Duplex Microsphere-Based Immunoassay for Detection of Anti-West Nile Virus and Anti-St. Louis Encephalitis Virus Immunoglobulin M Antibodies

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West Nile (WN) virus was introduced into the United States in 1999, when the first human cases of WN fever and encephalitis appeared in New York City. From there, the virus has spread throughout North America, in some areas cocirculating with the related flavivirus St. Louis encephalitis (SLE) virus. Public health laboratories currently use an immunoglobulin M (IgM) antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) as a primary test for human serodiagnosis, followed by a confirmatory plaque-reduction neutralization test (PRNT). The MAC-ELISAs take 2 days to perform; therefore there is a need for a more rapid test. This report describes a duplex microsphere-based immunoassay (MIA) that shortens the test processing time to about 4.5 h. The assay employs two sets of microspheres coupled to a single flavivirus group-reactive antibody, which are used to capture the WN and SLE viral antigens independently. Immunoglobulin G-depleted serum is concurrently assayed for IgM antibodies to each of the viral antigens. The results are standardized and classified by using quadratic discriminant analysis so that a single result, anti-WN IgM-positive, anti-SLE IgM-positive, negative, or nonspecific, can be determined. The duplex MIA results compared favorably to those of the plaque-reduction neutralization test and MAC-ELISA. The assay proved to be reproducible, produced accurate classifications as to the infecting virus, and was specific.

Since its introduction into the United States in 1999, West Nile (WN) virus has spread throughout most of the country. Human disease cases have been reported in all states except Alaska, Hawaii, and Washington as of October 2004. A total of 9175 human disease cases were reported to Centers for Disease Control ArboNET for 2003, as reported in the Centers for Disease Control West Nile website (http://www.cdc.gov/ncidod/dvbid/westnile/index.htm). The related flavivirus St. Louis encephalitis (SLE) virus is endemic in the United States. A total of 4482 confirmed human disease cases of SLE have been documented between 1964 and 2000 (http://www.cdc.gov/ncidod/dvbid/arbor/pdf/cases-sle-1964 to 2000.pdf). The last major outbreak of SLE in the United States occurred in 1974 to 1977, when more than 2500 human SLE disease cases were reported.

WN and SLE virus infections often present with similar clinical profiles. Symptoms common to both diseases may include sudden onset of fever, headache, and myalgia in mild cases and disorientation, meningitis, and encephalitis in severely affected patients. WN virus can produce a rash, and flaccid paralysis has been reported in some cases (5).

Both WN and SLE viruses belong to the Japanese encephalitis virus serocomplex of viruses (12). Not only do they share many clinical manifestations but they are serologically similar. Immunoglobulin G (IgG) antibodies to viruses within the serocomplex exhibit extensive cross-reactivity, whereas immunoglobulin M (IgM) antibodies are less cross-reactive (10, 15). The traditional serological method for identifying the infecting virus is the time-consuming and technically difficult plaque-reduction neutralization test (PRNT) (8). The serological testing algorithm that has been adopted by most of the United States state health departments uses the IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) (9) and IgG-ELISA (6) as primary tests. A confirmatory PRNT for positive samples is often performed by laboratories that have this capability. The MAC-ELISA is a 2-day test that requires about 4 h of hands-on time for a 40-sample test. The advent of a more rapid yet equally sensitive, single test to replace the WN and SLE MAC-ELISAs would be of benefit.

Microsphere-based immunoassays (MIAs) are becoming increasingly popular as a serological option for laboratory diagnosis of many diseases (4, 7). The technology involves the detection and analysis of a reaction attached to microspheres or beads. The detecting instrument is a simplified flow cytometer, and lasers simultaneously identify the microsphere sets (bead sets) and measure the fluorescence associated with the reaction. The speed at which these tests can be performed and the ability to multiplex make this methodology particularly attractive. MIAs have the potential to be especially applicable in arbovirus serology because tests for infection due to viruses of the same genus can share similar formats.

