Comparative Analysis of Immunoglobulin M (IgM) Capture Enzyme-Linked Immunosorbent Assay Using Virus-Like Particles or Virus-Infected Mouse Brain Antigens To Detect IgM Antibody in Sera from Patients with Evident Flaviviral Infections

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The use of immunoglobulin M (IgM) antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) serves as a valuable tool for the diagnosis of acute flaviviral infections, since IgM antibody titers are detectable early, peak at about 2 weeks postinfection, and subsequently decline to lower levels over the next few months. Traditionally, virus-infected tissue culture or suckling mouse brain (SMB) has been the source of viral antigens used in the assay. In an effort to provide a reliable source of standardized viral antigens for serodiagnosis of the medically important flaviviruses, we have developed a eukaryotic plasmid vector to express the premembrane/membrane and envelope proteins which self-assemble into noninfectious virus-like particles (VLPs). In addition to the plasmids for Japanese encephalitis virus, West Nile virus (WNV), St. Louis encephalitis virus (SLEV), and dengue virus type 2 (DENV-2) reported earlier, we recently constructed the DENV-1, -3, and -4 VLP expression plasmids. Three blind-coded human serum panels were assembled from patients having recent DENV, SLEV, and WNV infections to assess the sensitivity and specificity of the MAC-ELISA using VLPs or SMB antigens. In addition, serum specimens from patients infected with either Powassan virus or La Crosse encephalitis virus were used to evaluate the cross-reactivity of seven mosquito-borne viral antigens. The results of the present studies showed higher sensitivity when using SLEV and WNV VLPs and higher specificity when using SLEV, WNV, and the mixture of DENV-1 to -4 VLPs in the MAC-ELISA than when using corresponding SMB antigens. Receiver operating characteristic (ROC) curve analysis, a plot of the sensitivity versus false positive rate ($100 - \text{specificity}$), was applied to discriminate the accuracy of tests comparing the use of VLPs and SMB antigen. The measurement of assay performance by the ROC analysis indicated that there were statistically significant differences in assay performance between DENV and WNV VLPs and the respective SMB antigens. Additionally, VLPs had a lower cutoff positive/negative ratio than corresponding SMB antigens when employed for the confirmation of current infections. The VLPs also performed better than SMB antigens in the MAC-ELISA, as indicated by a higher positive prediction value and positive likelihood ratio test. Cell lines continuously secreting these VLPs are therefore a significantly improved source of serodiagnostic antigens compared to the traditional sources of virus-infected tissue culture or suckling mouse brain.

Members of the genus *Flavivirus* have an ~11-kb, single-stranded, positive-sense RNA genome which translates and encodes capsid (C), premembrane/membrane (prM/M), and envelope (E) structural proteins and seven nonstructural proteins. During natural flavivirus infection, noninfectious virus-like particles (VLPs) are produced in addition to infectious, mature virions (25). Flavivirus VLPs have structural and physiochemical properties similar to mature virus particles. VLPs have been characterized for several flaviviruses, including tick-borne encephalitis virus (27), Japanese encephalitis virus (JEV) (2, 14, 17), West Nile virus (WNV) (5), St. Louis

encephalitis virus (SLEV) (23), dengue virus type 2 (DENV-2) (3) and DENV-1, -3, and -4 (23), and Murray Valley encephalitis virus (18). We have previously described WNV, JEV, SLEV, and DENV-1 to -4 plasmid constructs that direct the expression of prM/M and E proteins and secretion of VLPs into the tissue culture media of plasmid-transformed cells. Plasmid DNA containing a eukaryotic transcriptional unit consisting of the human cytomegalovirus immediate early gene promoter, Kozak consensus ribosomal binding sequence, the signal sequence derived from the carboxy terminus of the C protein of JEV, and the prM/M and E gene regions is sufficient for production of VLPs. The transformation of tissue culture cells with plasmid DNA is therefore advantageous for antigen production, since these cells secrete viral prM and E proteins in VLPs having proper conformation and presentation of epitopes similar to those of virion particles.

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Dengue fever and/or dengue hemorrhagic fever (DHF), caused by four serotypes of DENV, is the most important arbovirus disease in terms of morbidity and mortality. Annually, it is estimated that 50 million to 100 million people may be infected with DENV worldwide, with more than 2.5 billion people living in areas where dengue is endemic and at risk of infection. DENV is spread by the bite of infected mosquitoes, with more than half of individuals infected being asymptomatic or having an undifferentiated fever (1, 7). In addition to the relatively mild form, dengue fever, an increase in the incidence of the more serious diseases DHF and dengue shock syndrome has been observed over the last 50 years, with an estimated 250,000 to 500,000 cases of DHF and 24,000 deaths reported annually in recent years (9).

The dengue serogroup consists of four antigenically related but distinct serotypes. Cross-reactive antibodies which react to similar epitopes presented on other flaviviruses, particularly for viruses within the same serogroup, are produced during flavivirus infection. For DENV, infection results in the production of neutralizing antibodies and lifelong immunity to the homologous serotype. In an early study, Albert Sabin demonstrated that the volunteers challenged with a second DENV serotype were fully cross-protected for only 2 months and partially protected for up to 9 months after infection with the first serotype but were not protected thereafter (26). Subsequent secondary infection by one or more of the three heterologous serotypes is generally accepted as a major risk factor for DHF and/or dengue shock syndrome due to antibody-dependent enhancement (ADE) (1, 10, 11, 12). Additionally, this ADE phenomenon has been observed within members of the JEV serocomplex under experimental conditions (19).

