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Differentiation of West Nile and St. Louis Encephalitis Virus Infections by Use of Noninfectious Virus-Like Particles with Reduced Cross-Reactivity▽†

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Differential diagnosis of St. Louis encephalitis virus (SLEV) and West Nile virus (WNV) infections can be complicated due to the high degree of cross-reactivity observed in most serodiagnostic assays. In an effort to provide a more specific diagnostic test, we developed virus-like particle (VLP) antigens with reduced cross-reactivity for both SLEV and WNV by identifying and mutating envelope protein amino acids within the cross-reactive epitopes of VLP expression plasmids. To determine the serodiagnostic discriminatory ability of the antigens with reduced cross-reactivity, a panel of 134 human serum samples collected predominately from North American patients with SLEV or WNV infections was used to evaluate the performance of these novel antigens in imunoglobulin M antibody-capture enzyme-linked immunosorbent assays. Positive/negative ratios and the resulting diagnostic classifications were compared between the mutant and the wild-type (WT) VLPs. The mutant VLP antigens were more specific, with higher positive predictive values and higher likelihood ratios than the WT VLP antigens. Both the SLEV and WNV mutant VLPs greatly reduced the observed cross-reactivity, significantly increasing the specificity and sensitivity of the assay. The use of these novel VLP antigens with reduced cross-reactivity in these serodiagnostic assays and others should lead to more accurate diagnoses of current infections, thereby reducing the need for time-consuming and cumbersome confirmatory plaque-reduction neutralization tests to differentiate between SLEV and WNV infections in North America.

Members of the genus *Flavivirus* have an ~11-kb single-stranded positive-sense RNA genome. The viruses belonging to this genus are the causative agents of diseases such as dengue hemorrhagic fever; dengue and yellow fever; and Japanese, St. Louis, West Nile, and tick-born encephalitis. Human infections range from asymptomatic to self-limiting mild flu-like illness to hemorrhagic fever or encephalitis (1).

A previously described immunoglobulin M (IgM) antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) and antigen-capture IgG ELISA were developed for the diagnosis of arbovirus-infected human serum samples (12, 16). While valuable tools for diagnosing presumptive flaviviral infections, these assays use antigens prepared from virus-infected suckling mouse brains (SMB). The procedure for producing SMB antigen is time-consuming and costly and requires personnel to use hazardous chemicals and infectious agents. During natural flavivirus infections, noninfectious virus-like particles (VLPs) consisting of viral premembrane/membrane (prM/M) and envelope (E) proteins are produced in addition to mature, infectious virions (23). Flavivirus VLPs have physiochemical and antigenic properties similar to those of mature virus particles; however, VLPs do not contain nucleocapsid or RNA and are not infectious (8). We have previously described an expression system developed for several flaviviruses to direct the production of secreted extracellular VLPs in mammalian cell culture (2, 3). Flavivirus VLPs have been shown to have higher sensitivity and specificity than SMB antigen in diagnostic assays and are safe, standardized, and cost-effective alternatives (7, 10, 11, 18).

Human flavivirus infections appear to provide lifelong immunity to the infecting virus, yet only temporally transient protection to heterologous flavivirus infections (14). Protective virus-specific antibodies are elicited by the E glycoprotein, which contains both virus-specific and flavivirus cross-reactive epitopes. Using mouse monoclonal antibodies (MAbs), the majority of the cross-reactive epitopes have been mapped onto domains I and II of the E protein (5, 19–22, 24). Although nonprotective, the large proportion of cross-reactive antibodies elicited from these immunodominant epitopes can complicate serodiagnosis, particularly in secondary infections: often necessitating the use of the time consuming, confirmatory plaque-reduction neutralization test (PRNT) (14, 25, 26). This "cross-reactivity problem" exists in all assays using wild-type (WT) diagnostic antigens, either SMB or VLP, because these antigens contain the same highly conserved immunodominant E glycoprotein epitopes responsible for eliciting the cross-reactive serum antibodies during viral infection.

Because the E proteins of VLPs can be readily manipulated via site-directed mutagenesis of VLP expression plasmids, we have applied a rational approach to systemically identify and ablate immunodominant cross-reactive E-protein epitopes (4). We performed extensive mutagenesis across the E glycoproteins of the two major encephalitic flaviviruses cocirculating in America, St. Louis encephalitis virus (SLEV) and West Nile

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virus (WNV). VLPs expressed from mutated plasmids were assayed to determine the mutational effects on both VLP secretion and antibody reactivity, exhibiting dramatic reductions in cross-reactivity (6, 27).

In this study, we selected SLEV and WNV VLPs with reduced cross-reactivity to evaluate as serodiagnostic antigens in IgM ELISA for the differentiation of WNV and SLEV infections. Differential antigen performance between the mutant and WT VLP antigens was evaluated using human serum samples from patients infected with SLEV or WNV and with other members of the flaviviruses (Japanese encephalitis virus [JEV], dengue virus [DENV], and Powassan virus [POWV]), alphaviruses (Eastern equine encephalitis virus [EEEV] and Western equine encephalitis virus [WEEV]), and a bunyavirus (LaCrosse virus [LACV]) as infected serum controls. Compared to their WT counterparts, the mutant antigens exhibited less cross-reactivity and increased assay specificity and sensitivity and resulted in higher positive predictive values (PPVs) and likelihood ratios. Incorporation of these novel antigens with reduced cross-reactivity into ELISA or other serodiagnostic assays should improve the ability to accurately and rapidly differentiate between WNV and SLEV infections in North America.