MIAs using microspheres coupled to recombinant envelope and nonstructural 5 proteins of WN virus have been described recently by Wong et al. (16, 17). Here we describe a different format that utilizes an antibody that, when coupled to bead sets, can be used to assay for human IgM antibodies directed
against any flavivirus, in this case WN and SLE viruses. The data transformation methodology constitutes a significant departure from those used in other serological methods for arbovirology.

**MATERIALS AND METHODS**

**Serum specimens.** A total of 990 frozen human serum specimens were used in this study. These were obtained from the specimen archive at Centers for Disease Control’s Division of Vector-Borne Infectious Diseases, Arboviral Diseases Branch, Diagnostics and Reference Laboratory (CDC/DVBD/ADB, Fort Collins, CO). The samples were from patients with a wide range of age and geographic distribution within the United States with approximately a 2:1 ratio of acute to convalescent specimens and as gifts from CDC/DVBD/Arboviral Diseases Branch (BZB, Fort Collins, CO); CDC/DVBD/Dengue Branch (DB, San Juan, Puerto Rico); Arizona Department of Health Services (AZDHS, Phoenix, AZ), and Focus Technologies (Cypress, Calif.). The sets and subsets of sera, and the numbers of samples are detailed in Fig. 1.

**Control sera.** Anti-WN IgM-positive, anti-SLE IgM-positive control human sera, and pooled antibody-negative sera, were obtained from the DVBD/ADB/DRL. These specimens were used throughout 2003 in the diagnostic MAC-ELISAs described in Martin et al. (9). Sera were diluted 1:400 in wash buffer; CSF specimens were used undiluted. The WN viral antigen used was a WN virus envelope-premembrane (E-prM) recombinant protein secreted in transformed COS-1 cells at the Centers for Disease Control (2); the inactivated SLE viral antigen was produced in suckling mouse brain as previously described (1). Control antigens were produced under the same conditions as the viral antigens.

**PRNT.** Neutralizing antibody titers for 316 sera from ADB were available from the ADB diagnostic database. The PRNT method was previously described (8).

**Microsphere coupling.** Carboxylated microspheres (lot B) were purchased from Luminex Corporation (Austin, Tex.). Purified flavivirus group-reactive SLE monoclonal antibody 6B6C-1 (14) was obtained as a gift from Hennessy Research Associates, LLC (Shawnee, Kans.). This monoclonal antibody was covalently coupled to nominal beadset numbers 32 and 57 using the lot B method provided by Luminex Corporation. Briefly, 5 million each of bead sets 32 and 57 were activated using 10 μl of 50 mg/ml sulfo-normal human serum (Pierce Chemical Co., Rockford, Ill.) and 10 μl of 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl at pH 6.3 in the dark for 20 min on a rotary mixer. Twenty-five micrograms of 6B6C-1 was coupled to each bead set at pH 6.0 with 2 h incubation in the dark on the rotary mixer. Unused sites on the coupled microspheres were blocked with 1% bovine serum albumin in PBN (phosphate-buffered saline with 0.05% bovine serum albumin and 0.02% sodium azide) for 30 min. Bead concentrations were adjusted to 2 × 10^7 beads/ml and stored in PBN at 4°C. To determine qualitatively if coupling of the antibody to the beads was successful, 100 beads/μl in MIA buffer (phosphate-buffered saline with 1%
bovine serum albumin [Sigma Chemical Company, St. Louis, Mo.] were reacted with 4 μg/ml phycoerythrin (R-PE)-conjugated anti-mouse IgG (PE) [Jackson Immunoresearch, West Grove, Pa.]. Reactions were read on a BioPlex instrument (Bio-Rad, Hercules, Calif.).

