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Performance of Immunoglobulin G (IgG) and IgM Enzyme-Linked Immunosorbent Assays Using a West Nile Virus Recombinant Antigen (preM/E) for Detection of West Nile Virus- and Other Flavivirus-Specific Antibodies

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Focus Technologies developed an indirect immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) and a mu-capture IgM ELISA for the detection of West Nile virus (WNV)-specific antibodies based on a WNV preM/E protein recombinant antigen. Normal and disease state serum panels were used to assess the performance characteristics of the two WNV ELISA kits. Totals of 807 and 1,423 sera were used to assess the IgG ELISA and IgM ELISA kits, respectively. The Focus Technologies IgG ELISA had a sensitivity of 97.6% and a specificity of 92.1% (excluding non-WNV flavivirus sera). The comparative method for WNV IgG may lack sensitivity in detecting IgG in early WNV infection, so the specificity of the Focus IgG ELISA may be higher than 92.1%. When sera from patients either infected with or vaccinated against other flaviviruses were tested on the WNV IgG assay, 35% of the sera reacted as positive for WNV IgG. Yellow fever and Japanese encephalitis vaccinees were less reactive in the IgG ELISA than St. Louis and dengue fever patients. The Focus Technologies IgM ELISA had a sensitivity and a specificity of 99.3% (excluding the non-WNV flavivirus sera). The overall cross-reactivity for the IgM ELISA to flavivirus sera was 12%, with 31% of St. Louis encephalitis patients found to be WNV IgM positive and no yellow fever vaccinees found to be WNV IgM positive. In a selected population of 706 sera, 15 false-positive WNV IgM sera were identified. The use of a background subtraction method for the IgM ELISA eliminated all 15 false-positive results, giving a specificity of 100% for the Focus IgM ELISA.

Since the initial outbreak of West Nile virus (WNV) in New York in 1999, WNV has spread rapidly across the entire continental United States in only 4 years (3, 7, 13, 16–18). WNV serology, in particular the detection of WNV immunoglobulin M (IgM) in both serum and cerebrospinal fluid, has become the primary tool for diagnosing human WNV infection. The detection of WNV IgM in serum represents a probable WNV infection, whereas the detection WNV IgM in cerebrospinal fluid is considered diagnostic of central nervous system involvement by WNV (13, 15, 24). Due to very low viremia at the time of clinical onset, nucleic acid detection methods and WNV culture are not useful diagnostic tools (10, 13). Only 20% of WNV-infected individuals are symptomatic; the majority of symptomatic patients present with a self-limited viral syndrome of fever, headache, malaise, and rash. Fewer than 1% of infected individuals progress to serious clinical disease, typically manifesting as either meningitis or encephalitis (19, 22).

WNV is a member of the *Flaviviridae* family and is in the Japanese encephalitis serocomplex that includes Japanese encephalitis (JE) virus and St. Louis encephalitis (SLE) virus. Other closely related flaviviruses include yellow fever (YF) virus and dengue virus types 1 to 4. The flavivirus antibody response is predominantly generated against the highly immunogenic envelope protein that contains both flavivirus cross-reactive epitopes and virus-specific epitopes (8, 23). Hunt et al. (8) developed a recombinant protein composed of the JE virus E-protein gene and the preM gene that resulted in the formation of noninfectious particulate JE virus antigen. The particulate nature of the recombinant protein allowed the antigen to maintain a tertiary structure similar to that of the native virus envelope protein and thus was a good candidate for both vaccine and serologic applications. Further development efforts resulted in the production of other flavivirus recombinant particulate antigens, including WNV (6). Studies at the Centers for Disease Control and Prevention (CDC) demonstrated the utility of the WNV particulate antigen for the detection of WNV antibodies and the diagnosis of WNV infection in humans. The recombinant WNV antigen was used to develop the Focus Technologies immunoassay for the detection of IgG and IgM WNV antibodies that recently received clearance from the U.S. Food and Drug Administration. We investigated the utility and performance characteristics of the Focus Technologies IgG and IgM assays in various healthy and diseased patient populations, as well as the reactivity with sera from various flavivirus vaccinees and infected individuals.

MATERIALS AND METHODS

Focus Technologies WNV IgG ELISA. The WNV IgG enzyme-linked immunosorbent assay (ELISA) uses the preM/E recombinant protein in a standard indirect ELISA. All sera were tested according to the package insert. Briefly, patient sera and controls were diluted 1:101 in the kit diluent, and 0.1 ml was added to microtiter wells containing the WNV preM/E recombinant protein. Negative and positive controls were included, as was a kit-supplied calibrator/cutoff control. After incubation for 1 h at room temperature and a washing step,

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peroxidase-labeled goat anti-human IgG conjugate was added to each well for 30 min. After the conjugation step, tetramethylbenzidine (TMB) was added, and the final reaction product was measured in a spectrophotometer at a wavelength of 450 nm. An index value was obtained for both control and patient samples by dividing the absorbance value of the patients and controls by the absorbance value of the calibrator (cutoff control). Patient index values of <1.3 were considered negative, values from 1.3 to 1.5 were considered equivocal, and values that were >1.5 were considered positive for WNV IgG.