MATERIALS AND METHODS

Cell culture, construction of plasmids, and antigen production. CHO-K1 cells (CCL-61; ATCC, Manassas, VA) were grown in a mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F-12) (Gibco Laboratories, Grand Island, NY). The medium was supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 110 mg/liter sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, penicillin (100 U/ml), and 100 μg of streptomycin per ml. Cells were grown for transformation to 90% confluence in 150-cm^2 culture flasks, trypsinized, and resuspended in ice-cold phosphate-buffered saline (PBS) to a final density of 1.5×10^7cells/ml. For the expression of SLEV and WNV VLPs, CHO cells were transiently transfected with the WT plasmids pCB8SJ2 and pCBWN, and the mutant E-protein plasmids with reduced cross-reactivity were derived from them. These WT plasmids and the mutants with reduced cross-reactivity derived from them have been previously described in detail (6, 7, 10, 11, 18, 27). A cell suspension (0.5 ml) and plasmid DNA (30 μg) were combined in a 0.4-cm electrode-gap cuvette. The cell-DNA suspension was electroporated with a Bio-Rad Gene Pulser II (Bio-Rad Laboratories, Hercules, CA) set at 250 V and 975 μF. A single 150-cm^2 culture flask containing 50 ml DMEM/F-12 was seeded with two reaction mixtures. The tissue culture medium was collected 48 to 60 h after electroporation.

Antigen characterization. The VLP-containing tissue culture fluids were clarified at 10,000 rpm for 30 min and concentrated from culture media at 4°C by overnight ultracentrifugation at 19,000 rpm in a Beckman Coulter type 19 rotor (Beckman Coulter, Fullerton, CA). The pellet was resuspended in TN buffer (50 mM Tris, 100 mM NaCl [pH 7.5]) to 1/100 the original volume, aliquoted into 250-μl samples, and stored at −70°C. These concentrated antigens were characterized in antigen-capture ELISA, based on levels of secretion and degrees of reactivity to a panel of flavivirus E-specific MAbs of various levels of cross-reactivity (6, 27).

Human serum. Previously characterized WNV- and SLEV-infected human serum specimens were obtained from the Diagnostic and Reference Laboratory (DRL), Arboviral Diseases Branch, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO. Non-WNV/SLEV-infected sera having serological evidence for DENV, JEV, POWV, LACV, EEEV, and WEEV and serum specimens of unknown etiologic origin were included as control panels. Based on the previously determined DRL serological results, we specifically selected a large number of sera exhibiting high levels of IgM and IgG cross-reactivity between WNV and SLEV in an attempt to maximize the difficulty of serodiagnostic discrepancy for our assays. These previously determined DRL diagnostic classifications were based on IgM and IgG ELISA results using only WT antigens, VLP or SMB for WNV, and SMB only for SLEV. Almost all of the selected sera had PRNT titers for both WNV and SLEV associated with them as this was the confirmatory test used by the DRL to diagnose these highly cross-reactive sera. The virus responsible for the most recent WNV or SLEV infection was defined by DRL as that having the higher IgM reactivity and a neutralizing antibody titer at least fourfold greater than that for any other virus tested or as that virus exhibiting similar results from the testing of paired acute/convalescent-phase sera. Positive/negative (P/N) absorbance ratios of these specimens for MAC-ELISA with SLEV SMB antigen, WNV SMB antigen, or WNV WT VLPs were determined by the DRL. Tables 1 and 2 show that DRL classified 56 of the 134 sera as WNV infected, whereas this study classified only 55 sera as WNV infected. This discrepancy occurs because three sera classified as WNV infected by DRL were classified as "unknowns" in this study (sera 93, 109, and 115 in Table 6 and see Table S1 in the supplemental material), and two DRL unknowns were classified as WNV infected in this study (sera 98 and 103 in Table 6 and see Table S1 in the supplemental material), resulting in one more WNV-infected serum in the DRL disease state classification than in this study. Similarly, the presence of additional SLEV-infected sera in the DRL disease state classification relative to this study's is explained by differential classification of sera 42, 57, and 62 (Table 6 and see Table S1 in the supplemental material). The JEV and DENV serum specimens provided by Center for Disease Control—Taiwan were obtained from the Taiwanese population, and the currently infecting virus was determined by virus isolation and/or virus-specific nucleic acid detection and antigen-capture IgM ELISA. Taiwan is in an area of JEV endemicity, and mandatory, nationwide JEV vaccination has been implemented since the late 1960s. Thus, some of the DENV-infected serum specimens could be from patients previously vaccinated or exposed to JEV.

ELISA protocols. SLEV-WT and both SLEV and WNV mutant VLPs were prepared as described above. Lyophilized preparations of WNV VLPs and normal COS-1 cell culture antigen, prepared as previously described, were rehydrated in 0.5 ml of distilled water (7). Antigens were independently titrated, in the same MAC-ELISA format described below, against a positive control serum sample with a twofold-dilution series and standardized by selecting the dilution yielding an A_{450} of 0.2 to 0.5. The antigen dilutions selected were 1:10, 1:200, 1:20, and 1:100 for SLEV-WT, SLEV-DRR, WNV-WT, and WNV-RH, respectively.