Isolation and characterization of virus, detection of genomic sequence, detection of virus-specific antigen(s), and detection of dengue virus-specific antibodies are the most commonly employed methods for the diagnosis of dengue virus infection (30). In the serodiagnosis of DENV infection, detection of virus-specific immunoglobulin M (IgM) and IgG antibodies by enzyme-linked immunosorbent assay (ELISA) is a simple method which facilitates the processing of numerous serum samples. Differentiation between primary and secondary infections has been suggested by determining the ratio of IgM to IgG in acute-phase sera (15, 29). Demonstration of a fourfold or greater increase in IgM and/or IgG antibody titers between paired sera collected during the acute and convalescent phases is a reliable method for determination of a recent DENV infection. The use of IgM antibody-capture (MAC)-ELISA serves as a valuable tool for the presumptive diagnosis of acute flaviviral infections, since IgM antibody titers are detectable early, peak at about 2 weeks postinfection, and subsequently decline to lower levels over the next few months. IgM antibodies are generally less cross-reactive than IgG in primary infections, and the serotype having the highest IgM titer is often the one responsible for current infection. In addition to increasing the risk of severe disease due to ADE following a secondary DENV infection, cross-reactive antibodies make differential diagnosis of DENV infection difficult. Determination of primary versus secondary infection and serotyping of the most recently infecting dengue virus, especially in areas where multiple serotypes cocirculate, require conducting both IgM and IgG ELISAs or testing paired serum samples simultaneously in

the same ELISA. However, definitive information about serum samples, such as the collection date, the date of onset of symptoms, or patient travel and/or vaccination history, is often not readily available for clinical diagnostic laboratories.

The majority of ELISA formats described for the detection of DENV-specific antibody use virus-infected cell culture supernatants or suckling mouse brain (SMB) preparations as the serodiagnostic antigens. The VLP antigens are excellent alternatives to these antigens for the same purpose. They are non-infectious, do not require the use of live virus or hazardous chemicals for preparation, and can be easily concentrated from the tissue culture fluid of transiently transformed cells or continuously secreting, clonally selected cell lines by ultracentrifugation. For detecting ant flaviviral antibodies in human serum, ELISAs employing WNV, JEV, and SLEV VLP antigens have sensitivities and specificities comparable to those using SMB antigens (5, 14, 23). We recently demonstrated that low cross-reactivity with anti-WNV IgM antibody makes the use of SLEV VLPs preferable to that of antigens derived from SMB preparations in MAC-ELISA screening of patient serum samples (23). In this paper, we report the use of VLP antigens for four DENV serotypes in MAC-ELISA detection of ant flaviviral IgM antibody and compare these results with assays using the conventional virus-infected SMB antigens. Additionally, the DENV-1 to -4 and previously developed JEV, WNV, and SLEV VLP antigens were tested in parallel using serum panels of patients with evident WNV, SLEV, or JEV infections to assess the sensitivity and specificity of the MAC-ELISA using VLPs or SMB antigens. Additionally, serum specimens from patients infected with either Powassan virus (POWV) or La Crosse encephalitis virus (LACV) were used to evaluate the cross-reactivity of seven mosquito-borne viral antigens. COS-1 cell lines continuously secreting these DENV VLPs were clonally selected in order to establish a simple and standardized method for producing VLP antigens.

MATERIALS AND METHODS

Cell culture and plasmids. Chinese hamster ovary (CHO-K1) cells (CCL-61; American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco Laboratories, Grand Island, NY). African green monkey kidney (COS-1) cells (ATCC CRL-1650) were grown in DMEM (Gibco Laboratories). All growth media were supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 1 mM sodium pyruvate, 1 mM sodium glutamate, 0.1 mM MEM nonessential amino acids, penicillin (100 U/ml), and 100 µg/ml streptomycin. The clonal selection medium is COS-1 growth medium supplemented with Geneticin (G418; Boehringer Mannheim, Mannheim, Germany) at a concentration of 500 µg/ml, which was used to select stably transformed cells. CHO and COS-1 cells were incubated at 37°C with 5% CO₂.

The expression plasmids pCB8D1J2, pCB8D2-2J-2-9-1, pCB8D3J2, and pCBD4 used in transient transformation of tissue culture cells and collection of DENV-1, -2, -3, and -4 VLP antigens, respectively, were described previously (3, 22). The KpnI-NotI expression cassettes from the original plasmids were subcloned into KpnI-NotI-digested pCDNA-3 (Invitrogen Corp., Carlsbad, CA), and constructs were designated pCD8D1J2, pCD8D2J2, pCD8D3J2, and pCDD4. Automated DNA sequencing was performed on a CEQ 8000 genetic analysis system (Beckman Coulter, Fullerton, CA) according to the manufacturer's recommended procedures. The plasmids that had the correct sequence were identified and used for cell transformation and clone selection using G418.

Electroporation of tissue culture cells with plasmid DNA. For transformation, tissue culture cells were grown to 90 to 100% confluence in 150-cm² culture flasks, trypsinized, and resuspended in ice-cold phosphate-buffered saline (PBS) to a final density of 1.5×10^7 cells/ml. For each reaction, 0.5 ml of this cell suspension was electroporated with 30 µg of plasmid DNA in a 0.4-cm electrode

gap cuvette using a Gene Pulser II (Bio-Rad Laboratories, Hercules, CA) set at 250 V and 975 μ F. Cells from two electroporation reactions were seeded onto a single 150-cm² culture flask containing 40 ml growth medium. Tissue culture medium was harvested 48 h following electroporation and clarified by centrifugation at 10,000 rpm for 10 min at 4°C. Antigen-capture ELISA (Ag-ELISA) was performed using flavivirus E-specific, group-reactive monoclonal antibodies 4G2 (13) and 6B6C-1 conjugated to horseradish peroxidase (24) to capture and detect secreted VLPs, respectively (2, 14).