Focus Technologies WNV IgM ELISA. The WNV IgM ELISA is a mu-capture assay format wherein patient and control samples were diluted 1:101, and 0.1-ml portions of the patient and control sera were added to microtiter wells containing goat anti-human IgM. The kit controls included a negative control, positive control, and a calibrator/cutoff control. After the IgM was captured on the microtiter well, the WNV preM/E recombinant protein was added to each well. After the wells were washed, the presence of WNV antigen bound to either the patient or control IgM was detected by using a peroxidase-labeled mouse anti-flavivirus monoclonal antibody. The chromogen TMB was added, and the final reaction product was measured in a spectrophotometer at a wavelength of 450 nm. Index values for the patient samples and controls were obtained by dividing the absorbance of the patient or control well by the absorbance of the calibrator (cutoff control). Index values of <0.9 were considered negative, values from 0.9 to 1.1 were considered equivocal, and values of >1.1 were considered positive for IgM antibody.

An additional step was evaluated to detect possible false-positive IgM results when the standard procedure described above was followed. Briefly, when a patient sample was positive for WNV IgM by using the standard assay procedure, the specimen was tested again by using the following background subtraction method. All calibrators and controls were run exactly as indicated in the package insert. The patient specimens were diluted 1:101 per the package insert, and 0.1 ml of the diluted serum was added to duplicate wells of the IgM-capture plate and incubated for 2 h at room temperature. After the wells were washed, 0.1 ml of specimen diluent was added to one well, and WNV antigen was added to the second well. After incubation for 1 h at room temperature and further washing, all wells received the peroxidase-labeled mouse monoclonal anti-flavivirus antibody; the remainder of the assay was performed as described above. The patient's final IgM index value was determined by using the net absorbance value, calculated by subtracting the absorbance value of the well receiving diluent only from the absorbance value of the well receiving the WNV antigen. The net absorbance was divided by the absorbance obtained for the kit's calibrator/cutoff control, and the index value was interpreted per the package insert (i.e., index values of <0.9 were considered negative, values from 0.9 to 1.1 were equivocal, and values of >1.1 were positive for WNV IgM antibodies).

CDC WNV IgG and IgM ELISA. The comparative methods used here were the CDC's WNV IgG and IgM ELISA procedures (9, 14). Sixteen separate state public health laboratories (SPHLs) all used the CDC procedure to generate the IgG and IgM results shown in the WNV (SPHL) panel outlined below. The comparative data generated at the New York Department of Health laboratory for the encephalitis/meningitis and SLE panels also used the CDC ELISA protocol. Briefly, the IgG method utilizes an anti-flavivirus monoclonal antibody immobilized to the microtiter plate well to capture WNV antigen. Next, the immobilized monoclonal antibody-antigen complex is incubated with diluted patient sera, and IgG antibodies to WNV in the patient sample are bound to the antigen. The presence of WNV IgG is detected by using a peroxidase-labeled anti-human IgG antibody. The IgM method is an antibody capture ELISA wherein the patient's IgM is captured in a microtiter plate. The antibody capture step is followed by the addition of WNV antigen derived from either suckling mouse brain or recombinant WNV envelope protein. A control antigen (either uninfected suckling mouse brain or recombinant protein control antigen, matched to WNV antigen used) well is included for all samples tested by both the IgG and the IgM ELISA methods. After the addition of WNV or control antigen to the sample wells, peroxidase-labeled flavivirus monoclonal antibody is added to each well, followed by the chromogen TMB (with appropriate wash steps in between). A negative serum or a panel of negative sera was run with each assay and a patient sample with an absorbance reading three times higher than the negative serum was considered positive for the presence of WNV antibody.

WNV PRNT. The plaque reduction neutralization test (PRNT) performed at Focus Technologies Reference Laboratory followed the protocol outlined by Blitvich et al. (2).

Serum samples. The following serum panels were utilized in the present study.

(i) WNV (SPHL) panel. Specimens from 583 possible WNV cases were included in this panel. The sera were initially tested at Focus Technologies Reference Laboratory using an in-house native WNV antigen-based ELISA (21). These in-house IgG and IgM assays were only similar to the Focus kit assays in that the IgG ELISA was an indirect ELISA format and the IgM assay was a mu-capture format incorporating the 6B6-C1 anti-flavivirus monoclonal antibody. The positive samples were then forwarded to the appropriate SPHL (n = 16), where the samples were tested by using the CDC WNV IgG and/or IgM ELISA protocols. A subset of the samples also had PRNT results provided (n = 126). SPHL IgM results were provided for all 583 samples and IgG results were provided for 103 samples. The 583 samples were then tested by using the Focus Technologies IgG and IgM ELISA kits.

(ii) Encephalitis/meningitis panel. Three hundred sequentially submitted serum samples forwarded to the New York State Department of Health laboratory for evaluation of an infectious source for encephalitis or meningitis were tested by using the Focus WNV ELISAs, as well as by using the CDC ELISA protocols. All New York samples were tested at the New York State Department of Health laboratory facility. An additional 50 sera submitted to the CDC, Ft. Collins, Colo., for evaluation for encephalitis/meningitis were also included in this panel. This panel was included to evaluate the Focus WNV kits for reactivity of sera from patients with clinical symptoms consistent with WNV infection; at the time the sera were submitted, the possible infectious source was unknown. The CDC WNV IgM ELISA protocol was performed on all 350 samples to determine the presence of WNV IgM antibodies, and the CDC WNV IgG ELISA protocol was performed on 327 samples.

(iii) Blood donor panel. Sera from 236 normal blood donors from Southern California were used in this panel. All samples were collected in 2001 prior to the identification of any WNV activity in Southern California; this population was thus considered naive for exposure to WNV.