For detection of the presence of IgM in serum panels with the VLPs, we performed MAC-ELISA as previously described with some modifications (16). Briefly, the inner 60 wells of Immulon II HB flat-bottom 96-well plates (Dynatech Industries, Inc., Chantilly, VA) were coated with 75 μl 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]). The plates were incubated overnight at 4°C in a humidified chamber. Wells were blocked with 300 μl of StartingBlock (PBS) blocking buffer (Pierce, Rockford, IL) according to the manufacturer's recommended procedure.

Patient sera and positive and negative control sera were diluted 1:400 in wash buffer (0.5% Tween 20, 9.25 g/liter FTA hemagglutination buffer [Becton Dickinson and Company, Sparks, MD] in distilled water), added to wells (50 μl/well), and incubated at 37°C for 1 h in a humidified chamber. Positive and negative control antigens were diluted appropriately in wash buffer. Both positive and negative antigens were tested with each serum sample in triplicate, 50 μl was added to the appropriate wells, and plates were incubated at 4°C overnight in a humidified chamber. SLEV and WNV murine hyperimmune ascitic fluids were diluted 1:1,000 in PBS containing 5% nonfat dry milk, 50 μl was added to appropriate wells, and plates were incubated for 1 h at 37°C in a humidified chamber. Horseradish peroxidase-conjugated goat anti-mouse IgG was diluted 1:5,000 in PBS containing 5% nonfat dry milk, and 50 μl was added to each well. Plates were incubated for 1 h at 37°C in a humidified chamber. Bound conjugate was detected by adding 75 μl of 3,3′5,5′-tetramethylbenzidine (Neogen Corp., Lexington, KY) substrate and incubating plates in the dark at room temperature for 10 min. The reaction was stopped with 50 μl of 2N H_2SO_4, and the reactions were measured at A_{450} using a Synergy HT Multi-Detection Microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Preliminary serum panel. Three different E proteins derived from three mutated plasmids each for WNV and SLEV were selected and compared to the WT for a preliminary serum panel to determine the optimal antigens for the test panel (6, 27). The plasmid constructs compared were WNV-G106R/L107R (WNV-RR), WNV-G106R/L107H (WNV-RH), WNV-G106V/L107R (WNV-VR), SLEV-G106D/L107R/H246R (SLEV-DRR), SLEV-G106D/L107D/H246R (SLEV-DDR), and SLEV-G106Q/L107F/H246R (SLEV-QFR). The preliminary panel consisted of four SLEV-positive sera, four WNV-positive sera, and two non-SLEV/WNV-infected sera. Each serum sample was tested with each antigen in triplicate in the MAC-ELISA. We selected mutant antigens exhibiting the greatest specificity and

TABLE 1. Summary of MAC-ELISA results for 134 sera tested with WT and mutant SLEV and WNV antigens[a]

Disease state	DRL diagnostic serum classification					This study's diagnostic serum classification				
	No. of sera	No. of IgM-positive sera				No. of sera	No. of IgM-positive sera			
		SLEV		WNV			SLEV		WNV	
		WT	DRR	WT	RH		WT	DRR	WT	RH
SLEV	38	34	36	17	9	37	35	37	17	9
WNV	56	27	10	53	53	55	28	11	55	55
Non-SLEV/WNV flavivirus (JEV, DENV, POWV):	10	0	0	4	0	10	0	0	4	0
JEV	4	0	0	2	0	4	0	0	2	0
DENV	4	0	0	2	0	4	0	0	2	0
POWV	2	0	0	0	0	2	0	0	0	0
Flavivirus	16	4	2	4	2	3	2	0	2	0
Unknown flavivirus	8	4	2	4	2	3	2	0	2	0
Previous SLEV, WNV, or flavivirus	8	0	0	0	0	NA[b]				
Nonflavivirus (WEEV, EEEV, LACV)	12	0	0	0	0	12	0	0	0	0
Unknown	2	0	0	0	0	17	0	0	0	0
Total	134	65	48	78	64	134	65	48	78	64

[a] All 134 sera were tested in this study with WT and mutant VLP antigens for both WNV and SLEV. Results are grouped here and for further analyses according to disease states determined either (i) previously by the DRL or (ii) by using this study's diagnostic classification. As described in detail in Materials and Methods, DRL disease state classifications are based upon MAC- or antigen-capture IgG-ELISA results using WT SLEV and WNV antigens only and PRNT data. This study's diagnostic classifications were based solely on MAC-ELISA results using WT and mutant VLP antigens without prior knowledge of the DRL classifications. The different numbers of WNV- and SLEV-infected sera classified by DRL and in this study are explained in detail in Materials and Methods and in Table 6.
[b] NA, not applicable.

least cross-reactivity, while maximizing VLP secretion for later analysis with the complete serum panel.

Test validation and calculation of P/N values. Test validation and P/N ratios were calculated as previously described (15). Briefly, an internal positive and negative serum control was included on each plate. For a plate to be considered valid, the average A_{450} for the positive serum control reacted with positive viral antigen must be at least two times greater than the same positive control sera reacted with negative antigen. Each patient sample was validated in this manner.