Addition of antigens to coupled microspheres. Antigens were added to the coupled bead sets prior to performing the duplex MIA. Recombinant WN virus E-prM protein expressed in COS-1 cells and negative COS-1 antigen control (i.e., untransformed COS-1 cells processed in the same manner as the transformed cells) were purified by ultracentrifugation, and were obtained as a gift from Focus Technologies. SLE suckling mouse brain and negative antigens were obtained from the DVBID/ADB reference collection. Two and a half million 6B6C-1-coupled bead set 32 were added to 125 μg of WN viral antigen in a 5-ml volume of MIA buffer (a ratio determined to be optimal via titration). Negative recombinant antigen was added to 2.5 million beads of the same set. These were incubated with rotation in the dark at room temperature for 1 h, and then stored for up to a month at 4°C. The same procedure was used to add the SLE viral and negative suckling mouse brain antigens to 6B6C-1-coupled bead set 57. The viral protein concentration of the SLE virus antigen was unknown; however, the optimal volume of antigen per the 5-ml preparation was 25 μl, as determined by titration. The same amount was used for the negative control antigen.

IgG depletion of serum samples. Positive and negative serum controls were processed to remove IgG antibodies using Mini Rapi-Sep units (PanBio, Baltimore, Md.) according to the manufacturer’s instructions, resulting in a 1:8 dilution of the serum. The processed control serum could be stored for up to a month at 4°C. The relative amounts of IgM in the WN and SLE positive controls were determined via an MIA test using microspheres coupled to goat anti-human IgM (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) and detected using an anti-human IgM, Fc R-PE (Jackson Immunoresearch). With this setup, the median fluorescent indices (MFIs) indicated that the IgM concentrations of the anti-WN and anti-SLE IgM-positive control sera were equivalent, and therefore, no adjustment to the concentrations of the controls was necessary. Prior to use in the duplex MIA the control serum samples were adjusted to a final dilution of 1:400 using MIA buffer.

The following method was used to deplete IgG from the test serum samples. A Sepharose matrix was washed twice with phosphate-buffered saline, and a slurry containing 5 μl per well of protein G Sepharose 4 fast flow (Amersham Biosciences, Uppsala, Sweden) was added. The Sepharose matrix was washed twice with phosphate-buffered saline using a vacuum manifold (Millipore Corp., Burlington, Mass.) and 100 μl of a 1:20 dilution of patient serum in phosphate-buffered saline was added. The matrix was resuspended into the serum and the mixture was shaken on a platform for 30 min at room temperature. The IgG-depleted serum was collected by filtration into a 96-well plate that was placed inside the vacuum manifold. The serum samples were adjusted to a final dilution of 1:400 (the optimum dilution as determined via titration) with MIA buffer before use. The success of IgG depletion using protein G was shown using WN and SLE IgG-ELISAs (6) for a few samples.

Duplex MIA for detection of anti-WN and anti-SLE IgM in serum. A 96-well plate was prepared in triplicate and subjected to the same duplex MIA procedure described above for serum specimens. No pretreatment of CSF samples with protein G was performed because of the low levels of IgG that are present in CSF. Previously tested, pooled negative human CSF served as a negative control.

RESULTS

Classification of the duplex MIA results. The unprocessed MFI values for serum specimens reacting with the viral antigens in the duplex MIA reached a maximum of about 10,000 for anti-WN IgM antibodies and 6,000 for anti-SLE IgM antibodies. The positive controls typically gave MFI values of around 2,000 on the viral antigens and less than 100 on the negative antigens; the negative serum controls gave MFIs less than 100 on both viral and negative antigens. The test therefore exhibited a significant dynamic range. QDA classification regions determined using 491 specimens are shown in Fig. 2, where each specimen’s true classification, based on the PRNT or negative MAC-ELISA result, is indicated.

Cross-validation results for the QDA are shown in Table 1. The number of anti-WN IgM-positive and negative specimens available for the study was far greater than the number of available anti-SLE IgM-positive samples. In summary, cross-validation estimates of the correct classification rates for the groups were: Negative 96.0% (192/200); WN 98.4% (242/248); and SLE 93.0% (40/43).