(iv) JE panel. This panel consisted of 20 sera from subjects who had been vaccinated with JE virus (supplied by Acambis, plc) and 20 sera from naturally infected individuals from Southeast Asia (supplied by Jan Groen, Erasmus University, Rotterdam, The Netherlands).

(v) Dengue panel. Nineteen sera were kindly supplied by the CDC Dengue Reference Laboratory, San Juan, Puerto Rico. The samples were from secondary dengue infections from the Caribbean region.

(vi) SLE panel. A total of 32 SLE sera from naturally infected persons were included in the study. The New York SPHL provided 22 sera; all samples had measurable PRNT titers, confirming that the flavivirus antibodies present in these patients were due to exposure to SLE virus. A second group of 10 SLE sera from infected individuals was provided by Acambis.

(vii) YF panel. Forty sera from subjects who had been vaccinated with YF virus were provided by Acambis (20 sera) and Matthias Neidrig, Robert Koch Institute (20 sera).

(viii) Autoimmune serum panel. Sera containing either anti-nuclear antibody (ANA) (n = 20) or rheumatoid factor (RF) (n = 21) were obtained from Focus Technologies Reference Laboratory. The ANA sera had titers of 1:40 or higher, as determined by indirect immunofluorescence with HEp-2 cells. The RF sera had >20 IU of rheumatoid factor/ml as determined by nephelometry.

(ix) IgM serum panel. Sera with IgM antibodies to one of four different infectious agents (cytomegalovirus [CMV], Epstein-Barr virus [EBV], herpes simplex virus [HSV], and *Borrelia burgdorferi*) that are associated with clinical symptoms similar to those seen in individuals infected with WNV were obtained from Focus Technologies Reference Laboratory. A total of 79 sera were included. The presence of IgM to each infectious agent was determined as follows: CMV IgM (n = 14), determined by using the Diamedex CMV IgM ELISA; EBV IgM (n = 19), determined by the Focus Technologies EBV VCA RIFA IgM; HSV IgM (n = 20), measured by the Diamedix HSV IgM ELISA; and *B. burgdorferi* IgM (n = 20), measured by using the Focus Technologies Lyme IgM immunofluorescence assay.

RESULTS

IgG ELISA performance. Of the 583 sera in the WNV panel, only 103 sera had IgG results generated by SPHL (Table 1). Although >90% of the samples in this panel were WNV IgM positive, only 42 of the 103 sera tested (41%) were IgG positive at SPHLs. The overall concordance between the Focus IgG and SPHL results was 66 of 100 sera (66%). Although the sensitivity of the Focus assay was 100% relative to the SPHL results, the relative specificity was 41%. Equivocal results were excluded from all calculations of concordance, sensitivity, and specificity throughout the present study. Thirty-four sera were Focus IgG positive and SPHL IgG negative; however, 29 of

TABLE 1. Concordance of IgG ELISA results for the WNV (SPHL) panel and for the encephalitis/meningitis panel[a]

Focus IgG ELISA result	SPHL-CDC IgG ELISA result (no.)				NY-CDC IgG ELISA result (no.)				
	Positive	Negative	Equivocal	Total	Positive	Negative	Equivocal	NS[b]	Total
Positive	42	34	0	76	41	0	0	5	46
Negative	0	24	0	24	2	228	4	46	280
Equivocal	0	3	0	3	0	0	0	1	1
Total	42	61	0	103	43	228	4	52	327

[a] Equivocal results were excluded from all concordance, sensitivity, and specificity analyses.
[b] NS, The specimen had nonspecific ELISA reactivity, and the presence of IgG could not be determined.

these 34 sera were determined to be positive for IgM by both the Focus IgM assay and the SPHL IgM assay. Of the 34 discrepant samples, 10 were available for PRNT analysis. Of these 10 sera, 2 had PRNT results provided by SPHL, and 8 of the 10 sera were tested at Focus (6 of the 8 samples tested at Focus were WNV IgM positive). All 10 sera contained WNV antibody as determined by PRNT. Due to the high number of consensus WNV IgM-positive sera and WNV PRNT-positive sera in the 34 IgG discordant sera, it appears that the apparent low IgG specificity of the Focus IgG assay in this serum panel in fact reflects lower sensitivity of the IgG ELISA as performed in the various SPHLs. If the 29 concordant IgM positive sera were excluded from the specificity calculation, the relative specificity was 82.7% for the Focus IgG ELISA.

Table 1 presents the results for 327 serum samples submitted to two SPHLs for identification of an infectious etiologic agent for patients with clinical symptoms consistent with encephalitis or meningitis. The comparative serology results were generated only at the two public health laboratories where the samples were submitted. Forty-one sera were determined to be positive for WNV IgG by both the public health laboratories and the Focus IgG ELISA, and an additional two sera were determined to be IgG positive by the public health laboratories. Both IgG ELISA methods determined that 228 of the samples were negative for WNV IgG. Of note, 52 sera in this panel demonstrated high background activity in the CDC IgG ELISA; thus, the presence of WNV IgG could not be determined for these specimens by this method. Forty-six of the fifty-two sera (88%) were determined to be negative by the Focus IgG ELISA, and all four of the sera determined to be equivocal by the CDC IgG ELISA were negative with the Focus IgG ELISA. If the equivocal and CDC IgG high background sera are not included in the analysis, the Focus IgG ELISA had a sensitivity and specificity of 95.3 and 100%, respectively, compared to the CDC IgG ELISA. If the CDC high background sera are considered negative for WNV IgG, the Focus IgG ELISA demonstrated 95.3% sensitivity and 98.2% specificity.