Positive values for each specimen were determined as the average A_{450} for the patient serum sample reacted with positive viral antigen. Negative values for each plate were determined as the average A_{450} of the normal human serum control reacted with positive viral antigen. A sample was classified as positive if the P/N ratio was ≥3.0. For each specimen, the ratios of WNV-P/N to SLEV-P/N (W/S ratio) and SLEV-P/N to WNV-P/N (S/W ratio) were calculated for both the WT and mutant antigens (17). For W/S and S/W ratios, where the numerator P/N value was <3.0 (and thus negative for WNV or SLEV IgM), the ratio was assigned a value of zero.

TABLE 2. MAC-ELISA results for WNV- and SLEV-infected sera[a]

Antigen and serum type based on disease state[a]	Total no. of sera	No. (%) of results for:			
		WNV-WT	WNV-RH	SLEV-WT	SLEV-DRR
WNV infected					
This study					
Positive	55	55 (100)	55 (100)	28 (51)	11 (20)
Negative		0	0	27 (49)	44 (80)
DRL					
Positive	56	53 (95)	53 (95)	27 (48)	10 (18)
Negative		3 (5)	3 (5)	29 (52)	46 (82)
SLEV infected					
This study					
Positive	37	17 (46)	9 (24)	35 (95)	37 (100)
Negative		20 (54)	28 (76)	2 (5)	0
DRL					
Positive	38	17 (45)	9 (24)	34 (89)	36 (95)
Negative		21 (55)	29 (76)	4 (11)	2 (5)

[a] See Table 1 and Materials and Methods for a description of how the different disease state classifications were determined.

Data and disease state classifications. All 134 sera were randomly coded and blind tested concurrently using WNV-WT and SLEV-WT antigens and the two antigens with reduced cross-reactivity selected from the preliminary panel: WNV-RH and SLEV-DRR. The ELISA results were interpreted without prior knowledge of DRL interpretation. It is important to note that all disease state classifications were made based on MAC-ELISA data obtained for the mutant antigens alone; interpretations made from both the mutant and WT-VLP ELISA data will be noted where appropriate. Both WNV and SLEV infections were classified based on the same criteria. For the sake of brevity, the criteria are outlined using the SLEV panel as the example. Samples producing positive P/N values (P/N, ≥3.0) for SLEV-DRR in the MAC-ELISA and negative P/N values (P/N, <3.0) for WNV-RH in the MAC-ELISA were classified as "SLEV infections." "Presumptive secondary SLEV infections" were positive for SLEV-DRR in the IgM assay, yet also produced positive P/N values with WNV-RH in the MAC-ELISA (although of a smaller magnitude). Sera producing positive P/N values in the MAC-ELISA when reacted on WT-VLPs but which were negative with the mutant antigens were classified as resulting from individuals exposed to an "unknown flavivirus." Sera exhibiting no reactivity in the MAC-ELISA with the WT or mutant antigens were classified negative for SLEV and WNV and of "unknown etiology" (Table 1 and see Table S1 in the supplemental material).

Statistical analysis. The receiver-operator characteristic (ROC) curve, a plot of sensitivity versus specificity, was applied to determine the discriminatory accuracy of the tests in question using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). The overall ability of the test's accuracy to discriminate between those cases with and without the disease is quantified by the area under the ROC curve (AUC). A nonparametric method was used to calculate the AUC and 95% confidence interval (95% CI). A test with an AUC of 1.0 has zero false positives and zero false negatives; a test with an AUC of 0.5 is neither sensitive nor specific. We determined the AUC two different ways: we defined the disease state for both WNV and SLEV cases based upon this study's

TABLE 3. Influence of WT and mutant antigens on performance of MAC-ELISA in distinguishing the infected serum panel from other arbovirus-infected serum panels[a]

Target panel	Control panels	Antigen	AUC (95% CI)	Critical ratio (*z*)	Discriminatory probability (95% CI)	
					% Sensitivity	% Specificity
WNV infections	SLEV and remaining groups	WNV-WT VLP	0.97 (0.95–1.00)	1.34	100.0 (93.5–100.0)	70.9 (59.6–80.6)
		WNV-RH VLP	0.99 (0.97–1.00)		100.0 (93.5–100.0)	88.6 (79.5–94.7)
SLEV infections	WNV and remaining groups	SLEV-WT VLP	0.94 (0.90–0.98)	2.18	94.6 (81.8–99.3)	69.1 (58.9–78.1)
		SLEV-DRR VLP	0.98 (0.96–1.00)		100.0 (90.5–100.0)	88.7 (80.6–94.2)

[a] Shown is the influence of WT and mutant antigens on performance of MAC-ELISA in distinguishing the infected serum panel (target panel) from other arbovirus-infected serum panels (control panels) using a P/N ratio of ≥3 as the cutoff for the presence of antigen-specific IgM. All statistical analyses are based upon the panels as defined by our disease state classification.

classifications and then again based upon the DRL classifications; the nondisease state consisted of all other serum samples. ROC curves derived from tests that were evaluated in the same group of patients and controls were compared to determine if the observed difference in AUC was random. We employed the method described by Hanley et al. (9). The general approach for this analysis involves calculating the critical ratio. Paired-ROC curves producing a critical ratio of ≥1.96 were determined to have ROC areas that are statistically different; the value of 1.96 indicates that the areas of the curves are 2 or more standard deviations apart, suggesting that there is only a ≤5% chance that the observed difference occurred by chance.