Clonal selection of G418-resistant transformed cells. In order to select a cell line that continuously secreted DENV prM and E antigens, transformed cells were trypsinized after reaching ~50 to 75% confluence (~4 days postelectroporation) and reseeded in 150-cm² culture flasks containing 40 ml selection medium. Tissue culture fluid was replaced with fresh selection medium every 4 days, and cells were incubated to ~75% confluence at each stage during the entire selection procedure. Cells were trypsinized again and incubated as described above in order to separate individual cells for a second round of transformed cell selection. G418-resistant cells were trypsinized, counted, diluted appropriately in 20 ml selection medium, and seeded on gridded cloning petri dishes (Greiner Labortechnik, Frickenhausen, Germany) at 125 to 700 cells per dish (0.25 to 1 cell per grid). Actively growing cloned cells from ~96 individual grids per DENV construct were expanded to 96-well plates, followed by subsequent expansion into 24-well plates, 6-well plates, and 25-cm² culture flasks. Clones were screened for the presence of E antigens in the culture medium by Ag-ELISA, preferably upon reaching ~75 to 100% confluence 48 h following passage from the 6-well plate into a 25-cm² culture flask. Clones having the highest titers were selected for further expansion to 75- and 150-cm² culture flasks.

Human serum. Serum specimens were obtained from the Diagnostic and Reference Laboratory, Arbovirus Diseases Branch (ADB-DRL), Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO. Serum was randomly numbered and tested as a blind panel. Panels were assembled by selecting serum specimens of North American origin collected from 1999 to 2003 having neutralizing antibody titers to WNV ($n = 10$), SLEV ($n = 5$), POWV ($n = 5$), or LACV ($n = 5$), as determined by DRL using the "gold standard" 90% plaque reduction neutralization test. The serum panel ($n = 27$) with the evidence of DENV infection was assembled by selecting specimens from United States residents who had a clear travel history to regions where dengue is endemic or epidemic and who were previously determined to be positive for anti-DENV IgM antibodies by DRL using a mixture of DENV-1-, DENV-2-, DENV-3-, and DENV-4-infected SMB antigens.

VLP antigen preparation. The DENV VLPs and normal CHO cell culture antigen were concentrated and partially purified from clarified tissue culture medium of transiently transformed CHO cells by ultracentrifugation at 19,000 rpm for 8 to 16 h in a Beckman Coulter type 19 rotor at 4°C. The pellet was resuspended in TN buffer (50 mM Tris, 100 mM NaCl, pH 7.5) to 1/50 the original volume, aliquoted into 1-ml samples, and stored at -70°C. For use in assays, aliquots were thawed once, stored at 4°C for up to 1 week, and then discarded. SLEV and JEV VLPs were prepared as described previously (14, 23) in a manner similar to that described above. Lyophilized preparations of WNV VLP antigen and normal COS-1 cell culture antigen, prepared as previously described (5), were resuspended in 0.5 ml distilled water. Antigens were independently titrated against a positive control serum sample using a twofold dilution series and standardized by selecting a dilution that yielded an absorbance of 0.8 to 1.2 at 450 nm (A_{450}). For preparation of the DENV-1 to -4 VLP antigen mixture, an appropriate volume of each undiluted antigen was added at a ratio corresponding to its individual working dilution as determined above. This antigen mixture was then titrated and standardized as described above.

ELISA protocols. MAC-ELISAs using the protocol described by Martin et al. (20) were performed by DRL with virus-infected SMB antigens. SMB antigens were titrated in the same manner against the same positive human control serum used for VLP titration and standardized by selecting a dilution that yielded an A_{450} of 0.8 to 1.2.

For detecting the presence of antiviral IgM antibody in serum panels using the VLPs, the MAC-ELISA described by Martin et al. was modified and performed as previously described (23). Briefly, the inner 60 wells of Immulon II HB flat-bottomed 96-well plates (Dynatech Industries, Inc., Chantilly, VA) were coated overnight at 4°C in a humidified chamber with 75 μ l of goat anti-human IgM (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:2,000 in coating buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6). Wells were blocked with 300 μ l of StartingBlock (PBS) blocking buffer (Pierce, Rockford, IL) according to the manufacturer's recommended procedure. Dried plates were stored with desiccant at 4°C until use. Patient serum specimens were randomly numbered and tested in triplicate as a blind panel. Patient sera and positive and negative antibody controls were diluted appropriately in wash buffer

(PBS-0.5% Tween 20), added to wells (50 μ l/well), and incubated at 37°C for 1 h in a humidified chamber. Test and negative human control sera were diluted 1:400 as recommended previously (16). Positive control sera were diluted 1:3,000 for SLEV, 1:400 for DENV, and 1:200 for WNV and JEV. Positive and negative control antigens were tested with each patient serum sample in triplicate by diluting appropriately in wash buffer and adding 50 μ l to appropriate wells for incubation at 4°C overnight in a humidified chamber. Horseradish peroxidase-conjugated 6B6C-1 was diluted 1:6,000 in PBS containing 0.5% Tween 20 and 5% nonfat dry milk; 50 μ l was added to each well, and wells were incubated at 37°C for 1 h in a humidified chamber. Bound conjugate was detected by adding 75 μ l of 3,3',5,5'-tetramethylbenzidine (Neogen Corp., Lexington, KY) substrate and incubating at room temperature for 10 min. The substrate reaction was stopped with 50 μ l of 1 N H₂SO₄, and reactions were measured at an A_{450} using an EL 312e Bio-Kinetics microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Test validation and calculation of P/N values. Test validation and positive-to-negative ratio (P/N) values were determined according to the procedure of Martin et al. (20) using internal positive and negative serum controls included in each 96-well plate. Positive (P) values for each specimen were determined as the average A_{450} for the patient serum sample reacted with positive viral antigen. Negative (N) values were determined for individual 96-well plates as the average A_{450} for the normal human serum control reacted with the positive viral antigen.

Statistical analysis. Sensitivity and specificity of a given assay are measured by the proportion of patients with evidence of infection who tested positive and the proportion of patients without evidence of infection or patients with clear evidence of having been infected by other arboviruses who test negative, respectively. We defined the evidence of infection by using the following criteria to transform the numeric P/N ratio into five discrete categories: 0, P/N < 2 (definitive negative); 1, P/N $\geq$ 2 and < 3 (presumptive positive); 2, P/N $\geq$ 3 and < 4 (probable positive); 4, P/N $\geq$ 4 and < 5 (positive); and 5, P/N $\geq$ 5 (definitive positive). These transformed data were applied to the following analysis and interpretation.