The presence of WNV IgG antibody in a previously "naive" blood donor population is shown in Table 2. Two hundred and thirty-six healthy blood donor sera from Southern California were screened for the presence of WNV IgG; seven sera (3.0%) were positive and four sera (1.7%) were equivocal, giving a specificity for the Focus IgG ELISA of 97.0% for this population. The seven IgG positive sera may represent either IgG false-positive sera or prior exposure to a flavivirus, either by natural infection or vaccination. Therefore, the Focus IgG specificity in this population may actually be >97.0%. The final serum group evaluated for IgG reactivity was from individuals either naturally infected or vaccinated to non-WNV flaviviruses, including JE virus, SLE virus, dengue virus, and YF virus (Table 2). The cross-reactivity in the Focus WNV IgG ELISA for this study population ranged from a high of 95% cross-reactivity with sera from secondary dengue infection to a low of 15% for sera from YF vaccinees. The overall IgG cross-reactivity for the 131 sera in this flavivirus group was 35%. It is interesting that only 41% of the sera from naturally infected SLE patients were WNV IgG positive, whereas nearly all of the secondary dengue infection sera were WNV IgG positive. Also, only 22.5% of the JE vaccine recipients were WNV IgG positive, even though WNV is part of the JE serogroup.

To determine the overall specificity of the Focus IgG ELISA, the data from Table 1 and the blood donor panel of Table 2 were combined to give a specificity of 92.1% (477 of 518). If the 28 IgM concordant but IgG discordant sera from Table 1 (SPHL-CDC IgG ELISA) are removed from the calculation, the specificity is 97.3%.

IgM ELISA performance. The lefthand columns of Table 3 present the Focus IgM results obtained for 583 sera tested at 16 different SPHLs during the 2002 WNV season. There was an overall concordance of 97.2% (566 of 582 samples) between the SPHL IgM results and the Focus IgM results. Although this sample set is heavily biased toward IgM-positive samples, it does allow for a good indication of the sensitivity of the Focus IgM assay (99.6% in this group). Three samples were SPHL positive, but Focus equivocal or negative; however, neither PRNT nor SPHL IgG results were available for these samples. One of the three samples was tested by Focus IgG ELISA and found to be negative. The specificity of the Focus IgM ELISA with this study group was only 68%; however, only

TABLE 2. Focus WNV ELISA IgG results for non-WNV sera[a]

Category	Focus IgG ELISA results (no. [%])			
	Positive	Negative	Equivocal	Total
Blood donor panel	7 (3)	225 (95.3)	4 (1.7)	236
Flavivirus serum panel				
JE virus	9 (22.5)	28 (70)	3 (7.5)	40
Dengue virus	18 (95)	1 (5)	0	19
SLE virus	13 (41)	18 (56)	1 (3)	32
YF virus	6 (15)	29 (72.5)	5 (12.5)	40
Total	46 (35.1)	76 (58)	9 (6.9)	131

[a] See Table 1, footnote *a*.

TABLE 3. Concordance of IgM ELISA results for the WNV (SPHL) panel and for the encephalitis/meningitis panel[a]

Focus IgM ELISA	SPHL-CDC-MAC-IgM ELISA results (no.)				NY-CDC IgM ELISA results (no.)			
	Positive	Negative	Equivocal	Total	Positive	Negative	Equivocal	Total
Alone								
Positive	536	14	0	550	44	2	0	46
Negative	2	30	0	32	1	299	1	301
Equivocal	1	0	0	1	1	2	0	3
Total	539	44	0	583	46	303	1	350
With background subtraction								
Positive					44	0	0	44
Negative					1	303	1	305
Equivocal					1	0	0	1
Total					46	303	1	350

[a] See Table 1, footnote *a*.

44 of the 583 samples were SPHL IgM negative, so very few samples were available to assess specificity in this panel. For the 14 sera that were Focus IgM positive and SPHL IgM negative, all remained Focus IgM positive after the background subtraction method described below. Neither SPHL IgG nor PRNT results were available for the 14 sera; only 2 of the 14 had Focus IgG results available, and both were IgG positive.

To better assess the specificity of the Focus IgM ELISA, two separate serum groups were tested. The first was comprised of 350 sera that had been submitted to either New York State Public Health ($n = 300$) or to the CDC ($n = 50$) for suspected cases of encephalitis and/or meningitis. The samples were sequentially received and tested at sites for the presence of WNV IgM antibodies by using the CDC IgM ELISA protocol (Table 3, righthand columns). Of the 350 samples, 46 were determined to be IgM positive by the CDC ELISA, and 44 of 46 were determined to be positive by Focus IgM ELISA (sensitivity = 95.6%). As a measure of specificity in this patient group, of 303 samples determined to be negative by the CDC IgM ELISA, 299 were Focus IgM ELISA negative, and 2 were Focus IgM ELISA equivocal (specificity of 99.3% with equivocal samples excluded from the calculations). The second serum panel used to assess specificity was the blood donor serum

TABLE 4. Focus WNV ELISA IgM results for non-WNV sera[a]