Calculation of PPVs and NPVs. A P/N ratio of <3 or ≥3 for a given specimen was classified as negative or positive, respectively. A 2-by-2 contingency table was prepared which categorized four quadrants as true positive, true negative, false positive, and false negative. The PPV is the fraction of people with positive tests who actually have the disease, and the negative predictive value (NPV) is the fraction of people with negative tests who do not have the disease.

RESULTS

Preliminary panel. Three different mutant VLPs with reduced cross-reactivity for WNV and SLEV were selected to compare as antigens in MAC-ELISA screening of the preliminary serum panel. WNV-RH and SLEV-DRR were selected as the final antigens in the assays because these mutants exhibited the greatest type specificity, the least cross-reactivity, and enhanced or comparable VLP secretion to the WT antigens. WNV-VR and SLEV-QFR, after analysis, were easily identified as the most cross-reactive and least type-specific of the antigens tested in the preliminary panel and were excluded from further statistical analysis. The remaining antigens were analyzed by ROC analysis and 2-by-2 contingency tables. WNV-RR and WNV-RH were not statistically different for sensitivity or specificity when analyzed. However, WNV-RH was selected for further analysis because when tested with anti-WNV antibody-positive sera it produced higher P/N values, indicating greater type specificity than WNV-RR antigen. ROC curve analysis also found no statistical difference between SLEV-DDR and SLEV-DRR mutant antigens, and SLEV-DRR was selected as the antigen in this study because cells transiently transformed with this construct exhibited higher VLP secretion than did DDR-transformed cells (27).

Detection of WN and SLE viral antibody by ELISA. A total of 134 acute-phase human serum specimens, consisting of WNV-, SLEV-, DENV-, JEV-, POWV-, LACV-, EEEV-, and WEEV-infected samples, samples infected with an undetermined flavivirus, and samples of unknown etiology, were used in the study.

Serum specimens were randomly coded and blind tested concurrently using WNV-WT, WNV-RH, SLEV-WT, and SLEV-DRR antigens in MAC-ELISA. The resulting ELISA data were classified without prior knowledge of the DRL diagnosis following the criteria specified in Materials and Methods. The WNV-infected serum panel consisted of 55 presumptive WNV-infected sera. The WNV-WT antigen detected anti-WNV IgM in 55/55 of these samples (P/N ratios of ≥3.0; range, 6.3 to 74.1; average, 30.3). Replacement of WNV-WT with the WNV-RH antigen in the MAC-ELISA also produced 55/55 positive samples (P/N ratios of ≥3.0; range, 3.7 to 47.7; average, 23.0 [Table 2]). Analyses using existing DRL disease state classifications produced qualitatively similar results (Table 2). Paired-ROC curve analysis revealed no statistical difference ($z = 1.34$) in the reported AUC between WT and mutant antigens in this assay (Table 3). However, the mutant WNV-RH antigen had a higher PPV and higher likelihood ratio than WNV-WT antigen (Table 4). The AUC assesses the tests' overall discriminatory ability, while the PPV is determined based on the specified cutoff of 3.0 and disease state classifications.

The SLEV-infected serum panel consisted of 37 presumptive SLEV-infected sera. The SLEV-WT antigen detected anti-SLEV IgM in 35/37 presumptive positive samples (P/N ratios of ≥3.0; range, 3.8 to 52.0; average, 15.1). Replacement of SLEV-WT with SLEV-DRR antigen detected 37/37 positive samples (P/N ratios of ≥3.0; range, 3.0 to 62.8; average, 14.2 [Table 2]). Again, analyses using the DRL disease state classifications were qualitatively similar (Table 2). Paired-ROC curve analysis revealed that the AUCs for each of these assays are statistically different ($z = 2.18$ [Table 3]). This result indicates that the assay using the SLEV-DRR antigen (AUC = 0.98) more accurately discriminated between positive and negative cases than did the SLEV-WT antigen (AUC = 0.94). Analysis of the 2-by-2 contingency table associated with this assay demonstrated that both the PPV and NPVs as well as the

TABLE 4. Predictive values and likelihood ratios for WT and mutant VLPs in the MAC-ELISA at the P/N ratio of ≥3[a]

VLP type	% PPV (95% CI)	% NPV (95% CI)	Likelihood ratio
WNV-WT	70.5 (59.1–80.3)	100.0 (93.6–100.0)	3.4
WNV-RH	85.9 (75.0–93.4)	100.0 (94.9–100.0)	8.8
SLEV-WT	53.9 (41.0–66.3)	97.1 (89.9–99.7)	3.1
SLEV-DRR	77.1 (62.7–88.0)	100.0 (95.8–100.0)	8.8

[a] All statistical analyses are based upon the panels as defined by our disease state classification.

