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Enzyme-Linked Immunosorbent Assays Using Novel Japanese Encephalitis Virus Antigen Improve the Accuracy of Clinical Diagnosis of Flavivirus Infections▽

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The cross-reactive antibodies induced by flavivirus infections confound serodiagnosis and pathogenesis, especially in secondary infections caused by antigenically closely related yet distinct flaviviruses. The envelope (E) glycoprotein fusion peptide contains immunodominant cross-reactive determinants. Using a recombinant Japanese encephalitis virus (JEV) premembrane and E expression plasmid producing JEV virus-like particles (VLPs), dramatic reductions in cross-reactivity were produced by the G106K-L107D (KD) double-mutant VLP against a panel of flavivirus murine monoclonal antibodies. Human serum panels from patients with recent flavivirus infections were analyzed to compare the accuracy of JEV wild-type (WT) and KD VLPs as serodiagnostic antigens in enzyme-linked immunosorbent assays. Statistical analysis demonstrated significant differences in assay performances for accurate determination of current JEV infections between WT and KD antigens by detecting immunoglobulin M antibodies at a serum dilution of 1:4,000 (likelihood ratios = 2.74 [WT] and 22 [KD]). The application and continued development of cross-reactivity-reduced antigens should improve both flavivirus infection serodiagnosis and estimates of disease burden.

Japanese encephalitis virus (JEV), a member of the genus *Flavivirus* in the family *Flaviviridae*, is the leading cause of endemic/epidemic viral encephalitis in Asia, including India, Thailand, Vietnam, Singapore, the Philippines, Taiwan, China, Korea, and Japan (40). It is also one of several mosquito-borne flaviviruses, in addition to four serotypes of dengue virus (DENV-1 to -4), that have experienced emergence and/or re-emergence throughout the world, especially in the tropical regions (22, 24). Sequential infection by multiple cocirculating flaviviruses in the affected population confounds serodiagnosis (20), disease burden estimation (23), and the impact on pathogenesis (10).

Flavivirus infections elicit protective antibody responses primarily against the envelope (E) glycoprotein (20). The E protein contains three structural and functional domains. E domain I (EDI) is an eight-stranded β-barrel; it contains two large insertion loops forming the elongate dimerization EDII and containing the highly conserved internal fusion peptide. EDIII has an immunoglobulin (Ig)-like structure and contains the primary receptor-binding motifs (16, 29). Murine monoclonal antibody (MAb) studies have demonstrated that EDI contains predominately type-specific nonneutralizing (non-Nt) epitopes, EDII contains cross-reactive epitopes eliciting both Nt and non-Nt antibodies, and EDIII contains the majority of the type-specific Nt epitopes (6, 31–34, 37, 38).

Diagnostic enzyme-linked immunosorbent assays (ELISAs) are common, relatively quick, and efficient assays for clinical diagnosis, traditionally requiring the use of suckling mouse brain-grown (SMB) antigen and more recently utilizing non-infectious recombinant virus-like particle (VLP) antigen. Studies have shown that VLP antigens have higher performance accuracy than SMB antigens when used in ELISA for diagnosing flaviviral infections (8, 13, 14, 28). However, both SMB and VLP antigens contain wild-type (WT) E proteins that exhibit the same cross-reactive epitopes as the virus responsible for the infection. The amino acids located in the highly conserved E glycoprotein fusion peptide, in particular Gly104, Gly106, and Leu107, have been identified as important flavivirus cross-reactive epitope determinants (6, 7, 37, 39). Thus, it is possible to develop cross-reactivity-reduced antigens by introducing substitutions for amino acids within the fusion peptide, thereby improving virus-specific diagnostic assays (7, 39). Recently, fusion peptide mutant VLPs for both St. Louis encephalitis virus (SLEV) and West Nile virus (WNV) demonstrated dramatic reductions in the observed cross-reactivity of immunoglobulin M capture (MAC) ELISA, producing more accurate differentiation of both current and past WNV and SLEV infections (30).

Here, we present results from mutagenesis in the fusion peptide region of the JEV E protein to identify and ablate cross-reactive E protein epitopes and utilize these mutant VLPs as improved serodiagnostic antigens. The JEV G106K/L107D (KD) VLP exhibited the most dramatic reductions in cross-reactivity of the JEV fusion peptide mutants. Thus, the JEV-KD and the previously described cross-reactivity-reduced WNV G106R/L107H (RH) VLP (30) were used as serodiagnostic antigens to test a diverse group of flavivirus-infected patients' sera and to compare their performances for the de-

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TABLE 1. Nucleotide sequences of mutagenic PCR primers for JEV VLPs

Primer	Length (nt)	Mutagenic primer sequence (5′–3′)[a]	Nucleotide mutation	Amino acid substitution	% VLP secretion[b]
G104H	38	CCCTTCCCGAAAAGTCCACA**ATG**GTTGCCCCACCCACG	GGA-CAT	Gly-His	0
G106Q	36	CTTCCCTTCCCGAAAAG**CTG**ACATCCGTTGCCCCAC	GGA-CAG	Gly-Gln	200
G106K	38	CTTCCCTTCCCGAAAAG**TTT**ACATCCGTTGCCCCACCC	GGA-AAA	Gly-Lys	400
G106V	37	CTTCCCTTCCCGAAAAG**CAC**ACATCCGTTGCCCCACC	GGA-GTG	Gly-Val	100
G106D	38	CTTCCCTTCCCGAAAAG**ATC**ACATCCGTTGCCCCACCC	GGA-GAT	Gly-Asp	200
L107F	37	TTCCCTTCCCGAA**GAA**TCCACATCCGTTGCCCCACCC	CTT-TTC	Leu-Phe	30
L107D	36	TTCCCTTCCCGAA**ATC**TCCACATCCGTTGCCCCACC	CTT-GAT	Leu-Asp	200
L107R	36	TTCCCTTCCCGAA**GCG**TCCACATCCGTTGCCCCACC	CTT-CGC	Leu-Arg	400
L107H	36	TTCCCTTCCCGAA**GTG**TCCACATCCGTTGCCCCACC	CCT-CAC	Leu-His	200
L107G	36	TTCCCTTCCCGAA**ACC**TCCACATCCGTTGCCCCACC	CCT-GGT	Leu-Gly	200
G106K-L107D	45	CAAGCTTCCCTTCCCGAA**ATCTTT**ACATCCGTTGCCCCACCCACG	GGA-AAA CTT-GAT	Gly-Lys Leu-Asp	100
G106K-L107R	41	CTTCCCTTCCCGAA**GCGTTT**ACATCCGTTGCCCCACCCACG	GGA-AAA CTT-CGC	Gly-Lys Leu-Arg	100
G106K-L107F	41	GCTTCCCTTCCCGAA**GAATTT**ACATCCGTTGCCCCACCCAC	GGA-AAA CTT-TTC	Gly-Lys Leu-Phe	50
G106V-L107D	41	CTTCCCTTCCCGAA**ATCCAC**ACATCCGTTGCCCCACCCACG	GGA-GTG CTT-GAT	Gly-Val Leu-Asp	100
G106V-L107R	40	TTCCCTTCCCGAA**GCGCAC**ACATCCGTTGCCCCACCCACG	GGA-GTG CTT-CGC	Gly-Val Leu-Arg	50
G106V-L107F	41	GCTTCCCTTCCCGAA**GAACAC**ACATCCGTTGCCCCACCCAC	GGA-GTG CTT-TTC	Gly-Val Leu-Phe	50
G106D-L107D	41	CTTCCCTTCCCGAA**ATCATC**ACATCCGTTGCCCCACCCACG	GGA-GAT CTT-GAT	Gly-Asp Leu-Asp	50
G106D-L107R	40	TTCCCTTCCCGAA**GCGATC**ACATCCGTTGCCCCACCCACG	GGA-GAT CTT-CGC	Gly-Asp Leu-Arg	50
G106D-L107F	41	GCTTCCCTTCCCGAA**GAAATC**ACATCCGTTGCCCCACCCAC	GGA-GAT CTT-TTC	Gly-Asp Leu-Phe	50

[a] Mutated nucleotides are shown in boldface.
[b] Standardized measurements of VLP secretion from transiently transformed COS-1 cells recovered at 37°C presented as percentages of the wild-type plasmid VLP secretion (arbitrarily set at 100%). The G104H substitution prevented VLP secretion from transformed COS-1 cells at 37°C, but not at 28°C.

tection of virus-specific IgM and IgG in ELISA with those of WT JEV and WNV VLP antigens.