The MAC-ELISA data for these same specimens were compared to their respective true classifications by plotting the log10 anti-WN MAC-ELISA positive-to-negative (P/N) ratios

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against the log_{10} anti-SLE MAC-ELISA P/N ratios (Fig. 3). A line was used to separate those results that were greater for anti-WN IgM than for anti-SLE IgM. The standard algorithm for the MAC-ELISA for a sample taken day 9 after onset or later classifies results with a P/N ratio of less than 2 as negative for IgM to that antigen, and a P/N of ≥3 as positive for IgM. A P/N falling in between 2 and 3 is termed equivocal.

Lines delineating the equivocal zone are shown in the graph. The MAC-ELISA results compared to the true classifications as follows: Negative 91% (with a P/N of <2) with 7 additional negative samples being classified as equivocal; WN 97.2%; SLE 74.4%. Table 1 shows the breakdown of these results. In addition, the QDA classifications of these samples were compared to MAC-ELISA results (Table 2). Of the eight samples that were incorrectly classified by the QDA as IgM-positive to either SLE or WN viral antigens (Table 1), four were in agreement with the MAC-ELISA results; i.e., results from the duplex MIA and MAC-ELISA methods disagreed with the PRNT values. None of these four specimens were collected less than 9 days after onset of symptoms, suggesting that a small percentage of patients were either unusually late in developing neutralizing antibody, failed to produce any at all, or were true false-positives.

Probabilities of correct classification. The QDA determines classification for a given sample by computing the probabilities that the sample should be classified into each group (anti-WN IgM-positive, anti-SLE IgM-positive, and negative) after which the classification is made to the group with the highest probability. The line in Fig. 2 represents the coordinates of log_{10}(W) and log_{10}(S) pairs where the group classification probability for WN is highest.

<table>
<thead>
<tr>
<th>Table 1. QDA cross-validation results and MAC-ELISA classifications compared to the true classifications of 491 samples that were used to generate the QDA classification rules</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Result</strong></td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>WN</td>
</tr>
<tr>
<td>SLE</td>
</tr>
</tbody>
</table>

*True classification generated by PRNT for 316 samples and by MAC-ELISA for 175 negative samples.*

*Correct classification of negative samples by MAC-ELISA was 94.3% when equivocal results were not included.*
bilities on either side of the line are equal. As the difference between the individual group classification probabilities increases, visualized in Fig. 3 by moving further away from the line, the certainty of correct classification increases. The surface shown in Fig. 4 was made by computing the correct classification probability over a grid of $\log_{10}(W)$ and $\log_{10}(S)$ pairs in the range of the observed data. The contour plot on the $\log_{10}(W)$-$\log_{10}(S)$ plane is an interpretation of the surface, where contour lines closer together indicate steeper grade on the surface. Samples associated with coordinates $\log_{10}(W)$ and $\log_{10}(S)$ which yield classification probabilities on the plateaus of the surface plot therefore have high probabilities (>95%) of correct classification. The steepness of the canyon walls in the surface reflect the relatively high discriminatory ability of the QDA classification scheme for these data, which in turn reflects the large separation of the $\log_{10}(W)$ and $\log_{10}(S)$ values for the different groups (see Fig. 2).

Independent comparison of the duplex MIA with the MAC-ELISAs. Serum specimens not included in the QDA were analyzed by the duplex MIA and by MAC-ELISA. The results from the duplex MIA for 351 samples were transformed and classified using the previously generated QDA to provide an independent classification using the duplex MIA. The details of the results are shown in Table 3. Not including samples that were found to be equivocal by MAC-ELISA (31/351) the agreement of the duplex MIA compared to the MAC-ELISA was 90% (288/320). The greatest discrepancy between the two methods occurred when one method classified a sample as anti-SLE IgM-positive and the other method classified it as one of the other two groups.