TABLE 5. MAC-ELISA W/S and S/W ratios for highly cross-reactive samples and the correlation with PRNT results

Specimen[a]	$PRNT_{90}$[b]		VLP S/W or W/S ratio[c]				Interpretation[d]
			Mutant		WT		
	SLEV	WNV	S/W	W/S	S/W	W/S	
17	81,920	2,560	**2.16**	0.46	**1.69**	0.59	SLEV-s
18	81,920	2,560	**2.24**	0.45	**4.22**	0.24	SLEV-s
40	20,480	320	**4.22**	0.24	**4.33**	0.23	SLEV-s
108	40,960	5,120	**1.82**	0.55	**3.38**	0.30	SLEV-s
82	1,280	10	**6.64**	0.15	**1.39**	0.72	SLEV-s
6	640	20	**6.80**	0.15	**2.13**	0.47	SLEV-s
35	2,560	640	**3.12**	0.32	**2.93**	0.34	SLEV-s
3	160	20	**2.52**	0.40	**1.36**	0.73	SLEV-s
91	160	1,280	0.18	**5.62**	0.77	**1.30**	WNV-s
101	40	1,280	0.12	**8.10**	0.13	**7.61**	WNV-s
105	20	320	0.23	**5.18**	0.14	**7.37**	WNV-s
64	80	640	0.26	**3.91**	0.14	**7.10**	WNV-s
84	20	640	0.10	**10.06**	0.07	**14.08**	WNV-s
38	80	5,120	0.11	**9.22**	0.39	**2.59**	WNV-s
33	40	2,560	0.09	**10.90**	0.12	**8.63**	WNV-s
15	10	1,280	0.07	**13.36**	0.07	**14.53**	WNV-s
116	10	160	0.36	**2.76**	0.18	**5.61**	WNV-s
56[c]	1,280	160	**1.43**	0.70	0.94	**1.07**	SLEV-s
103	5,120	2,560	0.96	**1.04**	0.74	**1.34**	WNV-s
50	ND[e]	80	0.34	**2.98**	0.28	**3.60**	WNV-s

[a] The values shown in boldface and italics indicate samples that exhibited conflicting results between WT and mutant antigens and/or could not be PRNT confirmed.
[b] $PRNT_{90}$, titers represent 90% plaque reduction endpoints reported by the DRL.
[c] Values represent ratios of SLEV P/N to WNV P/N and vice versa as described in Materials and Methods. The higher of the two ratios for both WT and mutant VLPs in both MAC-ELISAs are shown in boldface.
[d] -s, secondary infection.
[e] ND, not done.

likelihood ratio were higher using the SLEV-DRR antigen (Table 4).

Detection of cross-reactive antibodies in WNV- and SLEV-infected serum panels. As shown in Table 2, use of SLEV-WT antigen in MAC-ELISA with WNV-infected sera detected IgM antibodies in 28 (51%) of the 55 WNV-positive sera. The SLEV-DRR antigen detected IgM antibodies in 11 (20%) of the same 55 WNV-infected sera. Similarly, 37 sera were indicative of SLEV infections. When testing this panel for IgM antibodies, 17 of 37 (46%) SLEV-infected serum samples were detected with the WNV-WT antigen, compared to 9 of 37 (24%) positive with the WNV-RH antigen.

For all 134 samples, we employed the diagnostic algorithm previously described, whereby the WNV and SLEV P/N, W/S, and S/W ratios were calculated (17) (see Table S1 in the supplemental material). Of the 134 samples tested in the serum panel, 20 sera (15% [11 WNV- and 9 SLEV-infected specimens]) were particularly cross-reactive and were difficult to interpret based upon SLEV or WNV P/N ratio alone; to aid in classification of these sera, we exploited this algorithm. As shown in Table 5, with the exception of one sample, all of these sera had associated PRNT data for both SLEV and WNV. We considered these highly cross-reactive sera to be indicative of previous WNV or SLEV infections and based upon our ELISA data and resulting disease state classifications, we determined that the higher W/S or S/W IgM ratio was indicative of the current, secondarily infecting virus. For 17 of 18 PRNT-confirmed samples, application of the algorithm to both the WT and mutant antigens resulted in a disease state classification that was consistent with the PRNT data. The remaining sample, no. 56, had conflicting results after the application of the algorithm. For this sample, MAC-ELISA results with mutant antigens indicated an SLEV infection, whereas the WT antigens indicated a WNV infection. Of note, both the PRNT data and the DRL classification are in accord with the results observed using the mutant antigens with reduced cross-reactivity. Of the 20 samples, 2 could not be PRNT confirmed (no. 50 and 103). Application of the S/W and W/S ratios of the ELISA results from WT and mutant antigens with sample no. 50 indicated a WNV infection; however, this could not be PRNT confirmed due to the lack of reported SLEV PRNT data. Application of this algorithm to the other sample, no. 103, suggests a WNV infection; however, the PRNT data were again inconclusive because the neutralizing antibody titers against SLEV (1:5,120) and WNV (1:2,560) were only twofold different.

Detection of cross-reactive antibodies in the non-SLEV/WNV- and non-flavivirus-infected sera. A panel of 10 non-SLEV/WNV flavivirus-infected sera was tested with the WT and mutant WNV and SLEV antigens (Table 1 and see Table S1 in the supplemental material). This panel consisted of samples IgM positive for DENV ($n = 4$), JEV ($n = 4$), and POWV ($n = 2$). Use of the WNV-RH antigen in the MAC-ELISA detected no cross-reactive IgM antibodies compared to the WNV-WT antigen, which detected cross-reactive antibodies in 4 of 10 samples (40%). Neither SLEV-WT nor SLEV-DRR antigens detected cross-reactive IgM antibodies in MAC-ELISA.

A panel of 12 nonflavivirus samples was also tested with the WT and mutant WNV and SLEV antigens (Table 1 and see Table S1 in the supplemental material). The panel was composed of sera antibody positive for LACV ($n = 5$), EEEV ($n = 5$), and WEEV ($n = 2$). The use of the WT and mutant WNV and SLEV antigens in MAC-ELISA did not detect cross-reactive IgM antibodies in any of the samples tested.