MATERIALS AND METHODS

Cell culture, virus strain, and recombinant plasmid. COS-1 cells (ATCC CRL 1650; American Type Culture Collection, Manassas, VA) were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 110 mg of sodium pyruvate/liter, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 20 ml of 7.5% $NaHCO_3$/liter, 100 U of penicillin/ml, and 100 μg of streptomycin/ml.

We used the recombinant expression plasmid pVJE as the template DNA both for site-directed mutagenesis and for the transient expression of WT JEV recombinant antigen (see below). pVJE, derived from the pCBJE plasmid (4, 5, 14), includes the human cytomegalovirus early gene promoter, JEV signal sequence, JEV premembrane/membrane (prM/M) and E gene region in its entirety, and bovine growth hormone poly(A) signal. JEV strain SA14 was used as a template for amplification of JEV prM and E genes. The cloning procedures were described in detail in a previous publication (5). The ampicillin resistance gene in pCBJE was replaced by a kanamycin resistance gene derived from the pVAX plasmid (Invitrogen, Carlsbad, CA) to generate pVJE. In addition, the chimeric human β-globin gene intron sequence derived from the pCI expression vector (Promega, Madison, WI) was PCR amplified and inserted between nucleotides 1240 and 1241 of the E gene to generate pVJEi. The intron insertion increased the plasmid yield in the *Escherichia coli* host and enhanced VLP secretion in transiently transformed COS-1 cells (G. J. Chang and J. Kim, unpublished results).

Site-directed mutagenesis. A homology model for the JEV E protein was produced using the published atomic coordinates for DENV-2 and WNV and the Swiss-model workspace (http://swissmodel.expasy.org/workspace/). We focused on the amino acid substitutions at G106 and L107 of E protein based on a number of criteria, as previously described (6, 7, 39). Stability calculations ($\Delta\Delta G$) were determined for all possible substitutions at residues 106 and 107 using the PoPMuSiC server (http://babylone.ulb.ac.be/popmusic/) and Protein Data Bank coordinates from the JEV homology model. Four and five substitutions were selected at positions 106 and 107 to represent the diversity of biochemical and structural properties of amino acid side chains (e.g., basic, acidic, polar, nonpolar, small, and large). Individual substitutions maximizing stability (lower $\Delta\Delta G$ values) were selected from within each side chain class.

Site-specific mutations were introduced into the JEV E gene of the pVJE plasmid using a QuikChange multisite-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's recommended protocols. The sequences of the mutagenic primers used for all constructs are listed in Table 1. Four or five colonies from each mutagenic PCR transformation were selected and grown in 5-ml Luria-Bertani broth cultures, miniprepped, and sequenced across the intended substitution to identify the correct mutant clone(s). The transcription units, including prM/M and E gene regions and the transcriptional and translational regulatory elements, of all purified plasmids were sequenced in their entirety upon identification of the correct substitution(s). Automated DNA sequencing was performed with an ABI 3130xl genetic-analysis system (Applied Biosystems, Foster City, CA), and sequences were analyzed with Lasergene software (DNAStar, Madison, WI).

Electroporation of tissue culture cells with plasmid DNA. For transformation, COS-1 cells were grown to 90 to 100% confluence in 150-cm^2 culture flasks, trypsinized, and resuspended in ice-cold phosphate-buffered saline (PBS) to a final density of 1.5×10^7 cells/ml. For each reaction, 0.5 ml of this cell suspension was electroporated with 20 μg of plasmid DNA in a 0.4-cm-electrode-gap cuvette with a Bio-Rad Gene Pulser II (Bio-Rad Laboratories, Hercules, CA) set at 250 V and 975 μF. Two electroporation reaction mixtures were seeded onto a single 150-cm^2 culture flask containing 50 ml of growth medium and allowed to recover at 37°C overnight. The tissue culture flasks were continuously maintained at 37°C or at 28°C for an additional 1 to 4 days. We observed that substitutions at JEV E Gly104, which were previously found not to secrete at 37°C in other flavivirus systems (6, 7, 39), secreted to sufficient levels for MAb analysis when the transformants were seeded into COS-1 cells and maintained at 28°C. Tissue culture medium was harvested on day 2 (37°C) or day 5 (28°C) following electroporation,

TABLE 2. Comparison of antibody reactivities[a] for JEV WT and mutant VLPs

Mutation	Virus titer[a]										
	Polyclone[b]	Group				Subgroup		Complex		Subcomplex	Type
	MHIAF (JEV)[c]	6B6C-1 (SLEV)	4G2 (DENV2)	23-1 (WNV)	23-2 (JEV)	5-2 (JEV)	2B5B-3 (SLEV)	6B4A-10 (JEV)	16 (JEV)	1B5D-1 (SLEV)	J3 14 H5-2 (JEV)
None	4.9	6.3	≥6.8	≥6.8	≥6.8	5.4	≥6.8	4.9	5.9	5.4	3.0
G104H[d]	4.4	**<3.0**	**4.9**	6.3	6.3	**3.9**	6.3	4.4	**3.9**	4.9	<3.0
G106Q	4.9	**<3.0**	**<3.0**	≥6.8	≥6.8	**3.0**	**3.5**	***5.9***	6.3	**3.0**	3.0
G106K	4.9	**4.9**	**4.0**	≥6.8	≥6.8	**3.5**	**<3.0**	5.4	6.3	**<3.0**	3.0
G106V	4.4	**<3.0**	**<3.0**	≥6.8	**5.9**	**<3.0**	**5.9**	4.9	5.9	**<3.0**	3.0
G106D	4.9	**<3.0**	**3.0**	≥6.8	≥6.8	**<3.0**	**3.0**	5.4	6.3	**<3.0**	3.0
L107R	4.9	≥6.8	**<3.0**	**<3.0**	6.3	**<3.0**	**4.9**	***5.9***	6.3	5.9	3.5
L107D	4.9	5.4	**<3.0**	**3.0**	**4.9**	**<3.0**	≥6.8	***5.9***	6.3	5.9	3.9
L107H	4.4	6.3	**<3.0**	**5.4**	**5.9**	**<3.0**	≥6.8	5.4	5.9	5.4	3.5
L107A[e]	4.9	≥6.8	**3.0**	6.3	6.3	**<3.0**	≥6.8	5.4	6.3	5.9	3.9
L107G	4.9	≥6.8	**<3.0**	**5.4**	**5.9**	**<3.0**	≥6.8	***5.9***	5.9	5.9	3.5
L107F	4.4	**4.4**	**<3.0**	**4.9**	**5.9**	**<3.0**	**5.9**	**3.5**	**4.4**	**3.9**	3.0

[a] Ag-ELISA was used to determine the reciprocal end point titers ($\log_{10}$) for secreted VLP antigens. The numbers shown in boldface are the end point titers decreased below that of the WT by at least two threefold dilutions; the numbers in boldface italic are the titers increased over that of the WT by at least two threefold dilutions.