The receiver operating characteristic (ROC) curve analysis, a plot of the sensitivity versus false positive rate (100 - specificity), was applied to discriminate the accuracy of tests between VLPs and SMB antigens using the MedCalc (Berkeley, CA) statistical package (28). The performance of a diagnostic test, or the ability of a test to determine the evidence of infection in the target panel or no evidence of infection in the control panels, is evaluated using ROC curve analysis, and the accuracy of discrimination is measured by the area under the ROC curve (AUC). We selected a P/N of <3 or $\geq$ 3 to classify a given specimen as assay negative or positive, respectively, and to define the evidence of infection or noninfection, respectively. This P/N ratio is the current standard used in the DRL-ADB to interpret test results. A test with perfect discriminatory power (no overlapping in the sensitivity and specificity distributions) has a ROC plot that passes through the upper left corner (100% sensitivity and 100% specificity). Therefore, the closer the ROC plot is to the upper left corner, the higher the overall accuracy of the test. The comparative ROC was used to calculate the significance level and compare the paired-assay performance. The AUC and 95% confidence interval (95% CI) were calculated by a parametric method using a maximum likelihood estimator (31). An AUC approaching 1 indicates that the test is highly sensitive as well as highly specific; an AUC approaching 0.5 indicates that the test is neither sensitive nor specific. The P values that are less than 0.05 are considered to be statistically significant.

Since ROC curve analysis is not intuitively interpretable in terms of the probability of defining the evidence of infection for an individual specimen, the likelihood ratio (LR) is calculated to indicate the predicted probability of the infection based on the different cutoff levels (P/N ratio). The LR indicates the probability of a given test result among people with confirmed infection divided by the probability of that test result among people without the infection. Thus, the positive likelihood ratio (+LR) indicates the ratio between the probability of a positive test result given the presence of the infection and the probability of a positive test result given the absence of the infection. The magnitude of the LR provides intuitive meaning as to how strongly a given test result can be used to predict the likelihood of the evidence of infection or noninfection.

Additionally, the most significant public health impact of a new test versus the existing test is the positive predictive value (PPV) and negative predictive value (NPV) when applied to a large-scale seroepidemiologic study. PPV is the proportion of specimens with positive tests that have evidence of infection. NPV is the proportion of specimens with negative tests that do not have infection. The PPV and NPV are highly dependent on the proportions of the study population that do or do not have the infection in the study area. We applied the same cutoff criteria as used in the AUC calculation to define the evidence of infection or noninfection. A two-row by two-column table, which categorized four quadrants

TABLE 1. Results of MAC-ELISA detecting cross-reactive antibodies in human serum specimens with various evident arboviral infections

Panel and no.	P/N value from a MAC-ELISA using ^a																
	Virus-like particle							Suckling mouse brain antigen									
	DENV1-4	DENV-1	DENV-2	DENV-3	DENV-4	SLEV	WNV	JEV	DENV1-4	DENV-1	DENV-2	DENV-3	DENV-4	SLEV	WNV	POWV	LACV
SLEV																	
1	1.2	1.1	1.5	1.0	1.1	10.6	2.9	3.9	4.0	0.9	2.5	1.3	2.9	9.1	2.7		
2	1.5	1.3	1.7	1.3	1.3	9.9	2.8	4.0	4.3	1.8	*	1.8	3.7	8.5	2.3		
3	2.4	2.2	3.6	2.1	2.1	8.6	8.3	9.9	14.3	2.2	8.9	5.0	8.1	9.1	5.3		
4	1.3	1.1	1.5	1.2	1.3	8.4	4.3	6.3	8.1	2.1	5.0	3.0	6.1	8.3	4.6		
5	1.1	1.1	1.4	1.2	1.0	7.3	2.4	4.5	2.7	1.2	2.1	1.2	3.1	9.0	2.2		
Positive no.	(0)	(0)	(1)	(0)	(0)	(5)	(2)	(5)	(4)	(0)	(2)	(2)	(4)	(5)	(2)		
WNV																	
1	2.0	1.4	2.6	2.0	1.5	2.0	37.5	5.9	10.9	2.0	9.5	8.3	8.2	3.5	22.3		
2	1.2	1.2	1.3	1.1	1.2	2.1	24.5	8.9	4.2	*	3.8	2.7	2.1	4.3	8.9		
3	2.0	1.5	3.0	1.5	1.6	2.5	23.1	7.5	7.1	*	5.2	2.6	5.4	4.4	8.0		
4	1.5	1.3	2.7	1.3	1.2	2.8	20.0	3.4	4.1	1.6	4.5	1.8	3.0	2.9	9.0		
5	1.9	2.3	2.7	1.9	2.4	3.8	19.6	8.0	6.6	2.7	8.6	7.5	5.7	14.3	7.9		
6	1.0	1.1	1.1	1.0	1.1	2.4	16.7	3.4	4.2	3.1	4.1	2.5	2.7	4.8	9.9		
7	1.4	1.2	2.2	1.5	1.4	3.4	16.4	15.8	12.0	5.2	12.8	12.8	2.4	2.1	36.0		
8	1.0	0.9	1.1	1.0	1.0	1.3	9.3	1.8	1.6	1.4	1.3	0.8	1.1	*	1.9		
9	2.1	1.9	2.9	1.5	1.8	2.2	4.7	4.4	5.2	4.2	*	4.0	3.3				
10	1.2	1.1	1.2	1.0	2.0	1.7	20.1	3.1	2.4								
Positive no.	(0)	(0)	(1)	(0)	(0)	(2)	(10)	(9)	(8)	(3)	(7)	(4)	(5)	(5)	(7)		
POWV																	
1	1.1	1.1	1.2	1.0	1.1	2.4	1.8	2.4	3.1	1.2	2.6	2.1	2.5	5.0	3.1	9.9	
2	1.1	1.2	1.2	1.1	1.1	1.6	1.9	2.9	3.8	1.0	3.4	2.2	2.4	3.1	2.9	6.2	
3	1.0	1.0	1.1	1.0	1.1	1.2	1.1	2.9	1.3	0.7	1.1	0.8	1.2	1.1	0.8	5.4	
4	1.1	1.1	1.1	1.0	1.1	1.8	1.3	1.2	1.9	0.8	1.4	0.9	1.5	2.0	0.9	5.0	
5	1.3	1.4	1.0	1.0	1.5	1.1	1.1	1.6	1.5	1.0	1.0	0.7	1.3	0.6	0.5	2.3	
Positive no.	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(2)	(0)	(1)	(0)	(0)	(2)	(1)	(4)	
LACV																	
1	1.0	1.1	1.0	1.0	1.1	1.1	2.1	0.9	1.7	1.4	*	1.0	1.3	1.0	9.6	7.9	
2	1.2	1.3	1.0	1.2	1.1	1.1	1.0	1.0	1.4	1.4	1.4	0.8	1.8	1.1	1.3	6.6	
3	1.0	1.0	0.9	1.0	1.0	1.1	1.0	0.9	1.0	1.0	1.2	0.9	1.0	0.9	0.9	6.3	
4	1.1	1.1	1.0	1.0	1.2	1.0	1.0	1.0	0.9	0.9	1.0	1.1	1.2	1.0	0.5	5.5	
5	0.9	1.0	1.0	1.0	1.0	1.0	1.1	0.8	0.9	1.2	0.9	0.8	1.0	0.9	0.5	5.3	
Positive no.	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(1)	(5)	