Nonspecific reactors. A nonspecific reactor is defined as a sample that reacts with the negative antigen such that the result using the viral antigen cannot be interpreted. In the duplex MIA this situation could produce a false-positive result. Numerically, we defined a nonspecific reaction as follows: for the WN viral antigen, a specimen that had a $\log_{10}(W)$ value $>0.857$ and a raw MFI value of $>8$ times the mean value of the

![FIG. 3. Comparison of true classifications and MAC-ELISA results for the samples used in the QDA. The equivocal zone is defined as $2 \leq P/N < 3$.](http://cvl.asm.org/)

<table>
<thead>
<tr>
<th>MAC-ELISA classification</th>
<th>QDA classification</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>SLE</td>
</tr>
<tr>
<td>Negative</td>
<td>184</td>
<td>1</td>
</tr>
<tr>
<td>SLE</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>WN</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Equivocal</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>199</td>
<td>45</td>
</tr>
</tbody>
</table>

TABLE 2. QDA classifications versus MAC-ELISA classifications for the samples used in the generation of the classification rules.
negative control sera reacted on the WN viral antigen was considered to have a nonspecific reaction against the negative control recombinant antigen. For the SLE viral antigen a specimen that had a log10 (S) value of <0.549 and a raw MFI value of ≥5 times the mean value of the negative control sera reacted on the SLE viral antigen, was considered to have a nonspecific reaction against the negative control suckling mouse brain antigen. These values corresponded to the lowest values for samples used to generate the QDA classification rules that were classified as anti-WN or anti-SLE IgM-positive (confirmed by PRNT), respectively.

The definition was retrospectively applied to all the log10 (W) and (S) values of the specimens included in the QDA. Of the samples with both negative true classifications and negative classifications with the duplex MIA, 6% (12/200) of WN viral antigen reactions and 11% (23/200) of SLE viral antigen reactions were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific reactions. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific. A total of 7.1% (35/491) of the samples with both negative true classifications and negative classifications were classified as nonspecific.

Twenty-three serum specimens produced nonspecific background reactions with the negative control antigen in the WN MAC-ELISA, the SLE MAC-ELISA, or both, but were PRNT negative (true negative). These samples were tested to determine if similar nonspecific reactions were observed in the duplex MIA. The overall rate of nonspecific reactions among these samples was 26% (6/23).

Specificity. To test how specific the duplex MIA is in regard to antibodies produced by infections other than WN and SLE viruses, panels were assembled from a variety of sources (Fig. 1). The results are shown in Table 4. The panels that gave either positive, or nonspecific (according to the definition above) results were the dengue virus (low IgM) (1/14 nonspecific); dengue virus (high IgM) (1/18 WN; 3/18 SLE; 5/18 nonspecific); syphilis (1/21 WN); rheumatoid factor (1/13 nonspecific). Of 154 samples negative by MAC-ELISA to all arboviruses tested for, 6 gave nonspecific results. All other groups were 100% specific.

Reproducibility of results between plates and within plates. Thirty-seven serum specimens were subjected to the duplex MIA on different plates and read at different times to determine if the results were reproducible. The QDA classifications agreed for all 37 replicates. The ICC was 0.986 (95% CI, 0.974 to 0.993) for the log10 (W) values and 0.970 (95% CI, 0.942 to 0.984) for the log10 (S) values. A similar study using 40 different serum samples was performed to evaluate within-plate reproducibility. All QDA classifications agreed between the replicates. The ICC for the log10 (W) values was 0.994 (95% CI, 0.988 to 0.997), and for the log10 (S) values was 0.956 (95% CI, 0.918 to 0.976).