[b] Group, recognized by all flaviviruses examined; subgroup, recognized by more than one serocomplex; complex, recognized by all members of JEV complex; subcomplex, not recognized by all members of the serocomplex; type, recognized by JEV only.

[c] Virus against which antibody was raised.

[d] The G104H substitution prevented VLP secretion from transformed COS-1 cells at 37°C, but not at 28°C.

[e] The L107A mutation was obtained unintentionally due to misincorporation of sequence during the mutagenesis procedure.

clarified by centrifugation at 10,000 rpm for 30 min at 4°C in a Sorval F-16/250 rotor (Beckman Coulter), and stored at 4°C for further analysis.

Human serum. Serum specimens were obtained from the Diagnostic and Reference Laboratory, Arboviral Diseases Branch, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention. Panels were assembled by selecting serum specimens collected from 1999 to 2003 having Nt antibody titers to WNV ($n = 21$), SLEV ($n = 6$), or alphaviruses ($n = 12$), as determined by the 90% plaque reduction neutralization test. The serum panels with evidence of DENV ($n = 24$) or JEV ($n = 16$) infection were assembled from Taiwanese residents and provided by the Center for Disease Control—Taiwan. The dengue serotype responsible for the most recent infection was defined by virus isolation and/or virus-specific reverse transcriptase PCR, and the JEV infection status was determined by IgM and IgG ELISAs (35).

MAb panel. When selecting MAbs for use in antigen characterization, we specifically chose a variety of group-, subgroup-, complex-, and subcomplex-cross-reactive MAbs that had been raised against a diverse assortment of flaviviruses (L.-K. Chen, unpublished results; 9, 17, 18, 34). MAbs 4G2, 23-1, 23-2, and 6B6C-1 are flavivirus group cross-reactive (recognizing viruses from all major pathogenic serocomplexes of flaviviruses) and non-Nt to moderately Nt. MAbs 2B5B-3 and 5-2 are subgroup-reactive antibodies recognizing the JEV complex and yellow fever virus, and only JEV and DENV-1 and -2, respectively. MAbs 16, 6B4A-10, 1B5D-1, 109, and 203 exhibit various levels of cross-reactivity with viruses within the JEV complex and are non-Nt. J3 14 H5-2, 112, and 503 are the JEV-specific MAbs used in this study (17).

Antigen characterization. Antigen capture ELISA (Ag-ELISA) was performed to determine VLP secretion from plasmid-transformed cells and to determine reductions in MAb reactivity to mutant VLP antigens as previously described (6). Briefly, the inner 60 wells of an Immulon II HB flat-bottom 96-well plate (Dynatech Industries, Inc., Chantilly, VA) were coated with polyclonal rabbit-anti-JEV antibody in 50 μl of coating buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6) and incubated overnight at 4°C. The wells were blocked with 300 μl of Start Block (PBS) blocking buffer (Pierce, Rockford, IL) according to the manufacturer's recommended procedure. Secreted WT and mutant antigens were titered in PBS, captured with 1 h of incubation at 37°C, and detected with anti-JEV murine hyperimmune ascitic fluid (MHIAF) at a 1:8,000 dilution in PBS with 5% milk. Anti-JEV MHIAF was detected with horseradish peroxidase-conjugated goat anti-mouse HIAF at a 1:5,000 dilution in PBS containing 5% milk. Bound conjugate was detected by adding 75 μl of the 3,3′5,5′-tetramethylbenzidine (Neogen Corp., Lexington, KY) substrate and incubating the mixture at room temperature for 10 min. The substrate reaction was stopped with 50 μl of 2 N H_2SO_4, and the reactions were measured at an A_{450} with an EL 312e Bio-Kinetics microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Secreted antigen concentrations were standardized for MAb screening by selecting the antigen concentration producing an optical density of ~1.0 with the polyclonal MHIAF. The panel of MAbs was used to determine the end point MAb reactivities of mutated and WT antigens in Ag-ELISA (Tables 2 and 3). MAb affinity reductions were determined utilizing the same Ag-ELISA described above, except that the standardized Ag concentrations determined above were used to determine the end point titer of the MAb.

ELISA protocols. JEV VLPs and normal COS-1 cell culture antigen were prepared as described above, and WNV VLPs were prepared as described in a previous publication (30). Antigens were independently titrated against JEV or WNV positive control serum samples with a twofold dilution series and standardized by selecting a dilution that yielded an absorbance of ~1.0 at 450 nm (A_{450}). The MAC-ELISA was performed as described previously (14) with some modifications for detecting the presence of virus-specific IgM in patient's serum panels with the VLPs. Briefly, the inner 60 wells of Immulon II HB flat-bottom 96-well plates (Dynatech Industries, Inc., Chantilly, VA) were coated overnight at 4°C in a humidified chamber 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 wells were blocked with 300 μl of Start Block (PBS) blocking buffer (Pierce, Rockford, IL) according to the manufacturer's recommended procedure. Patient sera and positive and negative antibody controls were diluted appropriately in wash buffer (PBS with 0.05% Tween 20), added to wells (50 μl/well), and incubated at 37°C for 1 h in a humidified chamber. Test positive and negative human control sera were diluted 1:400 or 1:4,000. Positive and negative control antigens were tested with each patient serum sample in triplicate by diluting them appropriately in wash buffer and adding 50 μl to appropriate wells for incubation at 4°C overnight in a humidified chamber. WT and mutant JEV or WNV antigens were added and incubated at 37°C for 1 h in a humidified chamber. After being washed, the captured JEV or WNV antigens were detected with anti-JEV or anti-WNV MHIAF, respectively, at a 1:8,000 dilution. Anti-JEV and anti-WNV MHIAFs were detected with horseradish peroxidase-conjugated goat anti-mouse HIAF used at a 1:5,000 dilution. Bound conjugate was detected by adding 75 μl of the 3,3′5,5′-tetramethylbenzidine (Neogen Corp., Lexington, KY) substrate and incubating the mixture at room temperature for 10 min. The substrate reaction was stopped with 50 μl of 2 N H_2SO_4, and the reactions were measured at A_{450} with an EL 312e Bio-Kinetics microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

For detection of virus-specific IgG in patients' serum samples using VLPs, the IgG capture ELISA (GAC-ELISA) was performed as described above for MAC-ELISA with the exception that the plates were coated with anti-human IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:2,000 in coating buffer.

TABLE 3. Effect of antibody reactivity[a] on JEV double-amino acid mutant VLPs

Mutations	Virus titer[a]										
	Polyclone[b]	Group				Subgroup		Complex		Subcomplex	Type
	MHIAF (JEV)[c]	6B6C-1 (SLEV)	4G2 (DENV-2)	23-1 (WNV)	23-2 (JEV)	5-2 (JEV)	2B5B-3 (SLEV)	6B4A-10 (JEV)	16 (JEV)	1B5D-1 (SLEV)	J3 14 H5-2 (JEV)
None	5.9	6.3	≥6.8	≥6.8	≥6.8	4.9	6.3	5.4	5.9	5.9	3.0
G106K/L107D	5.4	**3.0**	**<3.0**	**<3.0**	**3.9**	**<3.0**	**<3.0**	5.4	5.9	**<3.0**	3.0
G106K/L107R	5.4	**5.9**	**<3.0**	**<3.0**	**4.9**	**<3.0**	**<3.0**	5.4	5.9	**<3.0**	3.0
G106K/L107F	**4.9**	**<3.0**	**<3.0**	**4.9**	≥6.8	**<3.0**	**<3.0**	5.4	5.9	**<3.0**	<3.0
G106V/L107D	5.4	**<3.0**	**<3.0**	**3.0**	5.9	**<3.0**	**<3.0**	5.4	5.9	**3.5**	3.0
G106V/L107R	5.4	**<3.0**	**<3.0**	**<3.0**	**4.4**	**<3.0**	**<3.0**	4.9	5.9	**3.5**	3.0
G106V/L107F	**4.9**	**3.9**	**<3.0**	**5.9**	**4.9**	**<3.0**	**<3.0**	5.4	5.4	**<3.0**	3.0
G106D/L107D	5.4	**<3.0**	**<3.0**	**3.0**	**3.0**	**<3.0**	**<3.0**	4.9	5.9	**<3.0**	3.5
G106D/L107R	**4.9**	**3.0**	**<3.0**	**<3.0**	6.3	**<3.0**	**<3.0**	4.9	5.9	**<3.0**	3.0
G106D/L107F	5.4	5.9	≥6.8	**5.4**	≥6.8	**<3.0**	6.3	5.4	5.9	4.9	3.0

[a] Ag-ELISA was used to determine the reciprocal end point titers ($\log_{10}$) for secreted VLP antigens. The numbers shown in boldface are the end point titers decreased below that of the WT by at least two threefold dilutions.