Panel	*n*	No. of results (%)					
		Focus IgM ELISA			Focus IgM ELISA with BS		
		Positive	Negative	Equivocal	Positive	Negative	Equivocal
Blood donor	236	2 (0.8)	234 (99.2)	0	0	236 (100)	0
Flavivirus							
JE virus	40	2 (5)	36 (90)	2 (5)	2 (5)	38 (95)	0
Dengue virus	19	4 (21)	10 (53)	5 (26)	3 (16)	12 (63)	4 (21)
SLE virus	32	10 (31)	21 (66)	1 (3)	ND	ND	ND
YF virus	40	0	40 (100)	0	ND	ND	ND
Total	131	16 (12)	107 (82)	8 (6)			
Autoimmune							
ANA	20	1 (5)	19 (95)	0 (0)	0	20 (100)	0
RF	21	4 (19)	17 (81)	0 (0)	0	21 (100)	0
Total	41	5 (12)	36 (88)	5 (12)	0	41 (100)	0
IgM panel							
CMV	14	1 (7)	13 (93)	0	0	14 (100)	0
EBV	19	0	19 (100)	0	0	19 (100)	0
HSV	20	1 (5)	18 (90)	1 (5)	0	20 (100)	0
B. burgdorferi	20	1 (5)	19 (95)	0	0	20 (100)	0
Total	79	3 (3.8)	75 (94.9)	1 (1.3)	0	79 (100)	0

[a] See Table 1, footnote *a*.

panel from Southern California, where no WNV IgM-positive sera would be expected. Table 4 shows 234 of the 236 blood donor samples (99.2%) were found to be IgM negative by the Focus IgM ELISA. The WNV IgM reactivity detected in the flavivirus serum panel is detailed in Table 4. Unlike WNV IgG reactivity for this panel, WNV IgM was detected in none of the YF and JE vaccinees and in only 2 of 20 individuals naturally infected with JE virus. A similar number of SLE virus-infected sera were positive for both WNV IgG and IgM antibodies (41 and 31%, respectively); however, only 4 of 19 secondary dengue sera (21%) were WNV IgM positive (compared to 95% WNV IgG positive). The autoimmune serum panel evaluated for WNV IgM reactivity was comprised of 41 sera containing either ANA ($n = 20$) or RF ($n = 21$); 5 and 19%, respectively, were found to be positive in the Focus WNV IgM ELISA. The final serum panel used to evaluate the Focus WNV IgM ELISA included 79 sera containing IgM to one of four different infectious agents (CMV, EBV, HSV, and *B. burgdorferi*) and is labeled the IgM panel in Table 4. Of the 79 sera in this panel, 3 (3.8%) were determined to be positive for WNV IgM by using the Focus IgM ELISA.

Detection of false-positive IgM sera. The CDC WNV IgG and IgM ELISA procedures incorporate control wells (no WNV antigen added) to measure nonspecific reactivity and/or background signal for each patient specimen tested. The patient specimen wells containing WNV antigen must give an absorbance value at least double the absorbance value of the background wells for the ELISA results to be considered interpretable. This procedure allows the CDC procedure to detect serum samples with high-background (i.e., noisy) signals that may otherwise be interpreted incorrectly as positive for either IgG or IgM. Preliminary data (not shown) indicated that, although many of these noisy IgM samples in the CDC IgM ELISA were negative in the Focus WNV IgM ELISA procedure, a limited number of noisy IgM sera did indeed yield a positive Focus IgM result. The use of a background subtraction well to detect noisy sera was thus evaluated in the Focus IgM ELISA. The background subtraction method was only used for sera that gave a positive WNV IgM result when initially screened by using the Focus IgM ELISA.

Preliminary studies using the background subtraction method were performed on known WNV IgM-positive and -negative sera. First, 35 consensus WNV IgM-negative sera, for which both the SPHL and the Focus results were IgM negative, were run in the Focus WNV IgM assay with the background subtraction method, and they remained IgM negative. Next, 36 sera positive for WNV IgM in the Focus assay and confirmed positive for WNV by PRNT results supplied by SPHLs were tested by the background subtraction method. All 36 sera remained Focus WNV IgM positive after background subtraction. Finally, 131 sera determined to be IgM positive by the CDC IgM ELISA were evaluated with or without the background subtraction modification of the Focus assay. On initial screening with the Focus kit, 130 sera were found to be positive for IgM, and one sample was equivocal with an index of 1.1. After the background subtraction method, 130 sera remained IgM positive and 1 sample was equivocal with the Focus IgM ELISA. The sample originally equivocal with an index of 1.1 became positive with an index of 1.2. Also, one sample originally positive with an index of 1.2 became equivocal with a post-background subtraction index of 1.1.

The background subtraction method was then applied to the following serum panels: a encephalitis/meningitis panel, a blood donor panel, a flavivirus panel, and the autoimmune panel. The IgM results for each serum panel when the background subtraction method was used are shown in Tables 3 and 4. For the encephalitis/meningitis panel, the two sera determined to be positive and two sera determined to be equivocal by Focus but negative by the CDC method were interpreted as negative after background subtraction. The 45 sera found to be positive by the CDC ELISA method retained the result obtained in the original Focus assay; i.e., 44 sera were IgM positive, and 1 serum remained equivocal after the background subtraction method was applied (Table 3). After the background subtraction method was applied, the Focus IgM ELISA still exhibited 97.8% sensitivity for the entire encephalitis/meningitis panel, and the specificity increased to 100%. The two sera in the blood donor panel that were IgM positive in the Focus IgM assay became negative after background subtraction; thus, all 236 donor sera were considered WNV IgM negative (Table 4).