Discrepancies in diagnostic interpretation. The results presented above are based upon the panels defined by the disease state classifications produced from this study's MAC-ELISA data alone. As can be seen in Tables 1 and 2, we also included disease panels based on the DRL classifications. Most of the discrepancies in diagnostic interpretation were within the control panels and therefore did not produce significant changes in the resulting statistical analyses. We observed discrepancies in the disease state classifications between DRL and our study for 11 of the 134 tested specimens (Table 6). Seven of the 11 discrepancies were due to P/N values of <3.0, below the positive cutoff value, in the MAC-ELISA with both our WT and mutant antigens (samples 115, 62, 34, 42, 93, 58, and 59). Of the remaining four samples, three produced discrepancies due to our antigens resulting in more specific diagnostic interpretations. For samples 57, 98, and 103, the inconclusive PRNT and ELISA data reported by the DRL indicate unspecified flaviviral infections, while our MAC-ELISA data produced more specific interpretations of the presumptive infecting virus. The remaining sample, no 109, had a P/N value of <3.0 when tested against both mutant antigens in the MAC-ELISA. However, the positive P/N value of ≥ 3.0 was observed when

TABLE 6. Samples resulting in different disease state classifications between DRL and this study

Specimen	$PRNT_{90}$[a]			P/N value[b]				Antigen[c]	Disease state classification[d]
				IgM		IgG			
	SLEV	WNV	DENV-2	SLEV	WNV	SLEV	WNV		
57	40	40		1.50	**5.31**	**4.98**	**4.23**	DRL	Flavivirus
				14.56	1.10	ND[e]	ND	WT	SLEV
				5.00	1.09	ND	ND	Mutant	
98	20	40		**3.50**	**5.50**	**6.80**	**4.30**	DRL	Flavivirus
				7.69	**10.11**	ND	ND	WT	WNV
				1.14	**3.73**	ND	ND	Mutant	
103	5120	2560		**5.90**	**5.00**	**7.60**	**15.50**	DRL	Flavivirus
				5.29	**7.11**	ND	ND	WT	WNV-secondary
				4.71	**4.91**	ND	ND	Mutant	
109	<10	<10		1.20	2.70	**4.60**	**4.60**	DRL	Recent WNV
				5.71	2.55	ND	ND	WT	Unknown flavivirus
				1.77	1.93	ND	ND	Mutant	
115	20	160		0.85	2.50	**3.60**	**8.20**	DRL	Recent WNV
				1.03	2.72	ND	ND	WT	Unknown etiology
				1.06	2.41	ND	ND	Mutant	
62	<10	<10	10	1.79	1.18	**5.71**	1.95	DRL	Recent SLEV
				1.59	1.43	ND	ND	WT	Unknown etiology
				1.12	1.31	ND	ND	Mutant	
34	2560	640		**6.90**	ND	**3.60**	**4.10**	DRL	Flavivirus
				1.93	1.37	ND	ND	WT	Unknown etiology
				1.95	1.43	ND	ND	Mutant	
42	20480	1280		**3.90**	2.00	**10.50**	**3.20**	DRL	SLEV
				1.14	1.74	ND	ND	WT	Unknown etiology
				1.18	2.03	ND	ND	Mutant	
93	<10	10	40	1.44	2.07	2.49	**3.27**	DRL	Flavivirus, WNV-secondary
				1.08	1.14	ND	ND	WT	Unknown etiology
				0.99	1.10	ND	ND	Mutant	
58	20	10	20	2.53	**4.72**	**8.11**	**7.00**	DRL	Flavivirus
				1.19	1.19	ND	ND	WT	Unknown etiology
				1.14	0.99	ND	ND	Mutant	
59	ND	ND		2.80	**6.52**	**9.03**	**8.00**	DRL	Flavivirus
				1.08	1.55	ND	ND	WT	Unknown etiology
				0.85	1.06	ND	ND	Mutant	

[a] $PRNT_{90}$, titers represent 90% plaque reduction endpoints reported by the DRL.
[b] P/N values of ≥3.0 are shown in boldface.
[c] ELISA data are presented from tests with three types of antigen: the DRL antigens, which include both SMB and VLP for WNV and SMB for SLEV; this study's WT VLP antigens; and the mutant antigens with reduced cross-reactivity of both WNV and SLEV.
[d] DRL disease state classifications are matched by row with the DRL antigen. Some of the DRL disease state classifications are based partially on additional data not presented in this table.
[e] ND, not done.

this sample was tested with SLEV-WT antigen; we classified this sample as coming from an individual with an unknown flaviviral infection. The DRL reported that this individual was recently infected with WNV; however, the PRNT titers were <1:10, the sample tested negative for IgM to both SLEV and WNV, and the IgG assays resulted in positive P/N values for both SLEV and WNV. In this case, the reported DRL classification was based on the results from testing a subsequent serum sample from this individual, which we did not have access to.