[b] Group, recognized by all flaviviruses examined; subgroup, recognized by more than one serocomplex; complex, recognized by all members of JEV complex; subcomplex, not recognized by all members of the serocomplex; type, recognized by JEV only.

[c] Virus against which antibody was raised.

Test validation and calculation of P/N absorbance ratio values. Test validation and positive/negative (P/N) ratio values were determined according to the procedure of Martin et al. (25). Briefly, internal positive and negative serum controls were included in each 96-well plate for test validation. For a testing plate to be considered valid, the average A_{450} for the positive serum control reacted with positive viral antigen had to be at least two times greater than the average A_{450} for the same positive serum control reacted with the negative tissue culture fluid antigen. Each patient serum sample was validated in the same manner. This verified that significant A_{450} values against viral antigens were not due to nonspecific binding of serum antibodies to tissue culture fluid components.

Positive values for each specimen were determined as the average A_{450} for the patient serum sample reacted with positive viral antigen. Negative 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. A specimen was classified as a validated positive sample if it had a P/N ratio of ≥3.0.

Statistical analysis. A plot of the sensitivity versus the false-positive rate (1 − specificity), the receiver operator characteristic (ROC) curve analysis, was applied to determine the discriminatory accuracies of the tests using WT or cross-reactivity-reduced JEV VLPs using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). The area under the ROC curve (AUC) was used to evaluate the performance of a diagnostic test to determine the evidence of infection. The comparative ROC was used to calculate the significance level and to compare the paired-assay performance according to the method described by Hanley and McNeil (11).

A P/N ratio of <3 or ≥3 for a given specimen was classified as negative or positive, respectively. A two-by-two contingency table was prepared that categorized four quadrants as true positive, true negative, false positive, and false negative. These transformed data were applied to calculate the sensitivity, specificity, and positive likelihood ratio.

RESULTS

Development of cross-reactivity-reduced JEV VLP antigens. A total of one, four, and six different amino acid substitutions were introduced at each of the JEV E protein fusion peptide residues Gly104, Gly106, and Leu107, respectively, into the WT JEV expression plasmid (Tables 1 and 2). VLP secretion levels from transiently transformed cells with G104 substitutions in previous DENV-2, WNV, and SLEV studies were below the detection level and thus excluded from MAb-mapping studies (6, 7, 39). Unexpectedly, we were able to detect the secretion of JEV G104H VLPs if the plasmid-transformed cells were initially recovered at 37°C and further incubated at 28°C.

Ag-ELISA was used to determine VLP secretion levels and to standardize VLP concentrations for MAb mapping. The initial MAb screening identified multiple E protein residues which, when mutated, resulted in altered recognition by MAbs of various levels of cross-reactivity (Table 2). The G104H substitution dramatically reduced the reactivities of two of four flavivirus group-reactive MAbs (4G2 and 6B6C-1), one subgroup-reactive MAb (5-2), and one complex-cross-reactive MAb (16). The reactivities of group (6B6C-1, 4G2, and 23-2)-, subgroup (5-2 and 2B5B-3)-, and subcomplex (1B5D-1)-reactive MAbs were reduced by G106Q, G106K, G106V, or G106D mutant VLPs. All of the L107 substitutions reduced the reactivities to the flavivirus group-reactive MAbs, with the exception of 6B6C-1. Only L107F dramatically reduced the reactivity of 6B6C-1. The group-reactive MAb 4G2 exhibited dramatically reduced reactivity for all substitutions introduced at G104, G106, or L107, as was the case for the subgroup-reactive MAb 5-2. The only L107 substitution that significantly reduced JEV complex- and subcomplex-cross-reactive MAbs was L107F. The reactivity of the JEV type-specific MAb (J3 14 H5-2), although not strongly reactive for the JEV-WT VLP, was not negatively affected by either the G106 or L107 substitution, but it was reduced by the G104H substitution. Since the G104H mutation reduced VLP secretion and JEV type-specific MAb reactivity, this mutation was eliminated from further analysis.

Based on MAb-mapping results for the individual fusion peptide mutants (Table 2), substitutions at Gly106 (K, V, or D) and Leu107 (D, R, or F) were combined into nine different double-mutant constructs in an effort to maximize both cross-reactivity reductions and antigen secretion (Table 3). The combinations KD, G106V/L107R (VR), and G106D/L107D (DD) produced the most dramatic reductions in cross-reactive MAb reactivity: these constructs either lost the ability to be detected or were severely reduced in recognition by all of the group-, subgroup-, and subcomplex-cross-reactive MAbs yet exhibited only minor reductions for the complex-reactive MAbs 6B4A-10 and 16. As expected, there was no reduction in the

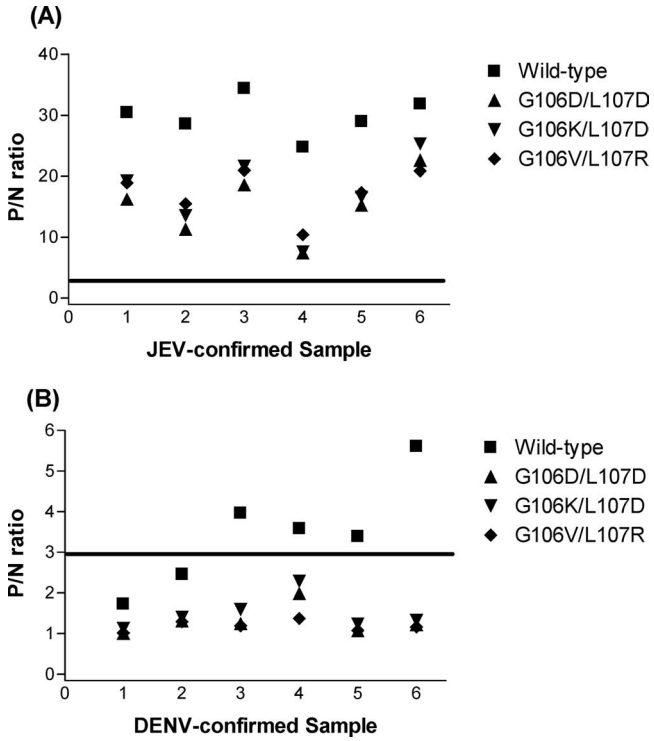

FIG. 1. Determination of cross-reactivity-reduced JEV VLPs for serodiagnosis. Six each of JEV-confirmed (A) and DENV-confirmed (B) serum samples were tested at 1:400 dilution by ELISA using WT and three G106 and L107 double-amino-acid-mutated JEV VLPs, DD, KD, and VR. The bold lines represent the P/N ratio cutoff value of 3.0 used for the positive detection of serum Ig.

reactivity of type-specific MAb J3 14 H5-2 relative to JEV WT (Table 3). These reactivity reductions were predictable in that the double mutants retained any reactivity reduction observed in either of the two corresponding single mutants. However, we also observed nonadditive phenomena in the L107F-containing double mutants. For example, G106D-L107F restored various degrees of WT reactivities against 6B6C-1, 4G2, 6B4A-10, and 1B5D-1 that were observed in their corresponding single-substitution antigens.