^a *, uninterpretable specimens due to high absorbent value against normal mouse brain antigen. The P/Ns that are ≥3.0 are shown in bold, and the values in parentheses are numbers of specimens with P/N values that were ≥3.0.

as true positive, true negative, false negative, and false positive, was prepared to calculate PPV and NPV.

RESULTS

Selection of COS-1 cell lines continuously secreting DENV VLPs. We recently described the construction of expression plasmids containing DENV prM/M and E protein-encoding regions that direct the expression and secretion of DENV-2 (3), DENV-1, DENV-3, and DENV-4 (22) VLPs when electroporated into tissue culture cells. In order to select stably transformed COS-1 cell clones which continuously secrete DENV VLPs, the expression cassette of each DENV construct was subcloned into a pCDNA-3 vector containing the neomycin antibiotic resistance gene, which confers resistance to G418. Cells transfected with these constructs were propagated in G418-containing media to select cell clones with G418 resistance phenotypes that expressed DENV VLPs. Extracellular VLP secretion into the cell culture fluid was monitored at various stages by Ag-ELISA during the clonal selection to identify the clones with the highest secreted VLP titers. The COS-1 clones designated D1C1, D2E7, D3C10, and D4A12 were selected as the DENV-1, -2, -3, and -4 clonal cell lines, respectively. These four clones had cell morphology similar to untransformed COS-1 cells, achieved 100% confluence approximately 1 day after passage at a ratio of 1:3, and had maximum Ag-ELISA titers of at least 1:32 by day 4 (data not shown). VLPs prepared from clonal cell lines were identical to the VLPs derived from transiently transformed cells in their antigenic reactivity. Both types of VLP preparations were used interchangeably in the MAC-ELISA.

Use of VLPs and SMB antigens in the MAC-ELISA. Arbovirus-infected human serum panels, which consisted of a WNV panel ($n = 10$), a SLEV panel ($n = 5$), a POWV panel ($n = 5$), a LACV panel ($n = 5$), and a DENV panel ($n = 27$), were obtained from the DRL. WNV, SLEV, and POWV are the medically important flaviviruses in North America. LACV, a member of *Bunyavirus* of the California serocomplex, was the most prominent arbovirus in the United States prior to the introduction of WNV in 1999. Panels were assayed for the presence of IgM antibody by the MAC-ELISA. The assay results, expressed as the P/N ratio using VLPs or virus-infected SMB antigens, are summarized in Tables 1 and 2.

Recombinant VLP antigens for the four DENV serotypes, SLEV, WNV, and JEV were employed in MAC-ELISAs to determine the virus-specific IgM as well as the extent of cross-reactive IgM antibodies in various human serum panels (Table 1). MAC-ELISAs employing the DENV-2 VLPs detected cross-reactive IgM antibody in one specimen each of the SLEV and WNV serum panels (P/Ns of 3.6 and 3.0, respectively). None of the other DENV VLP preparations detected cross-reactive antibodies in any non-DENV serum panel. As expected, VLP antigens for members of the JEV serogroup (JEV, WNV, and SLEV) exhibited high degrees of cross-reactivity to antibodies produced against other members of this group. WNV and JEV VLPs detected cross-reactive IgM antibodies in two and five of five SLEV-infected serum specimens, respectively. SLEV and JEV VLPs detected cross-reactive antibodies in 2 and 9 of 10 serum specimens in the WNV

panel, respectively. None of the seven VLP antigens detected the existence of cross-reactive antibodies in either the POWV or LACV panels. Compared with the corresponding VLP antigen, SMB antigens exhibited less specificity. The mixture of virus-infected SMB antigens for the four DENV serotypes, the current antigen mixture used by ADB-DRL in screening serum specimens for antidengue antibody, detected cross-reactivity in panels for SLEV (4 of 5 specimens; P/N values ≥ 3.0 ; range, 4.0 to 14.3; average, 7.8), WNV (8 of 10 specimens; P/N values ≥ 3.0 ; range, 4.2 to 12.0; average, 6.8), and POWV (2 of 5 specimens; P/N values ≥ 3.0 ; range, 3.1 to 3.8; average, 3.5).