Detection of anti-WN and anti-SLE IgM in CSF. The CSF sample results were classified using the QDA classification rules derived using the serum sample set. These were compared to the interpretations in the DVBID database, which were based on MAC-ELISA or PRNT results, or both. The duplex MIA yielded 20 negatives, 3 anti-SLE IgM-positives, and 58 anti-WN IgM-positives. Of the 81 samples, 80 samples had duplex MIA results that were consistent with the previous laboratory results. One sample was incorrectly classified as WN when it should have been negative. Original laboratory results

Table 3. QDA classifications versus MAC-ELISA classifications for samples not used to generate the classification rules

<table>
<thead>
<tr>
<th>MAC-ELISA classification</th>
<th>QDA classification</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>SLE</td>
</tr>
<tr>
<td>Negative</td>
<td>103</td>
<td>1</td>
</tr>
<tr>
<td>SLE</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>WN</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Equivocal</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 4. Human specificity control serum panels tested by WN/SLE duplex MIA

<table>
<thead>
<tr>
<th>Antibody identity</th>
<th>No. of sera</th>
<th>MIA result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WN</td>
<td>SLE</td>
</tr>
<tr>
<td>LaCrosse encephalitis</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Old flavivirus</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Dengue virus (low IgM)</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Dengue virus (high IgM)</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>YF vaccine</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Other arboviruses</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Syphilis</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Antinuclear antibody</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Lyme disease (IgM)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Lyme disease (IgG)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>154</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>312</td>
<td>2</td>
</tr>
</tbody>
</table>

* NS, nonspecific.

a Determined by a positive IgG ELISA/negative MAC-ELISA result.

b MAC-ELISA P/N > 2 < 9.

c MAC-ELISA P/N ≥ 9.

d Included Eastern equine encephalitis, Chikungunya virus, Colorado Tick Fever virus and Jamestown Canyon virus antibodies.
identified it as Powassan IgM-positive, so presumably, flaviviral cross-reactivity was responsible for the incorrect classification.

**DISCUSSION**

The duplex MIA described here provides a rapid, accurate method to differentiate between anti-WN and anti-SLE IgM antibodies, and to distinguish between these and IgM antibody-negative serum samples. Results management for the duplex MIA incorporates a data manipulation algorithm that has not previously been applied to arboviral serologic techniques, but is very appropriate for use in this application. The shortcomings of the MAC-ELISAs have been addressed where possible in the duplex MIA.

The duplex design of the MIA was preferable to individual antiviral tests from an efficiency standpoint. For decreased test preparation time, and reagent and sample use, the addition of the normal antigen reactions into the wells with the viral antigens would have been beneficial, but this design was decided against because different bead sets would be required for the normal antigen reactions, thus creating a potential troubleshooting dilemma.

The creation of a novel results algorithm required first that a gold standard with which to compare the results be chosen. The decision to use PRNT in this capacity was made due to the fact that in the current arboviral diagnostic algorithm, PRNT results take precedence over those of MAC-ELISA for samples ≥9 days post onset. In addition, PRNT gives a more definitive result. In practice, PRNTs are not usually performed on specimens that test negative for IgM by MAC-ELISA with both viral antigens; therefore, 175 MAC-ELISA-negative samples that were not examined by PRNT were included in the classifying data set. The MAC-ELISA-negative results were used as the true classifications for this sample set.

The decision to use QDA to classify the anti-WN IgM-positive, anti-SLE IgM-positive, and negative groups of specimens was made based on the applicability of the QDA method to the data set. Other potential methods were investigated: mean ± 3X standard deviation; 3-way receiver operator characteristic analysis (3, 11); and linear discriminant analysis (13). The QDA was the most flexible in that it optimized group sensitivities, considered the results simultaneously, and admitted different correlations for the groups, a combination of which could not be achieved via the other methods.

In order to transform raw MIA data to produce definitive results for diagnostic use, we created an add-in program for Excel (Microsoft Office 2003, Microsoft Corp., Seattle, Wash.). Therefore, although several steps are involved in the data transformation algorithm, the routine use of it is easily achievable by using the add-in program.

The transformation of the data from raw MFI values to standardized adjusted values includes three operations that are not addressed in the method used for MAC-ELISA data analysis. First, all MFI values for viral antigen reactions are adjusted by their respective negative antigen reactions. This is important when a comparison between antigens is being made, because individual specimens may have different reactions on different negative antigens. Second, differences between anti-WN and anti-SLE IgM-positive controls are adjusted for. Third, all data are standardized to other plates (a historical standard) so that results are comparable across plates. The decision was made to force the regression line used in this standardization through the origin based on the fact that negative MFIs do not occur in practice. The assumption is that the controls on a plate reflect what is happening to all the samples on a plate, and, therefore, the effect of an unusual mean control value would be captured by the slope of the standardization regression line, thus permitting sample values on the plate to be corrected.