Selection of cross-reactivity-reduced JEV VLP antigens for serodiagnosis. Because the long-term application of this work is to develop novel serodiagnostic antigens and VLP secretion from plasmid-transformed cells is critical for efficient antigen production, we focused on substitutions that did not interfere with, or that enhanced, VLP secretion relative to that of the WT plasmid-transformed cells. Three double-mutant JEV VLPs, KD, VR, and DD, that showed the most dramatic reductions in reactivity with cross-reactive MAbs were selected to compare their performances as ELISA antigens against a preliminary panel of JEV- and DENV-confirmed human sera.

Recombinant JEV VLP antigens for the WT, KD, VR, and DD were employed in MAC-ELISA to determine their different abilities to detect virus-specific IgM, as well as cross-reactive IgM antibodies, in JEV ($n = 6$)- and DENV ($n = 6$)-infected human sera. The assay results with a serum dilution at 1:400, expressed as the P/N ratio using mutant or WT VLPs, are summarized in Fig. 1. MAC-ELISA employing the JEV-WT antigen detected IgM antibody in all six JEV-infected sera and also positively detected cross-reactive IgM antibody in four of six DENV sera (Fig. 1A and B). None of the three mutant JEV antigens detected cross-reactive antibodies in the DENV serum panel (Fig. 1B), but all of the mutant antigens positively detected IgM antibody in the JEV serum panel (Fig. 1A). As expected, all the P/N values employing mutant JEV antigens were lower than those with WT antigen in either the JEV or DENV serum panels. In the JEV serum panel, the P/N values were very similar for all three double mutants; however, on average, the P/N values were highest with the KD antigen. These results suggested that the three G106 and L107 double amino acid mutants eliminated detection of cross-reactive IgM antibody in non-JEV DENV patient sera but maintained the capacity to detect JEV-specific IgM antibodies in JEV patient sera. In addition, the JEV-KD plasmid-transformed cells maintained the same high VLP secretion as the WT plasmid-transformed cells, unlike the other two G106/L107 constructs, which exhibited reduced VLP secretion levels (Table 1). Before proceeding with the serum screening, we decided to examine the reactivities of the JEV-KD antigen against a very limited supply of two JEV-specific Nt MAbs (112 and 503) (17). As expected, the KD antigen maintained the same high-level reactivity as the WT against MAbs 112 and 503 (data not shown). For these reasons, the KD VLP was selected as the single cross-reactivity-reduced JEV antigen analyzed in IgM and IgG serum screening.

Detection of JEV and WNV antibodies by MAC- and GAC-ELISA. A total of 79 arbovirus-infected human serum specimens were screened with four different VLP antigens in this study. JEV and DENV are the most medically relevant flaviviruses in Asia, and WNV, SLEV, and Powassan virus (POWV) are the medically relevant flaviviruses in North America. Additionally, there is significant geographic overlap between WNV and JEV in Southeast Asia, the Indian subcontinent, and Oceania. In order to better understand the potential complications in serodiagnosis due to antibody cross-reactivity, serum specimens were randomly coded and blind tested using JEV-WT, JEV-KD, WNV-WT, and WNV-RH antigens in MAC- and GAC-ELISA to test for the presence of IgM and IgG antibodies. Sera were tested concurrently for all four antigens at dilutions of 1:400 and 1:4,000. We determined a priori that a P/N ratio of $\geq$3.0 indicated the positive presence of serum antibody. This value has become generally accepted and has worked well for us in the past (30).

The JEV panel consisted of 16 presumptive JEV-infected acute patient serum specimens. The JEV-WT antigen detected anti-JEV IgM in 16/16 of these presumptive positive samples at either a 1:400 or 1:4,000 serum dilution (Table 4) (P/N ratios $\geq$ 3.0; range, 16.8 to 38.9, and average, 31.4 for 1:400 serum dilution; range, 5.7 to 25.3, and average, 15.0 for 1:4,000 serum dilution). Replacing JEV-WT with the JEV-KD antigen in the MAC-ELISA also detected 16/16 positive samples (P/N ratios $\geq$ 3.0; range, 10.3 to 31.7, and average, 23.4 for 1:400 serum dilution; range, 3.4 to 20.9, and average, 10.1 for 1:4,000 serum dilution). The JEV-WT antigen detected anti-JEV IgG in 15/16 of these presumptive JEV-infected sera at a 1:400 or 1:4,000 dilution (P/N ratios $\geq$ 3.0; range, 4.1 to 26.5, and average, 9.4 for 1:400 serum dilution; range, 3.3 to 23.6, and average, 9.0 for 1:4,000 serum dilution). Replacing JEV-WT

TABLE 4. Summary of MAC- and GAC-ELISA-positive serum results grouped by infecting virus

Virus[a]	Specimen source	Sample size	No. of samples in which Ig was detected															
			JEV VLP								WNV VLP							
			IgM				IgG				IgM				IgG			
			1:400		1:4,000		1:400		1:4,000		1:400		1:4,000		1:400		1:4,000	
			WT	KD	WT	KD	WT	KD	WT	KD	WT	RH	WT	RH	WT	RH	WT	RH
JEV	Taiwan-CDC	16	16	16	16	16	15	15	15	14	16	13	8	1	14	4	14	4
DENV	Taiwan-CDC	24	10	5	5	1	22	19	22	18	20	8	9	1	24	22	24	20
WNV	U.S. CDC	21	20	15	12	2	10	4	6	3	21	21	21	21	19	19	18	18
SLEV	U.S. CDC	6	5	1	2	0	2	2	2	2	6	3	2	2	4	2	3	2
Other flavivirus	U.S. CDC	9	2	2	0	0	2	1	2	1	7	5	2	0	6	2	6	1
POWV		2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Undefined flavivirus		7	2	2	0	0	2	1	2	1	7	5	2	0	6	2	6	1
Nonflavivirus (WEEV, EEEV, LACV)	U.S. CDC	3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Total		79	53	39	35	19	51	41	47	38	70	50	42	25	68	49	65	45

[a] WEEV, Western equine encephalitis virus; EEEV, Eastern equine encephalitis virus; LACV, La Crosse virus.

with the JEV-KD antigen in the GAC-ELISA detected 15/16 and 14/16 positive samples at 1:400 and 1:4,000 serum dilutions, respectively (P/N ratios ≥ 3.0; range, 3.2 to 19.3, and average, 7.2 for 1:400 serum dilution; range, 3.2 to 19.8, and average, 7.0 for 1:4,000 serum dilution).

The WNV panel consisted of 21 presumptive WNV-infected sera from North America. The WNV-WT antigen detected anti-WNV IgM in 21/21 of these presumptive positive samples at a 1:400 or 1:4,000 dilution (Table 4) (P/N ratios ≥ 3.0; range, 19.37 to 45.11, and average, 30.19 for 1:400 serum dilution; range, 6.73 to 30.6, and average, 23.93 for 1:4,000 serum dilution). Replacing WNV-WT with the WNV-RH antigen in the MAC-ELISA also detected 21/21 positive samples (P/N ratios ≥ 3.0; range, 16.88 to 36.83, and average, 24.7 for 1:400 serum dilution; range, 4.68 to 24.52, and average, 19.27 for 1:4,000 serum dilution). The WNV-WT antigen detected anti-WNV IgG in 19/21 or 18/21 of these presumptive WNV-infected sera at a 1:400 or 1:4,000 dilution (P/N ratios ≥ 3.0; range, 3.86 to 34.27, and average, 18.82 for 1:400 serum dilution; range, 8.75 to 27.65, and average, 20.92 for 1:4,000 serum dilution). Replacing WNV-WT with the WNV-RH antigen in the GAC-ELISA similarly detected 19/21 and 18/21 positive samples at 1:400 and 1:4,000 serum dilutions, respectively (P/N ratios ≥ 3.0; range, 3.1 to 22.5, and average, 16.02 for 1:400 serum dilution; range, 4.78 to 26.62, and average, 17.51 for 1:4,000 serum dilution).