When the background subtraction method was applied to the flavivirus serum panel (Table 4), the IgM results for the JE sera remained unchanged. For the dengue sera, three of the four WNV IgM-positive sera remained positive, four of the five originally WNV IgM-equivocal sera remained equivocal, and the remaining two sera were negative after background subtraction. Only three WNV IgM-positive sera from the SLE group were available for background subtraction studies, and all three remained WNV IgM positive. Finally, the five WNV IgM-positive sera in the autoimmune serum panel, as well as three sera from the IgM panel, were all interpreted as negative when the background subtraction method was applied. Table 5 provides the details of the pre- and post-background subtraction index values for the sera tested in Tables 3 and 4. Two sera (PRNT identification [ID] numbers M-1 and M-2) are included in Table 5 as examples of the absorbance and index values obtained by the background subtraction method with consensus IgM ELISA- and PRNT WNV-positive samples. The pre- and post-background subtraction index values for the 44 consensus IgM-positive sera from Table 3 are not included in Table 5.

DISCUSSION

To date, the clinical utility of serologic tools for the diagnosis of WNV infection has been limited to the CDC IgG and IgM protocols with inactivated native antigen obtained from suckling mouse brain, with WNV recombinant antigen, or with cell culture-derived WNV (9, 14, 20, 21, 24). Focus Technologies developed, and in 2003 received Food and Drug Administration clearance for, WNV IgG and IgM ELISA kits based on the WNV recombinant viral particulate antigen developed by Davis et al. (6). Although the Focus Technologies assays used the same recombinant antigen as the latest version of the CDC protocol, enhancements in both the processing of the recombinant antigen and the ELISA format itself resulted in a more user-friendly ELISA with fewer steps than the CDC protocol. The present study describes the performance characteristics of

TABLE 5. Absorbance and index values with or without background subtraction for IgM ELISA

Panel	ID no.	A_{450}[a]			Index	
		With Ag	No Ag	Net	Screen	Net
PRNT	M-1	2.064	0.050	2.014	4.2	4.1
	M-2	1.698	0.076	1.622	3.5	3.3
Blood donor	512	0.656	0.526	0.130	1.9	0.4
	I	0.424	0.276	0.148	1.2	0.4
Encephalitis/meningitis	NY 174	0.588	0.481	0.077	1.1	0.2
	NY 192	1.020	0.978	0.042	2.1	0.1
	NY 210	0.689	0.682	0.007	1.0	0.0
	NY 1	0.628	0.187	0.441	1.1	0.9
	C33	1.920	1.898	0.022	4.2	0.0
JE virus	5-4	0.496	0.075	0.421	1.0	0.8
	5-5	0.499	0.064	0.435	1.0	0.8
	5-8	0.634	0.037	0.597	1.2	1.2
	5-10	1.194	0.088	1.106	2.3	2.2
Dengue virus	D-1	0.576	0.029	0.547	1.1	1.0
	D-3	0.475	0.069	0.406	0.9	0.8
	D-4	0.572	0.068	0.504	1.1	1.0
	D-5	0.558	0.070	0.488	1.1	0.9
	D-13	1.084	0.208	0.876	2.1	1.7
	D-15	1.230	0.044	1.186	2.4	2.3
	D-17	0.624	0.197	0.427	1.2	0.8
	D-19	1.512	0.033	1.479	2.9	2.9
	D-20	0.581	0.049	0.532	1.1	1.0
ANA	6071	0.762	0.737	0.25	1.4	0.0
RF	5616	1.016	0.877	0.139	2.0	0.3
	6059	0.872	0.854	0.018	1.6	0.0
	6303	1.279	1.242	0.037	2.4	0.0
	9029	0.589	0.528	0.061	1.1	0.1
CMV	7351s	0.612	0.589	0.023	1.2	0.1
HSV	410	0.591	0.568	0.023	1.3	0.1
B. burgdorferi	L7875	1.080	1.015	0.065	2.1	0.2

[a] With Ag, WNV Ag was added to the patient well; No Ag, no WNV Ag was added to the patient well.

the Focus Technologies WNV assays versus the well-characterized CDC ELISA protocol, as well as with PRNT-characterized sera.

Three serum panels were used to determine the performance characteristics of the Focus IgG and IgM ELISAs: the SPHL panel composed primarily of WNV-positive samples, the meningitis/encephalitis panel composed primarily of WNV-negative samples, and a blood donor panel from an area where WNV is not endemic. The first two panels included sera that were accurate indicators of the sensitivity of both the IgG and the IgM assays. The Focus IgG kit had a sensitivity of 97.6% with these two panels (100 and 95.3%, respectively), whereas the IgM kit had a combined sensitivity of 99.3% (99.6% in the SPHL panel and 95.6% in the meningitis/encephalitis panel). All three panels were used to determine the specificity of both the Focus IgG and IgM kits; the values ranged from 41 to 97.4% for the Focus IgG kit and from 68 to 99.2% for the Focus IgM kit. Both of the low specificity values for the IgG and IgM kits were obtained with the SPHL serum panel. This panel proved problematic for determining the specificity of the Focus kits for two reasons. First, only 44 of the 583 samples in the SPHL panel were negative for WNV IgM by the CDC protocol and, second, only 103 of the 583 samples had IgG results generated by the CDC protocol. For WNV IgG, the Focus IgG kits found 76 WNV IgG-positive samples, whereas only 42 of these 76 samples were determined to be positive by the CDC IgG protocol. However, 29 of the 34 CDC IgG-negative, Focus IgG-positive samples were positive for IgM by both the CDC IgM protocol and the Focus IgM assay, and 10 of 10 samples tested by PRNT were positive for WNV antibody. It may also be possible that the Focus IgG ELISA may be giving false-positive results for some or all of 24 sera where no PRNT results were available. The concordance of positive IgM results for these 29 sera may indicate a lack of sensitivity for determining IgG by the CDC IgG protocol rather than a lack of specificity by the Focus IgG assay. The SPHL panel had only 44 IgM negatives of the 583 tested by using the CDC protocol; however, 14 of the 44 CDC IgM protocol negative samples were positive by the Focus IgM assay. As with the IgG samples in the SPHL serum panel, the apparent lack of IgM specificity by the Focus assay may be due to lack of sensitivity of the CDC protocol performed at 16