Table 1 presents the results as percent correct classification. This term was used as opposed to the term sensitivity, because the latter refers to a dichotomy consisting generically of a “positive” and a “negative” group. Here there are three groups; therefore percent correct classification avoids ambiguity. The percent correct classifications of the duplex MIA were superior overall to those of the MAC-ELISAs, with the most improvement seen for the anti-SLE IgM-positive group. The percent correct classifications were computed by cross-validation, which involves leaving one data point at a time out of the data set, fitting the QDA to the remainder of the data points, and then classifying the data point in question. This is done for each data point in the set. This is a useful way to assess anticipated predictive performance of the classification algorithm, because it classifies specimens that were not used in the data set used to create the classification rule. This method is superior to the plug-in method (13), where the sample that is being classified has been used in the construction of the classification rule. Coincidentally, with these data, the correct classification rates for all groups were identical for both methods.

The percent correct classifications reported in this analysis relate primarily to samples taken less than 50 days after onset of symptoms, because this was the specimen set available. The duplex MIA and the MAC-ELISA both have high correct classification percents for anti-WN IgM-positive samples and negative samples. From this information it can be extrapolated that anti-WN IgM is detected with similar accuracy by the duplex MIA in samples from 0 to 50 days after onset of symptoms. Only 6 samples were obtained >50 days past onset of symptoms, and so a precise statement about the anticipated performance of the duplex MIA cannot be made for such samples.

The specificity data identified 2 groups of samples having results of particular interest when tested in the WN/SLE duplex MIA. The dengue virus (high IgM) category gave 4/18 results that were either positive for WN- or SLE IgM or both, and 5 that gave nonspecific results. The same 18 samples were analyzed by WN and SLE MAC-ELISA (data not shown), with 11/18 being positive for WN- or SLE IgM or both. A further 5 samples were equivocal or nonspecific. Flaviviruses exhibit significant cross-reactivity with one another (6) and therefore these results are unsurprising; however, results suggest that the MIA may be less prone to generation of positive results for samples containing dengue virus antibodies than the MAC-ELISAs.

The addition of dengue virus to the QDA analysis may provide for easier discrimination between primary flaviviral infections; the anamnestic responses seen with some secondary flaviviral infections would likely confound the picture, however. Wong et al. (16) reported positive reactions with a syphilis panel when using a polyvalent (IgG, IgA, and IgM) detec-
tion antibody in their recombinant WN virus envelope protein-MIA. None of the cross-reactors tested positive by WN PRNT. Here, specificity data revealed 1 sample from a patient diagnosed with late latent syphilis gave a positive reaction to WN in the MIA. This result was confirmed using the WN MAC-ELISA and also by PRNT (WN 1:1280; SLE 1:40). This indicated that the patient was carrying antibodies from a coinfection. Further clinical information or follow-up specimens were unavailable, but the patient was known to be resident in an area experiencing WN virus activity.

The criteria used to identify samples that reacted nonspecifically with negative antigen were necessarily different from those used in the MAC-ELISA, which are directed at positive results. In the MIA, false-negatives could potentially exist because nonspecific reactions with the negative control antigen could generate artificially low numbers in the first step of the data transformation scheme. The criteria to define a nonspecific reaction presented here are quite stringent, and are based purely on the data set under evaluation. As more experience is gained with the duplex MIA, these criteria may be modified. The samples that were identified retrospectively as having nonspecific reactions to either of the negative control antigens remained an integral part of the QDA. Removal of these samples would have marginally improved the reported correct classification rates, but the practical effects would be negligible. Conversely, the inclusion of these samples improved the robustness of the analysis, particularly of the SLE component.