Use of JEV-WT antigen in MAC-ELISA with non-JEV patient serum panels (Table 4; DENV, 24; WNV, 21; SLEV, 6; other flavivirus, 9; nonflavivirus, 3) detected JEV-cross-reactive IgM antibodies in 10/24 (DENV panel; 41.7%), 20/21 (WNV panel; 95.2%), 5/6 (SLEV panel; 83.3%), 2/9 (other flavivirus panel; 22.2%), and 0/3 (nonflavivirus panel; 0%) sera at a 1:400 serum dilution. For the same serum panels, when tested at a 1:4,000 dilution using the JEV-WT antigen in MAC-ELISA, the number of JEV-positive sera was reduced to 5/24 in the DENV panel (20.8%), 12/21 in the WNV panel (57.1%), 2/6 in the SLEV panel (33.3%), 0/9 in the other flavivirus panel, and 0/3 in the nonflavivirus panel. As expected, these false-positive detection rates for JEV-cross-reactive IgM antibodies were reduced further when the JEV-KD antigen and a serum dilution of 1:4,000 were used in the assay (Table 4). However, 1 of 24 DENV-positive and 2 of 21 WNV-positive serum specimens remained JEV-IgM positive, respectively. A similar serodiagnostic improvement was observed when WNV-WT antigen was replaced with WNV-RH antigen in the MAC-ELISA and the non-WNV serum panels were tested at a 1:4,000 dilution. Only 1/16 JEV, 1/24 DENV, 2/6 SLEV, 0/9 other flavivirus, and 0/3 nonflavivirus serum specimens, diluted 1:4,000, remained IgM positive with WNV-RH antigen (Table 4).

Similar reductions in the detection of cross-reactive antibodies were less pronounced when the JEV-KD or WNV-RH antigens were used in GAC-ELISA for non-JEV or non-WNV panels at a 1:4,000 serum dilution (Table 4). The JEV-KD antigen maintained positive P/N ratios for IgG in 18/24 DENV-infected and 3/21 WNV-infected patient sera. WNV-RH antigen detected cross-reactive IgG antibodies in 4/16 and 20/24 JEV- and DENV-infected patient sera, respectively. The DENV-infected patient serum specimens, provided by the Center for Disease Control—Taiwan, were obtained from the Taiwanese population, and the currently infecting DENV was determined by virus isolation and/or virus-specific nucleic acid detection and MAC-ELISA. Taiwan is in a JEV endemic area, and mandatory nationwide JEV vaccination has been implemented since the late 1960s (41). Thus, the presence of highly cross-reactive IgG antibodies against JEV-KD and WNV-RH is consistent with the observation that some of the DENV-infected sera could be from patients previously vaccinated against or exposed to JEV. Acute DENV infection

TABLE 5. MAC- and GAC-ELISA P/N ratios for highly cross-reactive serum specimens diluted 1:4,000

Specimen no.	P/N ratio							
	JEV				WNV			
	IgM		IgG		IgM		IgG	
	WT	KD	WT	KD	WT	RH	WT	RH
JEV panel								
2	92	7.85	4.63	7.59	3.2	4.52	1.29	
3	21.71	12.75	6.17	2.93	6.15	2.53	5.26	1.21
4	21.00	13.73	7.85	4.24	3.99	1.61	4.78	1.09
5	12.16	5.54	6.27	5.85	4.07	2.82	6.76	4.6
6	22.26	14.54	5.96	3.95	4.99	1.97	4.52	1.26
9	15.46	13.26	9.13	6.39	4.21	2.15	7.44	1.92
11	20.64	13.18	8.49	5.41	7.47	2.54	8.86	1.41
15	11.19	10.26	9.42	6.19	3.71	1.97	6.47	1.47
DENV panel								
3	1.6	1.19	10.48	6.54	4.19	1.45	19.63	12.89
7	4.87	2.14	24.13	19.54	6.56	2.56	22.84	23.34
11	3.6	2.47	7.22	1.8	5.9	2.13	18.4	2.95
12	18.52	3.74	24.28	13.42	14.07	3.88	28.65	17.52
14	2.55	1.7	24.58	19.17	3.2	1.78	27.93	23.52
16	3.27	1.34	1.78	1.35	5.97	1.59	3.13	1.08
20	2.45	1.4	21.88	7.53	3.24	1.64	15.38	12.27
21	2.39	1.48	28.58	23.15	3.79	1.69	18.73	18.28
24	5.17	1.95	29.91	24.9	6.68	1.98	17.68	16.37
WNV panel								
6	5.61	3.01	5.08	2.45	26.78	23.26	20.47	19.05
8	3.57	1.66	5.48	1.33	23.87	18.8	23.97	21.59
9	3.93	2.45	3.27	1.05	28.51	24.17	26.50	26.01
10	3.11	1.48	2.02	1.05	20.00	15.89	12.28	8.95
12	4.61	2.08	5.78	1.42	24.78	20.33	26.39	25.00
13	3.81	2.36	7.42	1.79	20.62	16.08	17.20	11.34
14	6.79	2.65	3.39	1.48	28.40	22.62	17.64	15.29
11	5.20	1.73	7.44	11.89	20.04	13.93	25.34	24.60
17	9.08	1.74	27.10	6.13	26.14	19.26	25.72	12.89
19	4.55	1.87	2.39	1.24	25.78	22.07	19.16	19.11
20	9.91	2.12	11.74	2.14	30.60	23.74	26.27	26.62
21	6.06	3.78	17.49	9.99	6.73	4.68	23.95	19.69

in JEV-immune individuals would be expected to increase the concentration and/or the relative avidity of cross-reactive IgG antibodies.

Results from the most cross-reactive sera, which were IgM positive at 1:4,000 against WNV-WT (8 of 16 in the JEV panel), JEV-WT (12 of 21 in the WNV panel), and WNV-WT or JEV-WT (9 of 24 in the DENV panel), are summarized in Table 5. Two SLEV patient specimens, although JEV-KD negative, remained positive against WNV-RH in the MAC-ELISA and were classified as primary WNV and acute SLEV infections in a previous study (30).

Replacing the WT antigens with JEV-KD or WVN-RH antigens in MAC-ELISA at a 1:4,000 serum dilution resolved the IgM cross-reactivity and produced more accurate disease classifications in 7 of 8 specimens in the JEV panel and 10 of 12 specimens in the WNV panel. The remaining apparently false-positive specimen, number 2 in the JEV panel, had a much higher P/N value with JEV-KD than with WNV-RH antigen, for which it was barely positive (Table 5) (20.92 versus 3.2, respectively). Similarly, specimens numbers 6 and 21 in the WNV panel had higher P/N values with WNV-RH than with JEV-KD antigen (Table 5) (23.26 versus 3.01 and 4.68 versus 3.78, respectively). As in previously published assays, the higher P/N ratio appears to be indicative of currently infecting virus (30); thus, the viruses responsible for the current infection could be accurately identified for these three specimens.

