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Evaluations of Commercial West Nile Virus Immunoglobulin G (IgG) and IgM Enzyme Immunoassays Show the Value of Continuous Validation

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West Nile virus was introduced into the United States in 1999 and in only four seasons has become endemic east of the Rocky Mountains. Recently, immunoglobulin M (IgM)-capture enzyme immunoassays for the detection of West Nile virus-specific IgM and indirect IgG enzyme immunoassays for the detection of IgG antibodies against West Nile virus were made available from Focus Technologies and PANBIO, Inc. We evaluated these commercial IgG and IgM test systems and determined agreement, sensitivity, and specificity for the assays, compared to immunofluorescence assay and the Centers for Disease Control and Prevention's IgM-capture enzyme-linked immunosorbent assay (ELISA). Initially, the Focus and PANBIO IgM enzyme immunoassays had at least 95% agreement, sensitivity, and specificity, and, based on the 95% confidence intervals, both IgM-capture assays performed similarly. The IgG assays also performed well, although the Focus IgG assay demonstrated greater specificity (98.8%) and the PANBIO IgG assay demonstrated greater sensitivity (99.3%). However, for 400 samples consecutively submitted for West Nile virus antibody testing during 2 days of the 2003 West Nile virus season, agreement, clinical sensitivity, and clinical specificity were 93.1, 98.0, and 92.4%, respectively, for the PANBIO IgM assay and were 97.4, 100.0, and 97.1%, respectively, for the Focus IgM assay. The specificities observed in this second evaluation equates to an overall false-positivity rate of 6.3% in the PANBIO West Nile virus IgM-capture ELISA versus 2.5% with the Focus West Nile virus IgM-capture ELISA. This experience demonstrates the importance of continuously evaluating the performance of an assay in order to detect any changes in assay performance as the test population evolves.

West Nile virus (WNV), an arbovirus first identified in Uganda in 1937 (11), has caused over a dozen epidemics of West Nile fever and meningoencephalitis during the past eight decades (1, 7, 8). WNV was introduced into the United States in 1999 in New York and has spread westward across the continental United States and into Canada. In only four seasons, WNV has permeated areas east of the Rocky Mountains, probably spread by migrating birds (5, 10) from geographic areas of infection between pools of *Culex* mosquitoes (14). Last year's outbreak of WNV infection was the largest thus far; during 2002, 4,156 human cases of infection were reported in 39 states and the District of Columbia (data found on the Centers for Disease Control and Prevention's (CDC's) website [http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount03.htm]). ^ It is believed that WNV is still establishing its geographic distribution, possibly resembling the pattern of St. Louis encephalitis (1), which suggests that large epidemics could continue during the next several seasons and that smaller outbreaks will occur intermittently.

Following the 1999 WNV outbreak, reference laboratories began developing in-house assays for WNV antibody detection (9), although these assays were not available commercially. In 2001, PANBIO, Inc. (Columbia, Md.), introduced WNV immunofluorescence assay (IFA) slides that performed well compared to the CDC's immunoglobulin M (IgM) antibody–capture enzyme-linked immunosorbent assay. While the IFA has high sensitivity and specificity (4), the test is relatively labor intensive and it became increasingly difficult to perform as the number of samples increased during the outbreak. At the peak of the 2002 season, our laboratory was performing WNV antibody testing by IFA on approximately 400 samples per day. With such high testing volumes, it is preferable to use a more automated, less labor-intensive, and more objective format, such as ELISA.

In preparation for future outbreaks, commercial assays using the ELISA format for the detection of IgG and IgM antibodies against West Nile virus have been developed and are now available. Both Focus Technologies (Cypress, Calif.), using flavivirus and WNV recombinant protein technology licensed from the CDC, and PANBIO, Inc., using inactivated purified native WNV antigen, have formulated IgM-capture immunoassays for the detection of WNV-specific IgM. IgG enzyme immunoassays are also available from both companies. The Food and Drug Administration (FDA) has granted clearance for the PANBIO WNV IgM assay and the Focus Technologies WNV IgM and IgG assays. These assays appeared to offer highly sensitive and specific testing platforms with decreased turnaround time, while providing a method to effectively test high numbers of samples.