different SPHLs. All 14 IgM discordant sera in this panel remained positive after the background subtraction method was used in the Focus IgM assay, indicating that these samples were not false positive by the Focus IgM assay. It should be noted that the SPHL serum panel represents WNV results generated from 16 different public health laboratories; discrepancies would be expected when such a large number of laboratories are involved. The impact of such a large number of laboratories submitting results and the consistency of the results could not be determined in the present study. In fact the relatively low number of discordant results obtained in this panel of nearly 600 sera demonstrates good concordance among the 16 public health laboratories. For the reasons outlined above, the encephalitis/meningitis panel and the blood donor panel were the most appropriate sera panels to determine the specificity of the Focus assays. The former panel included sera from meningitis/encephalitis symptomatic patients, most of who do not have WNV infection, and the latter panel includes sera from an WNV-naive healthy population. The specificity for the two combined panels was 98.4% (100 and 97.0%, respectively) for the Focus IgG assay, and 99.3% for the Focus IgM assay (99.3 and 99.2%, respectively). As noted previously, nearly 16% of the samples in the meningitis/encephalitis panel did not have the IgG results reported with the CDC IgG ELISA due to high background signal. These samples were primarily negative in the Focus IgG ELISA.

Heterophile antibodies in human sera react with proteins from other mammalian species; examples include human anti-mouse immunoglobulin, human anti-horse immunoglobulin, and human anti-bovine albumin (5, 11, 25). The presence of heterophile antibodies is known to interfere in numerous serologic assays, in particular IgM assays (5, 11). Although the Focus IgM assay gave a very high specificity with the panels investigated, when sera known to be problematic for IgM assays were tested, an increased number of false positives were encountered. This observation was especially true for rheumatoid factor-positive sera. Further, although the Focus IgM assay was >99% specific, one false-positive IgM result would be expected for approximately every 200 sera screened for the presence of WNV IgM. Internal studies have indicated the occasional false-positive IgM samples encountered with the Focus IgM assay are typically due to heterophile antibodies; however, these heterophile antibodies are heterogeneous and have reactivity to multiple mammalian proteins. To reduce the level of false-positive IgM results caused by heterophile antibodies to zero, a background subtraction method was used. The method did not adversely affect the sensitivity of the Focus IgM assay and produced a specificity of 100%. All false-positive results in the meningitis/encephalitis panel, the blood donor panel, the autoimmune panel, and the IgM panel were eliminated. Since the background subtraction step is only performed on samples initially IgM positive and the number of WNV IgM-positive samples routinely encountered is relatively low, only a small proportion of samples typically require testing by the background subtraction method.

The serologic detection of WNV or other flavivirus antibodies is confounded by the variable cross-reactivity detected by using the typical antibody screening methods such as ELISA and immunofluorescence assay (1, 12). The cross-reactivity is highest among viruses within a serocomplex, but cross-reactivity also occurs between flaviviruses from different serocomplex groups (4, 15, 26). Typically, the PRNT is required to determine the infecting flavivirus, since virus-specific neutralizing antibodies are detected in the PRNT. The Focus IgG and IgM assays demonstrated cross-reactivity with other flaviviruses, especially for the IgG assay, which had an overall 35% cross-reactivity. For the Focus IgM assay, 26% of patients with either secondary dengue or SLE infection were WNV IgM positive, whereas only 3% of JE and YE vaccinees were IgM positive.

Overall, the Focus Technologies IgG and IgM assays gave highly concordant results with the CDC WNV IgG and IgM protocols, and sensitivity and specificity values of 96 to >99%. The Focus IgM assay will yield specificity values of 100% when the background subtraction method is used.