The DENV VLPs were then applied to the DENV panel ($n = 27$) in the MAC-ELISA for IgM antibody detection. The P/N ratios of 21 specimens from this DENV panel were determined previously by the ADB-DRL employing the mixture of DENV-1 to -4-infected SMB antigens (P/N values ≥ 3.0 ; range, 3.1 to 33.0; average, 9.3) (Table 2). The dengue panel numbers 22 to 27 were retested in this study using individual as well as a mixture of DENV SMB antigens. The mixture of DENV-1 to -4 VLP antigens detected IgM antibody in 21 of 27 samples (P/N values ≥ 3.0 ; range, 4.1 to 14.4; average, 7.1). The VLP mixture did not detect IgM in 6 of 27 positive specimens; however, three additional serum specimens (specimen nos. 22, 23, and 24) tested positive by DENV-1, -2, or -4 VLP antigens. Three remaining specimens (nos. 25, 26, and 27) reported as positive by ADB-DRL as well as after retest in this study were completely negative by all seven VLP antigens (SLEV, WNV, JEV, and DENV-1 to -4). Unfortunately, due to insufficient volume of serum we were not able to perform the plaque reduction neutralizing assay to confirm the presence of virus-neutralizing antibodies. Thus, these three serum specimens were grouped as the unknown panel (Table 2) and excluded from further analysis.

Determination of the best cutoff for the P/N ratio by ROC analysis. ROC curve analysis was applied to determine the best cutoff for the P/N ratio to discriminate the evidence of infection in SLEV, WNV, or DENV serum panels, respectively, versus the combined panels of LACV, POWV, and negative serum specimens (Fig. 1). For SLEV VLP, the best cutoff for the P/N ratio to determine the SLEV infections was 2.0, which had 100% sensitivity and 100% specificity. For SLEV SMB, the cutoff was set at 3.0 to achieve 100% sensitivity and 90% specificity. For WNV VLP, the best cutoff for the P/N ratio to determine the WNV infection was 2.0, which turned out to give 100% sensitivity and 100% specificity. For WNV SMB, a cutoff of 3.0 was required to achieve 87.5% sensitivity and 90% specificity. For use of the mixture of DENV-1 to -4 VLPs to determine the DENV infection, the best cutoff P/N ratio of 2.0 achieved 95.8% sensitivity and 100% specificity. For the mixture of DENV-1 to -4 SMB antigens, a cutoff of 3.0 was required to achieve 95.8% sensitivity and 100% specificity. For individual DENV VLPs, the best cutoffs for DENV-1, -2, -3, and -4 were at 1.0, which all resulted in 100% sensitivity and 100% specificity (Fig. 2).

Influence of VLPs and SMB antigens on assay performance measured by comparative ROC analysis. Based on the calculation of the best cutoff for the P/N ratio, we transformed the numeric P/N ratio into five discrete categories: 0, P/N < 2 (definitive negative); 1, P/N ≥ 2 and < 3 (presumptive positive); 2, P/N ≥ 3 and < 4 (probable positive); 4, P/N ≥ 4 and

TABLE 2. Comparison of the recombinant virus-like particle and virus-infected mouse brain dengue antigens in detecting IgM antibodies in human serum specimens with the evidence of dengue virus infection

Panel and no.	P/N ^a														
	Virus-like particle								Suckling mouse brain antigen						
	DENV1-4	DENV-1	DENV-2	DENV-3	DENV-4	SLEV	WNV	JEV	DENV1-4	DENV-1	DENV-2	DENV-3	DENV-4	SLEV	WNV
DENV panel															
1	14.4	14.4	11.4	9.0	11.9	2.7	5.9	9.1	5.8						
2	12.5	13.2	8.9	14.2	14.9	6.3	23.4	32.9	26.8						
3	10.6	9.4	9.8	14.4	11.3	2.3	5.1	4.0	23.7	27.6	19.0	18.9	10.8		
4	10.0	14.8	4.1	11.7	2.8	1.1	1.8	1.3	33.0						
5	9.5	9.1	10.9	13.4	6.6	1.9	3.6	5.0	6.1						
6	9.3	12.6	9.2	5.4	4.3	1.3	2.0	3.2	5.4						
7	7.7	10.8	7.7	4.0	5.2	1.7	2.4	4.0	5.9						
8	6.6	4.2	7.6	3.9	5.0	1.8	4.3	3.4	7.1						
9	6.5	4.4	12.3	4.1	3.4	1.4	1.9	1.8	5.3						
10	6.3	1.6	10.9	1.6	1.5	1.1	1.3	1.3	7.9						
11	5.9	5.9	6.3	1.7	5.2	1.1	1.6	1.3	4.2						
12	5.9	6.1	7.1	6.7	7.0	5.3	19.3	17.1	25.0						
13	5.5	8.3	3.0	4.1	2.0	1.6	1.6	1.5	3.1						
14	5.4	3.2	3.7	6.9	2.3	1.6	3.1	2.8	7.9						
15	5.2	4.6	7.4	2.5	4.1	2.0	9.7	8.2	4.8						
16	5.1	4.9	6.2	6.9	4.2	1.4	2.2	3.5	8.7						
17	5.0	5.0	4.9	3.1	5.2	10.1	35.9	25.4	8.9						
18	4.8	2.2	6.2	2.3	2.4	1.2	1.5	1.5	8.9						
19	4.6	3.0	5.6	2.4	3.2	2.2	5.1	4.3	6.6						
20	4.4	3.6	9.1	3.2	4.4	3.4	11.4	11.9	4.4						
21	4.1	1.7	6.8	1.3	1.4	1.1	1.3	1.3	4.5						
22	2.8	3.4	2.0	2.6	1.9	1.5	1.6	2.0	6.7	11.1	4.7	5.1	2.8		
23	2.8	3.0	3.5	2.7	1.7	1.4	1.6	1.4	5.0	7.7	3.8	4.6	2.7		
24	2.0	1.9	2.7	1.4	3.7	2.3	5.8	5.3	7.9	6.8	7.4	8.6	5.5		
Positive no.	(21)	(20)	(22)	(15)	(16)	(4)	(12)	(14)	(24)						
Unknown panel															
25	2.1	0.9	1.0	0.9	1.1	1.0	0.9	0.9	4.6	1.3	1.1	1.1	5.6	1.2	1.0
26	1.3	1.1	1.2	1.1	2.1	1.1	0.9	0.9	5.5	1.2	1.0	1.0	5.4	1.3	1.2
27	1.1	1.1	1.0	1.0	1.1	1.3	1.5	1.4	7.5	*	*	*	*	3.9	*
Positive no.	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(3)	(0)	(0)	(0)	(2)	(1)	(0)
Negative control panel															
1	2.1	1.0	1.3	1.0	1.1	1.1	1.1	1.0	1.9						
2	1.2	1.1	1.0	1.0	1.1	1.0	1.0	1.0	1.0						
3	2.1	0.9	1.0	0.9	1.4	1.1	1.1	1.0	2.6					1.1	1.2
Positive no.	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)						