Use of JEV-KD or WNV-RH in MAC-ELISA with the DENV-infected patient sera at a 1:4,000 serum dilution also reduced the observed cross-reactivity, producing more accurate disease state classifications in eight of nine specimens, with the exception of specimen number 12 (P/N ratios, 3.74 and 3.88 against JEV-KD and WNV-RH, respectively) (Table 5). This specimen was reverse transcriptase-PCR positive for DENV-2. It also had the highest P/N ratios against JEV-WT (18.52) and WNV-WT (14.07) antigens (Table 5). Thus, the relatively low positive P/N ratios with JEV-KD and WNV-RH antigens in this patient specimen may have resulted from a small portion of DENV-2-induced IgM antibodies recognizing a conserved JEV serocomplex-cross-reactive epitope(s) in both mutant antigens.

Statistical comparison of antigen performances. The assay results, expressed as the P/N ratio, using JEV-WT or JEV-KD antigens were analyzed in a continuous rating scale by ROC curves for overall assay performance and are shown in Fig. 2. For MAC-ELISA at either a 1:400 or a 1:4,000 serum dilution, paired-ROC-curve analysis revealed no statistical difference

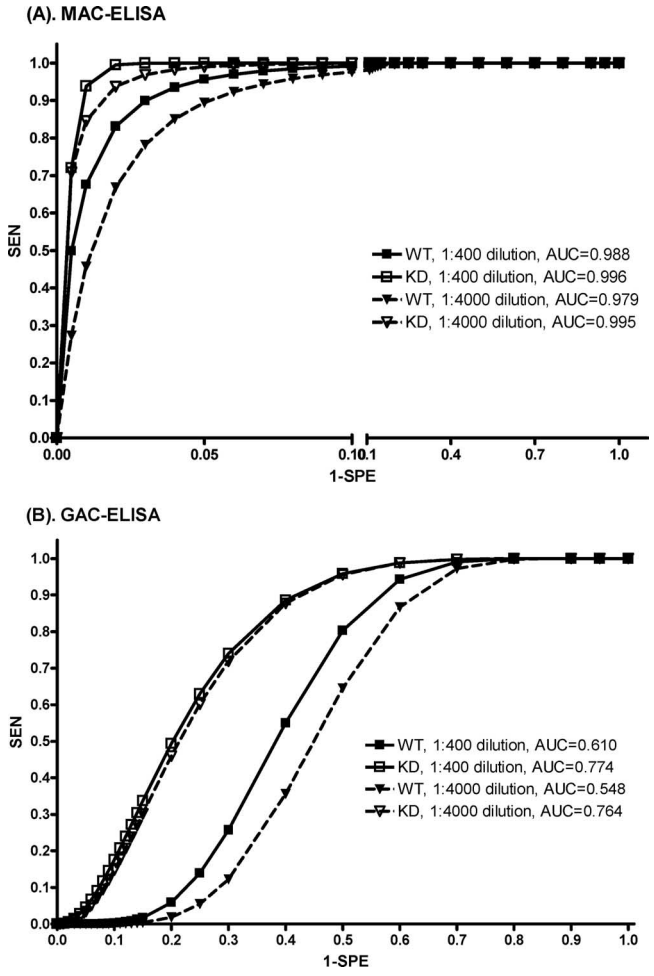

FIG. 2. Fitted ROC curves using P/N ratios for JEV WT and KD mutated VLP antigens. A JEV-infected target serum panel and five arbovirus-infected control serum panels were determined by MAC-ELISA (A) and GAC-ELISA (B).

($P > 0.05$) in the reported AUC between WT and KD antigens in this assay (Fig. 2A). Paired-ROC-curve analysis revealed that the AUC for each of the GAC-ELISAs was statistically different at a 1:400 or 1:4,000 serum dilution ($P < 0.05$) (Fig. 2B). Overall, the GAC-ELISA results indicated that the assay using the JEV-KD antigen (AUC = 0.774 and 0.764 at 1:400 and 1:4,000 serum dilutions, respectively) more accurately discriminated between true JEV IgG-positive and -negative serum specimens than did the JEV-WT antigen (AUC = 0.610 and 0.548 at 1:400 and 1:4,000 serum dilutions, respectively).

Two-by-two contingency tables were prepared to analyze the diagnostic accuracies of WT and cross-reactivity-reduced antigens for both JEV and WNV, including the sensitivity, specificity, and likelihood ratio (Table 6). In the MAC-ELISA with the JEV panel sera, the sensitivities of JEV-KD and JEV-WT antigens were 100%. The overall specificities at the 1:400 serum dilution were 41.27% and 63.64% for WT and KD antigens, respectively; at the 1:4,000 dilution, the specificities increased to 63.49% and 95.45%, respectively. The sensitivities of WNV-RH and WNV-WT antigens in the MAC-ELISA with the WNV serum panel were 100% at both 1:400 and 1:4,000 dilutions. The overall specificities at the 1:400 dilution were 17.24% and 52.63% for WT and RH antigens; at the 1:4,000 dilution, the specificities increased to 57.78% and 91.11%, respectively. The likelihood ratio test further indicated that the JEV-KD and WNV-RH antigens had higher propensities to correctly determine the disease state when the serum specimen was tested at a 1:4,000 dilution (Table 6) (likelihood ratios, 22 and 11.25, respectively, for JEV-KD and WNV-RH versus 2.74 and 2.37 for JEV-WT and WNV-WT, respectively).

The GAC-ELISA results obtained from sera tested at the 1:4,000 dilution are summarized in Table 6. The most accurate antigens using these testing procedures were JEV-KD and WNV-RH. Although these antigens had sensitivities of 87.5% for JEV-KD and 100% for WNV-RH, the assay specificities were relatively low: 45.45% for JEV-KD and 51.72% for WNV-RH. The likelihood ratios were 1.6 and 2.07 using

TABLE 6. Influences of WT and cross-reactivity-reduced JEV and WNV VLPs on the performances of MAC- and GAC-ELISAs[a]

Disease panel	Assay		Performance				
	Antigen	Serum dilution	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
JEV	MAC-ELISA						
	JEV-WT	1:400	100	79.4–100.0	41.27	29.01–54.38	1.7
		1:4,000	100	79.4–100.0	63.49	50.41–75.27	2.74
	JEV-KD	1:400	100	79.41–100.0	63.64	47.77–77.59	2.75
		1:4,000	100	79.41–100.0	95.45	84.53–99.44	22
	GAC-ELISA						
	JEV-WT	1:4,000	86.36	72.65–94.83	6.25	0.1581–30.23	0.92
	JEV-KD	1:4,000	87.5	61.65–98.45	45.45	30.39–61.15	1.6
WNV	MAC-ELISA						
	WNV-WT	1:400	100	83.89–100.0	17.24	8.590–29.43	1.21
		1:4,000	100	78.20–100.0	57.78	42.15–72.34	2.37
	WNV-RH	1:400	100	83.89–100.0	52.63	38.97–66.02	2.11
		1:4,000	100	78.20–100.0	91.11	78.78–97.52	11.25
	GAC-ELISA						
	WNV-WT	1:4,000	100	78.20–100.0	17.24	8.590–29.43	1.21
	WNV-RH	1:4,000	100	78.20–100.0	51.72	38.22–65.05	2.07

[a] Influence on abilities to distinguish JEV and WNV serum panels (disease panels), respectively, from other arbovirus-infected serum panels (control panels) using the positive-cutoff criterion (P/N ≥ 3) as the evidence of infection.

JEV-KD and WNV-RH, respectively (Table 6), indicating that the presence of JEV serocomplex-cross-reactive antibodies in JEV-, WNV-, and SLEV-infected patients or JEV-immune, DENV-infected patients complicated the disease state interpretation by using GAC-ELISA results alone. Thus, further research into the identification and ablation of a complex-cross-reactive epitope(s) and the incorporation of this mutation(s) into JEV-KD and WNV-RH antigens is critical for improving the GAC-ELISA for accurate disease burden studies in the future.