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REFERENCES

1. **Besselaar, T. G., N. K. Blackburn, and N. Aldridge.** 1989. Comparison of an antibody-capture IgM enzyme-linked immunosorbent assay with IgM-indirect immunofluorescence for the diagnosis of acute Sindbis and West Nile infections. J. Virol. Methods **25:**337–346.
2. **Blitvich, B. J., N. L. Marlenee, R. A. Hall, C. H. Calisher, R. A. Bowen, J. T. Roehrig, N. Komar, S. A. Langevin, and B. J. Beaty.** 2003. Epitope-blocking enzyme-linked immunosorbent assays for the detection of serum antibodies to West Nile virus in multiple avian species. J. Clin. Microbiol. **41:**1041–1047.
3. **Briese, T., X. Y. Jia, C. Huang, L. J. Grady, and W. I. Lipkin.** 1999. Identification of a Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis. Lancet **345:**1261–1262.
4. **Cardosa, M. J., S. M. Wang, M. S. H. Sum, and P. H. Tio.** 2002. Antibodies against prM protein distinguish between previous infection with dengue and Japanese encephalitis viruses. BMC Microbiol. **2:**9–14.
5. **Carpenter, A. B.** 1997. Enzyme-linked immunoassays, p. 20–29. *In* N. Rose, E. Conway de Macario, J. Folds, H. C. Lane, and R. Nakamura (ed.), Manual of clinical laboratory immunology, 5th ed. American Society for Microbiology, Washington, D.C.
6. **Davis, B. S., G. J. Chang, B. Cropp, J. T. Roehrig, D. A. Martin, C. J. Mitchell, R. Bowen, and M. L. Bunning.** 2001. West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and express in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J. Virol. **75:**4040–4047.
7. **Guptill, S. C., K. G. Julian, G. L. Campbell, S. D. Price, and A. A. Marfin.** 2003. Early-season avian deaths from West Nile virus as warnings of human infection. Emerg. Infect. Dis. **9:**483–484.
8. **Hunt, A. R., C. B. Cropp, and G. J. Chang.** 2001. A recombinant particulate antigen of Japanese encephalitis virus in stably-transformed cells is an effective noninfectious antigen and subunit immunogen. J. Virol. Methods **97:**133–149.
9. **Johnson, A. J., D. A. Martin, N. Karabatos, and J. T. Roehrig.** 2000. Detection of anti-arboviral immunoglobulin G by using a monoclonal antibody-based capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. **38:** 1827–1831.
10. **Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, and J. T. Roehrig.** 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. **38:**4066–4071.
11. **Levinson, S. S., and J. J. Miller.** 2002. Towards a better understanding of heterophile (and the like) antibody interference with modern immunoassays. Clin. Chim. Acta **325:**1–15.
12. **Malan, A. K., P. J. Stipanovich, T. B. Martins, H. R. Hill, and C. M. Litwin.** 2003. Detection of IgG and IgM to West Nile virus: development of an immunofluorescence assay. Microbiol. Infect. Dis. **119:**508–515.
13. **Marfin, A. A., and D. J. Gubler.** 2001. West Nile encephalitis: an emerging disease in the United States. Clin. Infect. Dis. **33:**1713–1719.
14. **Martin, D. A., D. A. Muth, T. Brown, A. J. Johnson, N. Karabatsos, and J. T. Roehrig.** 2000. Standardization of immunoglobulin M capture enzyme-

linked immunosorbent assays for routine diagnosis of arboviral infections. J. Clin. Microbiol. **38:**1823–1826.
15. **Martin, D. A., B. J. Biggerstaff, B. Allen, A. J. Johnson, R. S. Lanciotti, and J. T. Roehrig.** 2002. Use of immunoglobulin M cross-reactions in differential diagnosis of human flavivirus encephalitis infections in the United States. Clin. Diagn. Lab. Immunol. **9:**544–549.
16. **Nash, D., F. Mostashari, A. Fine, J. Miller, D. O'Leary, K. Murray, A. Huang, A. Rosenberg, A. Greenberg, M Sherman, S. Wong, and M. Layton.** 2001. The outbreak of West Nile virus infection in the New York City area in 1999. N. Engl. J. Med. **344:**1807–1814.
17. **O'Leary, D. R., R. S. Nasci, G. L. Campbell, and A. A. Marfin.** 2002. West Nile virus activity–United States 2001. Morb. Mortal. Wkly. Rep. **51:**497–501.
18. **Petersen, L. R., and J. M. Hughes.** 2002. West Nile virus encephalitis. N. Engl. J. Med. **347:**1225–1226.
19. **Petersen, L. R., and A. A. Marfin.** 2002. West Nile virus: a primer for the clinician. Ann. Intern. Med. **137:**173–179.
20. **Prince, H. E., and W. R. Hogrefe.** 2003. Performance characteristics of an in-house assay system used to detect West Nile virus (WNV)-specific immunoglobulin M during the 2001 WNV season in the United States. Clin. Diagn. Lab. Immunol. **10:**177–179.
21. **Prince, H. E., and W. R. Hogrefe.** 2003. Detection of West Nile virus (WNV)-specific immunoglobulin M in the reference laboratory setting during the 2002 WNV season in the United States. Clin. Diagn. Lab. Immunol. **10:**764–768.
22. **Sejvar, J. J., A. A. Leis, D. S. Stokic, J. A. Van Gerpen, A. A. Marfin, R. Webb, M. B. Haddad, B. C. Tierney, S. A. Slavinski, J. L. Polk, V. Dostrow, M. Winkelmann, and L. R. Petersen.** 2003. Acute flaccid paralysis and West Nile virus infection. Emerg. Infect. Dis. **9:**788–793.
23. **Seligman, S.J. and D. J. Busher.** 2003. The importance of being outer: consequences of the distinction between the outer and inner surfaces of flavivirus glycoprotein E. Trends Microbiol. **11:**108–110.
24. **Tardei, G., S. Ruta, V. Chitu, C. Rossi, T. F. Tsai, and C. Cernescu.** 2000. Evaluation of immunoglobulin M (IgM) and IgG enzyme immunoassays in serologic diagnosis of West Nile virus infection. J. Clin. Microbiol. **38:**2232–2239.
25. **Willman, J. H., H. R. Hill, T. B. Matins, T. D. Jaskowski, E. R. Ashwood, and C. M. Litwin.** 2001. Multiplex analysis of heterophile antibodies in patients with indeterminate HIV immunoassay results. Am. J. Clin. Pathol. **115:**764–769.
26. **Yoshii, K., D. Hayasaka, A. Goto, M. Obara, K. Araki, K. Yoshimatsu, J. Arikawa, L. Ivanov, T. Mizutani, H. Kariwa, and I. Takashima.** 2003. Enzyme-linked immunosorbent assay using recombinant antigens expressed in mammalian cells for serodiagnosis of tick-borne encephalitis. J. Virol. Methods **118:**171–179.