Porstmann, T., and S. T. Kiessig.** 1992. Enzyme immunoassay techniques. An overview. *J. Immunol. Methods* **150**:5–21.
20. **Russell, P. K., W. E. Brandt, and J. M. Dalrymple.** 1980. Chemical and antigenic structure of flaviviruses, p. 503–529. *In* R. W. Schlesinger (ed.), *The togaviruses*. Academic Press, New York, N.Y.
21. **Shu, P. Y., L. K. Chen, S. F. Chang, Y. Y. Yueh, L. Chow, L. J. Chien, C. Chin, H. H. Yang, T. H. Lin, and J. H. Huang.** 2002. Potential application of nonstructural protein NS1 serotype-specific immunoglobulin G enzyme-linked immunosorbent assay in the seroepidemiologic study of dengue virus infection: correlation of results with those of the plaque reduction neutralization test. *J. Clin. Microbiol.* **40**:1840–1844.
22. **Shu, P. Y., and J. H. Huang.** 2004. Current advances in dengue diagnosis. *Clin. Diagn. Lab. Immunol.* **11**:642–650.
23. **Vaughn, D. W., S. Green, S. Kalayanaroop, B. L. Innis, S. Nimmannitya, S. Suntayakorn, T. P. Endy, B. Raengsakulrach, A. L. Rothman, F. A. Ennis, and A. Nisalak.** 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J. Infect. Dis.* **181**:2–9.
24. **Wilson, M. B., and P. K. Nakane.** 1978. Recent developments in the peroxidase method of conjugating horseradish peroxidase (HPR) to antibodies, p. 215–224. *In* W. Knapp, K. Holubar, and G. Wicks (ed.), *Immunofluorescence and related staining techniques*. Elsevier, Amsterdam, The Netherlands.
25. **World Health Organization.** 1997. *Dengue haemorrhagic fever: diagnosis, treatment, prevention and control*. World Health Organization, Geneva, Switzerland.
26. **Young, P. R., P. A. Hilditch, C. Bletchly, and W. Halloran.** 2000. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. *J. Clin. Microbiol.* **38**:1053–1057.
27. **Zhang, F. C., Y. Q. Chen, Y. C. Lu, J. Wang, W. S. Chen, and W. X. Hong.** 2005. Analysis on clinical and epidemiological characteristics of 1032 patients with Dengue fever in Guangzhou. *Zhonghua Liu Xing Bing Xue Za Zhi* **26**:421–423.