We evaluated both of these commercial IgG and IgM ELISA systems using samples collected during the 2002 WNV season and determined agreement, clinical sensitivity, and clinical specificity for these assays, compared to IFA and the CDC

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TABLE 1. Comparison of initial percentages of agreement, sensitivities, and specificities between Focus and PANBIO MAC-ELISA[a]

Test	Comparison test	% Agreement	% Sensitivity (95% CI)	% Specificity (95% CI)
Focus MAC-ELISA	CDC MAC-ELISA	95.3	94.6 (82–99)	100 (54–100)
	IgM IFA	97.3	96.7 (94–98)	98.0 (95–99)
PANBIO MAC-ELISA	CDC MAC-ELISA	95.2	94.4 (81–99)	100 (54–100)
	IgM IFA	96.3	96.7 (94–98)	95.9 (93–98)

[a] MAC-ELISA, IgM antibody-capture ELISA.

IgM-capture ELISA. Following the evaluations and the implementation of the PANBIO assays, a revalidation of both assays was undertaken using samples from the 2003 WNV season to assure that the performance of the assays continued to be acceptable. Observations from this additional testing are also presented herein.

MATERIALS AND METHODS

Clinical samples. Two panels of sera were included in the original evaluation of the WNV IgM-capture enzyme immunoassays, and one panel of sera was used in the evaluation of the WNV IgG assays. IgM panel 1 consisted of 43 serum samples that were tested by IgM IFA and the CDC IgM-capture ELISA. Twenty-two of these samples were confirmed positive by plaque reduction neutralization test (PRNT). IgM panel 2 consisted of 332 serum samples tested by IFA. The IgG panel consisted of 325 serum samples previously tested only by IFA. All specimens were collected during the 2002 WNV season under approval of the University of Utah Institutional Review Board (IRB 10972). An additional 100 negative control sera collected from random healthy donors in the Salt Lake City, Utah, area were also tested by both IgM and IgG enzyme immunoassays. These samples were collected from donors in 2002. Specimens were stored at −20°C until ELISA testing commenced and were then stored at 2 to 8°C while the evaluations were performed.

IgG and IgM IFA. The clinical serum specimens used in these evaluations were first submitted to our laboratory for WNV IgG and/or IgM antibody testing by IFA. The IFA protocol was formulated by using WNV IFA slides (PANBIO) and validated against the Focus Technologies and the CDC WNV IgG ELISA and IgM-capture ELISA (4). As described by Malan et al. (4), agreement, clinical sensitivity, and clinical specificity of the IgG IFA were 92, 100, and 90%, respectively, and were 98, 96, and 100% for the IgM IFA, respectively, in comparisons against IgG and IgM-capture ELISA test systems. Briefly, serum samples were diluted 1:16 and added to IFA slides coated with WNV-infected Vero cells. Following a 37°C incubation of 30 (IgG testing) or 90 min (IgM testing), the slides were washed and either IgG or IgM fluorescein-labeled anti-human–anti-mouse conjugate (Focus Technologies) was added to each well. The slides were incubated for 30 min at 37°C and were then washed, and a coverslip was applied. Slides were examined by fluorescence microscopy at ×400. Samples with fluorescence of 2+ or greater (IgG testing) or 1+ or greater (IgM testing) were then serially diluted to the end point. Fluorescence pattern and intensity were graded based on comparison with positive and negative control sera.

IgM-capture immunoassays. Three hundred thirty-two specimens from both IgM panels and 100 random negative control sera were tested for IgM antibodies against WNV by the Focus Technologies and PANBIO WNV IgM-capture ELISAs. Testing was performed according to the manufacturers' instructions. In the Focus assay, serum samples, along with the controls and cutoff calibrator, were diluted 1:100 in sample diluent and then added to microwells coated with anti-human IgM antibodies. Following a 1-h incubation, the wells were washed and reconstituted recombinant WNV antigen was added. An incubation of 2 h occurred, followed by a second wash and the addition of horseradish peroxidase (HRP)-conjugated mouse anti-flavivirus conjugate. After 30 min, a third wash step was employed, and a substrate consisting of tetramethylbenzidine (TMB) and hydrogen peroxide was added to each well. After 10 min, 1 M sulfuric acid was added to each well to stop the reaction, and the absorbance of each well was determined spectrophotometrically at 450 nm.