^a *, uninterpretable specimens due to high absorbent value against normal mouse brain antigen. P/N values that are ≥ 3.0 are shown in bold, and the values in parentheses are numbers of specimens with P/N values that were ≥ 3 .

< 5 (positive); and 5, $P/N \geq 5$ (definitive positive). We then used these transformed data to compare the influence of VLPs and SMB antigen on overall assay performance and to determine whether there was any statistical significance between them in terms of sensitivity and specificity (Table 3). A P/N of <3 or ≥ 3 was used in classification of a given specimen as assay negative or positive, respectively, and to define the evidence of infection (target panel) or noninfection (control panels), respectively. The overall performance of the MAC-ELISA using VLPs was significantly better than the MAC-ELISA using SMB antigens, as determined in pairwise comparison. The AUCs were 0.99 (95% CI, 0.91 to 1.00) and 0.83 (95% CI, 0.70 to 0.92) for the mixture of all four DENV VLPs and SMB antigens, respectively, with $P = 0.02$. The AUCs were 1.00 (95% CI, 0.84 to 1.0) and 0.94 (95% CI, 0.75 to 0.99) for SLEV VLP and SMB antigens, respectively, with $P = 0.44$. The WNV VLPs and SMB antigen pair was statis-

tically different. The AUCs were 0.96 (95% CI, 0.78 to 0.99) and 0.85 (95% CI, 0.64 to 0.96) for WNV VLP and SMB, respectively, with $P = 0.05$.

Determination of the status of infection and seroprevalence.

The positive likelihood ratio can be used to predict the evidence of infection for an individual based on the P/N ratio reading of the assay from the patient's serum specimen. The +LR is influenced by the best cutoff for P/N of the assay (Table 4). A P/N of ≥ 3 was used as an example to define the evidence of infection in the individual's serum specimen by the MAC-ELISA. The +LRs were 20, 7.5, and ∞ for SLEV, WNV, and DENV infection, respectively, when the respective SLEV, WNV, and mixture of DENV VLPs were used; however, the +LRs were 3.4, 4.4, and 12.5 using respective SMB antigens. Use of VLPs thus significantly improved the predictive value of determining the evidence of infection compared to use of SMB antigens.

FIG. 1. Determination of the best cutoff positive/negative (P/N) ratio in the MAC-ELISA using either VLPs or virus-infected SMB antigens. DENV1-4, mixture of dengue virus types 1, 2, 3, and 4. P/N ratios (y axis) of each tested specimen obtained by the MAC-ELISA using VLPs (left panels) or SMB antigens (right panels) is indicated by $\square$. The disease or nondisease states of tested specimens are indicated in the x axis. The best cutoff ratio (the horizontal line intersecting the y axis) was predicted by ROC analysis (details in Materials and Methods). The test performance is indicated by the percentage of sensitivity (Sens) and specificity (Spe).

FIG. 2. Determination of the best cutoff positive/negative (P/N) ratio in the MAC-ELISA using VLPs of individual dengue serotype viruses (DENV). The P/N ratio (y axis) of each tested specimen obtained by the MAC-ELISA is indicated by □. The disease or nondisease states of tested specimens are indicated in the x axis. The best cutoff ratio (the horizontal line intersecting the y axis) was predicted by ROC analysis (details in Materials and Methods). The test performance is indicated by the percentage of sensitivity (Sens) and specificity (Spe).

In addition, when a P/N of ≥ 3 was used to define the evidence of infection (the current criteria used by the DRL) and applied to a large-scale seroepidemiologic study, the SLEV and WNV VLPs also had higher PPVs (71.4% and 83.3%, respectively) and NPVs (100% and 100%, respectively) than

the PPVs (41.7% and 63.6%) and the NPVs (100% and 78.6%) for tests using SMB antigens of SLEV and WNV. The mixture of DENV VLP had 100% PPV and 81.3% NPV, compared to the mixture of DENV SMB (92.3% PPV and 100% NPV) (Table 5).

TABLE 3. The influence of VLPs or SMB antigens on the performance of MAC-ELISA to distinguish the infection serum panel (target panel) from other arbovirus-infected serum panels (control panels)

Target panel	Control panels	Antigen and type	AUC (95% CI) ^a	Discriminatory probability (95% CI)	
				Sensitivity	Specificity
DENV	WNV+SLEV+POWV+LACV	DENV-1 to -4 VLP SMB	0.99 (0.91–1.00)* 0.83 (0.70–0.92)	87.5 (67.6–97.2) 100.0 (85.6–100.0)	100.0 (87.5–100.0) 50.0 (30.7–69.3)
SLEV	WNV+POWV+LACV	SLEV VLP SMB	1.00 (0.84–1.00) 0.94 (0.75–0.99)	100.0 (48.0–100.0) 100.0 (48.0–100.0)	90.0 (68.3–98.5) 58.8 (33.0–81.5)
WNV	SLEV+POWV+LACV	WNV VLP SMB	0.96 (0.78–0.99)† 0.85 (0.64–0.96)	100.0 (69.0–100.0) 87.5 (47.4–97.9)	86.7 (59.5–98.0) 73.3 (44.9–92.0)

^a *, $P = 0.02$; †, $P = 0.05$.