DISCUSSION

Two mutant antigens with reduced cross-reactivity along with their WT SLEV and WT WNV counterparts were tested with four serum panels, a WNV-infected panel, an SLEV-infected panel, a non-SLEV/WNV flavivirus-infected panel, and a nonflavivirus panel, to compare the performances of the mutant and WT antigens in the MAC-ELISA for clinical diagnosis. The sometimes confounding WNV-SLEV cross-reactivity reported in flavivirus serodiagnostic assays (14, 15, 17, 18) was reduced using the mutant antigens, producing a more accurate diagnostic assay. Overall, the WNV-RH antigen with reduced cross-reactivity outperformed the WNV-WT antigen in all four panels. In the WNV panel, the WNV-RH antigen was more specific than the WNV-WT antigen (Table 3) and it exhibited higher PPVs (Table 4). The increase in specificity indicates that more negative samples were correctly identified as negative using the WNV-RH antigen; and the increased PPV indicates that more samples that are identified as positive are actually positive. Taken in conjunction, these analyses demonstrate successful reduction in assay cross-reactivity with the WNV-RH VLP antigen. This reduction in cross-reactivity is also demonstrated by the results from the non-WNV sera panels. When tested with presumptive SLEV-infected sera, the WNV-RH antigen identified 21 to 22% fewer false-positive sera than did the WNV-WT antigen (Table 2).

The mutations introduced to the SLE VLP also produced an improved serodiagnostic antigen. In SLEV-infected sera analyzed in the MAC-ELISA, the discriminatory power using the SLEV-DRR antigen was significantly increased over that of the SLEV-WT antigen (Table 3). The SLEV-DRR antigen also produced both a more sensitive and a more specific assay with higher PPVs and NPVs than SLEV-WT antigen (Table 4). Similar to the WNV-RH antigen, we also observed dramatic reductions in cross-reactivity with the SLEV-DRR antigen when tested with WNV-infected sera: the SLEV-DRR antigen reduced the number of false positives by 30 to 31% compared to the SLEV-WT antigen (Table 2).

Although our mutant antigens with reduced cross-reactivity significantly improved the diagnostic performance of these assays, we were unable to completely eliminate cross-reactivity. This is not surprising because the mutant antigens presented here focus on cross-reactive epitopes centered on the highly conserved fusion peptide region of the E protein. Recent studies indicate that this is an immunodominant region eliciting predominately nonneutralizing, broadly cross-reactive antibodies making up a large proportion of the total antibody response (24, 26). However, serocomplex-cross-reactive antibodies are also stimulated from epitopes at a distance from the fusion peptide, most likely from epitopes in structural domain III (6, 19, 27). This residual cross-reactivity of the WNV-RH and SLEV-DRR antigens could be beneficial for the detection of previous WNV/SLEV exposure in secondary infections and/or in seroprevalence studies. Through analysis of WNV/SLEV ratios of P/N values and PRNT-confirmed samples, we propose that these highly cross-reactive samples are the result of previous WNV or SLEV infections and that the higher ratio of P/N values in the MAC-ELISA was indicative of the current, secondarily infecting virus. The sera analyzed in this study were obtained between the years 1999 and 2005, mostly from WNV epidemic or SLEV epidemic foci. Because some of these WNV foci occurred in areas of SLEV endemicity, and vice versa, secondary infections are not unexpected. Moreover, because these sera were specifically selected for high levels of cross-reactivity between SLEV and WNV, based on the existing DRL data, this would increase the probability of having secondary infections above that expected in the population at random.

Recently, Johnson et al. introduced a duplex microsphere-based immunoassay (MIA) for differential detection of anti-WNV and anti-SLEV IgM (13). This MIA uses WT WNV VLP and SLEV SMB antigens and is the only assay currently available that has improved specificity for distinguishing current WNV or SLEV infection over the existing MAC-ELISA (17). The MIA significantly reduced the time needed to perform the assay; however, it requires IgG depletion of the specimens prior to performing MIA to improve assay performance, an expensive new instrument for data acquisition, and proprietary data transformation and analysis software for its improved differential diagnostic capability. Although its multiplex and high-throughput capabilities are very attractive to a centralized laboratory; it may be less suitable for small laboratories, which do not require such high throughput and/or do not have a sufficient budget for purchasing the expensive instrument and retooling its standard operation protocol. Thus, despite the advantages of this MIA, there will continue to be a need for MAC-ELISA. The WNV and SLEV VLP antigens with reduced cross-reactivity presented here significantly improved the serodiagnostic discrepancy when used in MAC-ELISA, and they should similarly improve the specificity of other serodiagnostic assays, such as MIA or dipstick approaches, when utilized instead of highly cross-reactive WT antigens.

We have clearly outlined our diagnostic criteria, which, without the need for a PRNT, produce clear and accurate diagnostic conclusions. We feel that the use of these new antigens and diagnostic criteria is a significant improvement over current methods and reagents. Both the WNV and SLEV antigens with reduced cross-reactivity exhibited higher PPVs and improved specificity, aiding in interpretation and thereby reducing the need for a time-consuming confirmatory PRNT. In addition, the antigens with reduced cross-reactivity presented here are not only more reliable in identifying virus-specific antibody from the current infection, but we believe that when used to detect both IgM and IgG, these new antigens will provide more accurate insight into past flavivirus exposures. We are presently applying the approach presented here towards the development of antigens with reduced cross-reactivity for the other medically important flaviviruses. With the

implementation of these antigens with reduced cross-reactivity in the future, the improved assay performance not only will be important for clinical serodiagnosis in regions with multiple cocirculating flaviviruses, but also will provide the necessary tools to accurately address estimates of disease burden in geographic regions such as China, Southeast Asia, and Africa.

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