Not only was there strong statistical support for increased diagnostic accuracy using the JEV-KD and WNV-RH antigens versus the WT antigen, there was also a dramatic and statistically significant improvement in assay performance when patient sera were tested at a 1:4,000 instead of a 1:400 dilution. Although this was true regardless of the antigen used, it is best demonstrated in the MAC-ELISA with the cross-reactivity-reduced antigens. The overall specificity with the JEV-KD antigen increased from 63.64% to 95.45% when sera were diluted to 1:4,000 (Table 6) (95% confidence interval [CI] = 47.77% to 77.59% at 1:400 and 84.53% to 99.44% at 1:4,000 dilution). This performance improvement was further supported by the increase in the likelihood ratio for JEV-KD antigen from 2.75 to 22 when the serum dilution was increased from 1:400 to 1:4,000 (Table 6). Similar performance improvements in specificity and in likelihood ratios were observed with the WNV-RH antigen when the serum dilutions were increased from 1:400 to 1:4,000 (Table 6) (95% CI = 38.97% to 66.02%, likelihood ratio = 2.11 for 1:400; 95% CI = 78.78% to 97.52%, likelihood ratio = 11.25 for 1:4,000).

DISCUSSION

The presence of cross-reactive serum antibodies developed from sequential heterologous flavivirus infection or previous vaccination can dramatically complicate flavivirus serodiagnosis. Currently, the most accurate serologic method is the plaque reduction neutralization test, performed by testing paired acute- and convalescent-phase serum specimens (20). We applied a structure-based mutagenesis algorithm for the development of cross-reactivity-reduced WNV and SLEV mutant antigens that could be used in the MAC-ELISA with single serum specimens for the accurate identification and differentiation of WNV and SLEV infections (6, 7, 30, 37, 39). This strategy was applied to the development of a cross-reactivity-reduced JEV VLP antigen in the present study.

Substitutions for Gly104, Gly106, or Leu107 of the JEV E protein could significantly reduce the reactivities of flavivirus group-, subgroup-, complex-, and subcomplex-reactive MAbs (Tables 2 and 3). None of these substitutions significantly altered the reactivities of JEV-specific MAbs. Combinations of Gly106 and Leu107 substitutions in VLP antigens further decreased cross-reactive MAbs' reactivities. In most cases, the MAb reactivity reductions observed in the single mutants combined additively in the double mutants. However, in some of the double-mutant constructs, MAb reactivity reductions were much greater than those observed in either of the single-substitution VLPs. Similar synergistic effects on cross-reactive MAb reactivities were previously observed in WNV and SLEV Gly106/Leu107 mutants (7, 39). For example, in the RH VLP, the JEV complex-reactive MAbs 16 and 6B4A-10 were reduced dramatically, yet these same two substitutions alone showed little or no reduction in the reactivities of these MAbs. In this study, the only substitutions reducing the reactivities of MAbs 16 and 6B4A-10 were G104H and L107F. Unfortunately, these mutations also resulted in decreasing the secretion of mutant VLPs by an unknown mechanism. Nevertheless, when the L107F substitution was combined with Gly106 substitutions, the resultant double-mutant VLPs reverted to JEV-WT levels of reactivity for both of these JEV complex-reactive MAbs. We have not observed such negatively synergistic effects on MAb reactivity when single substitutions were combined in either WNV or SLEV studies (7, 39).

Gly104, Gly106, and Leu107, like many of the flavivirus fusion peptide residues, are almost completely invariant across the flaviviruses. Interestingly, L107F is known to occur in a few different flavivirus isolates; most relevant here is its occurrence in the JEV attenuated vaccine strain SA-14-14-2, DENV-2 strain PUO-280, POWV, and deer tick virus (3, 21, 26). It is likely that the L107F substitution does not interfere with flavivirus viability. However, the L107F substitution has been shown to reduce cross-reactive antibody recognition, not only in JEV, but also in WNV, SLEV, and tick-borne encephalitis virus (1, 7, 39).

Among the JEV Gly106/Leu107 double mutants, the JEV-KD combination exhibited the greatest reductions in cross-reactivity and the highest levels of type specificity. Similar results, but with different specific substitutions, were observed in WNV with RH and in SLEV with G106D/L107R substitutions (7, 39). These findings and previous reports suggest that certain fusion peptide residues can act as epitope determinants for flavivirus subgroup- and JEV complex-reactive MAbs, in addition to flavivirus group-reactive MAbs (6, 7, 27, 37–39).

The serological cross-reactivity between WNV and SLEV in MAC-ELISA was the primary reason why the 1999 outbreak of WNV in New York City was initially thought to be caused by SLEV (22). Strategies to differentiate current flavivirus infections have been proposed, such as by determining the IgM-to-IgG ratio (15, 36), by using recombinant EDIII or nonstructural protein 1 (NS1) as an antigen (2, 36), or by epitope-blocking ELISA (12, 19). All of these assays have limitations, including requiring the simultaneous testing of serum specimens for IgM and IgG, requiring paired acute- and convalescent-phase serum specimens, or the observation that not all infected individuals develop antibody against EDIII or NS1 antigen. Here, we have documented that the JEV-KD antigen proved to be superior to the JEV-WT antigen; it exhibited greater sensitivity and specificity, demonstrated by the higher AUC values in both the IgM and the IgG assays (Fig. 2). Moreover, the specificity of MAC- and GAC-ELISA was improved significantly using JEV-KD antigen to differentiate five distinct non-JEV-infected serum panels (Table 4). Nevertheless, the results of this and previous studies (30) demonstrate the appropriateness of using these cross-reactivity-reduced antigens to successfully differentiate JEV-specific from WNV-specific serum antibodies with single acute-phase serum samples and paired KD and RH antigens in the MAC-ELISA.

In the GAC-ELISA, the majority of DENV-infected patients' sera were IgG positive whether we used the JEV and

WNV WT or the cross-reactivity-reduced antigens (Table 4). The DENV patient sera were obtained from the Taiwanese population, where JEV is endemic. Thus, it is expected that a high percentage of the Taiwanese population would have IgG antibodies against JEV. Acute dengue infection in the presence of JEV immunity would be expected to enhance the production of flavivirus-cross-reactive antibodies derived from shared antigenic epitopes and thus be detected by WT and cross-reactivity-reduced JEV and WNV antigens. Nevertheless, even with these highly cross-reactive DENV patients' sera, both the JEV-KD and WNV-RH antigens exhibited improved specificity compared to the WT antigens. In the WNV- and SLEV-infected serum panels, the cross-reactivity of IgG antibody against JEV was dramatically reduced using JEV-KD antigen and serum tested at a 1:4,000 dilution. Thus, it is possible to estimate the disease burden more precisely using our JEV-KD and WNV-RH antigens in the GAC-ELISA, and assay specificity is further improved by screening all sera at a 1:4,000 dilution. The assay improvements noted at a 1:4,000 versus a 1:400 serum dilution resulted from the observations that broadly cross-reactive antibodies make up a large proportion of the flavivirus antibody response and yet the smaller proportion of virus-specific antibodies exhibits the highest avidities.

The results presented here confirm previous reports that the conserved fusion peptide region constitutes an immunodominant antigenic hot spot and forms a region of multiple overlapping cross-reactive epitopes (6, 7, 27, 37, 39). We have demonstrated that specific substitutions in this region can be utilized to construct novel cross-reactivity-reduced serodiagnostic antigens with improved assay performance for diagnosing and differentiating JEV-infected from other flavivirus-infected human serum specimens. This work, therefore, not only has important implications for furthering the basic understanding of immunological responses to flavivirus infections but should also strengthen the public health response to spreading flavivirus disease by improving serodiagnostic specificity and estimates of the global disease burden.

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