TABLE 4. Influence of VLPs and virus-infected SMB antigens on the MAC-ELISA and the positive/negative ratio used to predict the evidence of infection

P/N ratio	+LR					
	DENV 1-4		WNV		SLEV	
	VLP	SMB	VLP	SMB	VLP	SMB
<2	6.5	4.3	2.5	1.6	2.2	1.7
≥2	6.2	6.5	7.5	3.3	10.0	2.4
≥3	∞	12.5	7.5	4.4	20.0	3.4
≥4	∞	∞	15.0	6.6	∞	8.5
≥5	∞	∞	∞	13.1	∞	17.0

DISCUSSION

Antigens secreted as VLPs by cloned cell lines are easily collected from the culture medium, concentrated by polyethylene glycol or ultracentrifugation, and lyophilized for long-term storage. Such preparations function well in both MAC-ELISA and IgG-ELISA formats (5, 14, 23). The results of the present study showed a higher specificity with VLPs in the MAC-ELISA than with SMB antigens and simultaneously showed degrees of sensitivity similar to those seen in a previous study with DENV (21). The higher specificity with VLPs than with SMB antigens in the MAC-ELISA could be due to the better quality and higher purity of VLPs compared to SMB antigens. Viral infectivity in the SMB preparation is eliminated by chemical inactivation, and the lipid components in the mouse brain are removed by the acetone extraction procedures. There is no additional purification procedure involved in preparing SMB antigens. Conversely, VLPs are prepared from cell culture fluids by ultracentrifugation, which concentrates particulates or protein aggregates. Some nonviral proteins were coconcentrated with VLPs by this procedure; however, the viral E protein was clearly visible as the major protein band in a total protein stain gel (22).

The measurement of assay performance by ROC analysis indicated a statistically significant difference between the use of VLPs and SMB antigens for WNV and the mixture of DENV-1 to -4. VLPs of all seven flaviviruses had lower cutoff P/N ratios than the corresponding SMB antigens. The higher PPV and +LR for VLPs also indicated that these antigens performed better than SMB antigens in the MAC-ELISA. Cell lines continuously secreting VLPs are therefore significantly improved sources of flaviviral serodiagnostic antigens compared with the traditional sources of virus-infected tissue culture or suckling mouse brain.

The presence of antibodies developed to previous flavivirus infection or vaccination complicate flavivirus serodiagnosis. Differentiation between primary and secondary infections is

possible by determining the ratio of IgM to IgG in acute-phase sera (15, 29). This requires the simultaneous testing of serum specimens for both IgM and IgG by ELISA. Determination of a fourfold or greater increase in IgM and/or IgG antibody titers between paired sera collected during the acute and convalescent phases is the most reliable method for determination of an active flavivirus infection, but this requires the collection of at least two serum samples from an infected individual, which may not be possible in all cases. A protocol to determine the IgG antibody avidity of a single serum sample has been proposed to differentiate primary and secondary DENV infection (6). This protocol involves treatment of antigen-antibody complexes with a urea solution as the dissociation agent to determine the avidity index of samples. In primary tick-borne encephalitis virus infections, specific IgG antibodies with low avidity are initially produced, with the avidity generally increasing during the maturation of the humoral immune response (8). If the virus-specific IgG antibody present in a secondary response has higher avidity than that of cross-reactive antibody, it might be possible to reduce the detection of cross-reactive antibodies in ELISA by treatment with urea. However, the urea treatment increases the complexity of the assay without addressing the fundamental problem of cross-reactive antibodies that recognize cross-reactive epitopes on the current serodiagnostic antigens, VLPs as well as virus-infected antigens.

Reduction of cross-reactive epitopes on the VLPs by structure-based mutagenesis (4) may allow for use of a single assay in DENV serotyping and differentiation between primary and secondary infections. We demonstrate here that some flavivirus VLP antigens are more specific than SMB antigens. Replacement of the virus-infected SMB antigen preparations in conventional ELISAs with VLP antigens may be the first step in reducing the detection of cross-reactive antibodies. This cross-reactivity may be further reduced by identifying and modifying cross-reactive epitopes in these VLPs. The E protein, the major determinate for eliciting neutralizing antibodies and inducing protective immunity, consists of three structural domains designated DI, DII, and DIII. DI and DIII contain type- and subtype-specific epitopes, and DII contains the major flavivirus group-, complex-, and subgroup-cross-reactive epitopes. Six residues forming three distinct flavivirus cross-reactive epitopes recognized by the murine monoclonal antibodies in the E glycoprotein DII have been recently characterized in DENV-2 (4). Modification of these sites reduced the cross-reactivity of VLPs to flavivirus group-reactive antibodies while retaining reactivity to type-specific antibodies. Application of this experimental design to other dengue serotypes might facilitate the identification of serotype-cross-reactive epitopes and the development of more type-specific VLP antigens for use in single MAC-ELISAs for DENV serotyping as well as differentiation between primary and secondary infections. A similar approach is currently being applied to the members of the Japanese encephalitis serocomplex.

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TABLE 5. Predictive values at P/Ns of ≥3

Predictive value	Value (%) at P/N of ≥3					
	DENV1-4		WNV		SLEV	
	VLP	SMB	VLP	SMB	VLP	SMB
PPV	100.0	92.3	83.3	63.6	71.4	41.7
NPV	81.3	100.0	100.0	78.6	100.0	100.0

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