The duplex MIA will be implemented as an experimental diagnostic technique in the ADB Diagnostics and Reference Laboratory and is currently in the process of internal and external validation. The initial analysis of the duplex MIA by using samples not used in the derivation of the duplex MIA classification rules is shown in Table 3. To get a more accurate classification, samples with a maximum absolute difference in classification probabilities of <80% are currently being analyzed by WN and SLE virus PRNT, and this cutoff may later be revised if necessary. The duplex MIA results, however, will not be reported as equivocal because such a result does not help in diagnosis.

Results from the WN/SLE duplex MIA will be interpreted as follows. Classification of a sample as WN or SLE virus IgM-positive will indicate a presumptive infection with that virus; negative will indicate an absence of antiviral IgM to either virus; nonspecific will indicate either that the results could not be differentiated or that background reactions on the negative antigen inhibited interpretation, and that a different test should be performed.

The classification of the CSF samples using the QDA classification rules generated by the serum specimens appeared to be successful despite the fact that ideally we would have developed CSF classification criteria using CSF data in a QDA. The volume of CSF necessary for testing in the duplex MIA is only 1/5 of the volume needed in the MAC-ELISAs, a significant advantage because of the limited volume of CSF that is often received.

The cost of testing samples by the duplex MIA was compared to the cost of the equivalent SLE and WN MAC-ELISAs, averaged over 40 samples. Including hands-on technician time, the cost was approximately US$2.90 for the duplex MIA when beads were commercially coupled, compared to US$3.60 for the WN and SLE MAC-ELISAs. The major equipment required for the test is more expensive for the duplex MIA than for the MAC-ELISAs. The most important factor for most laboratories to consider, however, is the decreased turnaround time for the duplex MIA (approximately 4.5 h) as opposed to the MAC-ELISAs (2 days).

We intend that the duplex MIA will eventually replace the MAC-ELISAs in the ADB Diagnostic and Reference Laboratory. There is also an expectation that some other laboratories will implement the method as a routine diagnostic method for WN and SLE viral IgM serologies. As a reference laboratory, results generated as part of the confirmatory process are often compared to the results generated by the other labs. The ability to compare results directly when similar controls are used is now possible by using the Excel add-in program developed as a component of this study. Such comparison is not possible with the MAC-ELISAs currently in use. Additionally, the use of different antigen lots or different test performances among labs should be accounted for by the data transformations, though this expectation will need to be verified in practice.

To date the only other MIAs for arboviruses are those reported by Wong et al. (16, 17). The main differences between these and the duplex MIA reported herein are 1) that the duplex MIA positively identifies anti-SLE IgM in samples as opposed to distinguishing it by process of elimination and 2) that the duplex MIA here uses statistically more stringent methods for classification in the analysis. The use of a capture system whereby antigen is captured on to monoclonal antibody-coated microspheres allows for the use of different antigen preparation methods, so that very pure antigens are not required for the test to function. Thus, positive identification of IgM to other arboviruses such as dengue, LaCrosse, and eastern equine encephalitis viruses, should be possible within a relatively short time period using this method. The ability to classify additional antiviral reactions successfully in a multiplex assay using the method described herein will depend on the distributional separation of the viral groups’ responses in the QDA. In addition, in-house preliminary data suggest that tests to measure IgG can be designed based on the same assay format, with the substitution of the anti-human IgM-PE for anti-human IgG-PE.

As the duplex MIA is introduced and its use becomes routine, there will inevitably be modifications to the method described here as more experience is gained. Nevertheless, the duplex MIA represents a significant improvement over the way in which WN virus and SLE virus serology is currently performed, especially with respect to decreased turnaround time, and the generation of a single result. The use of the QDA is novel to both arbovirology and microsphere assays and is a promising method of analysis for these fields of study.

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Patent applications for the technology described herein have been submitted to the patent and trademark offices of the United States, Canada, and Australia, CDC ref. no. I-032-04; license 533,922.

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