In the PANBIO assay, serum samples, positive and negative controls, and the cutoff calibrator were diluted 1:100 in sample diluent and added to microwells coated with anti-human IgM antibodies. After a 1-h incubation at 37°C, wells were washed and an HRP-conjugated anti-WNV monoclonal antibody–reconstituted WNV antigen solution was added to each well. Following a second 1-h incubation at 37°C and wash step, TMB substrate was added and, after 10 min, 1 M phosphoric acid was added and the absorbance of each well was determined spectrophotometrically at 450 nm. List prices for 96 tests are $800 for the Focus WNV IgM assay and $600 for the PANBIO IgM assay.

WNV IgG enzyme immunoassays. Three hundred forty-eight specimens from the IgG panel and 100 random negative-control sera were tested for IgG antibodies against WNV by the Focus Technologies WNV IgG ELISA and the PANBIO Flavivirus Indirect IgG ELISA (first generation). Testing was performed according to the manufacturers' instructions. In the Focus assay, samples were diluted 1:100 in sample diluent and added to microwells coated with recombinant WNV antigen and incubated for 60 min. After the wells were washed, HRP-conjugated Fc fragment-specific anti-human IgG was added and the wells were incubated for 30 min. A second wash was performed, and TMB substrate was incubated in the wells for 10 min. To stop the reaction, 1 M sulfuric acid was added to each well, and the absorbance of each well was determined spectrophotometrically at 450 nm.

In the PANBIO assay, samples were diluted 1:100 in sample diluent, added to microwells coated with flavivirus antigen (dengue virus, serotypes 1, 2, 3, and 4), and incubated at 37°C for 30 min. Dengue virus antigen was used for the IgG WNV ELISA by PANBIO based on its ability to detect antibodies against all viruses in the flavivirus group, including WNV, St. Louis encephalitis virus, dengue virus, etc. Following the first wash step, HRP-conjugated sheep anti-human IgG was added to each well and the wells were incubated at 37°C for 30 min. After the wells were washed a second time with wash buffer, TMB substrate was incubated in the wells for 10 min. Phosphoric acid was added to stop the enzymatic reaction, and the resulting absorbance of each well was determined spectrophotometrically. List prices for 96 tests are $800 for the Focus IgG assay and $575 for the PANBIO IgG assay.

Statistical analysis. Using two-by-two contingency tables (12), agreement, clinical sensitivity, and clinical specificity were determined for the Focus Technologies and PANBIO WNV IgM-capture ELISAs and IgG ELISA. The 95% confidence intervals (CI) for the clinical sensitivity and clinical specificity were also determined for each assay. Results from the commercial IgM-capture ELISAs were compared to results from the CDC's WNV IgM-capture ELISA for the samples contained in IgM panel 1. Results from the commercial IgM-capture ELISAs were compared to results from WNV IgM IFA for the samples contained in IgM panel 2. Results from the commercial IgG ELISAs were compared to IgG IFA results for the samples contained in the IgG panel. Equivocal results were excluded from the calculations. In the second evaluation, results from the PANBIO WNV IgM-capture ELISA were compared to results from the Focus WNV IgM-capture ELISA, and discordant samples were retested by the Utah Department of Health (UDOH) using the CDC-approved procedure (6).

RESULTS

Initial comparison of commercial IgM enzyme immunoassays. We compared commercial IgM-capture ELISA and CDC IgM-capture ELISA results for 43 samples contained in IgM panel 1. The Focus assay had agreement, clinical sensitivity, and clinical specificity of 95.3, 94.6 (95% CI, 82 to 99%), and 100.0% (95% CI, 54 to 100%), respectively (Table 1). The PANBIO assay had calculated agreement, clinical sensitivity, and clinical specificity of 95.2, 94.4 (95% CI, 81 to 99%), and 100.0% (95% CI, 54 to 100%), respectively (Table 1). Two samples were positive by CDC IgM-capture ELISA, negative

TABLE 2. Comparison of initial IgM results between Focus and PANBIO MAC-ELISA[a] and CDC MAC-ELISA and IgM IFA

Test	Result	No. of samples with indicated result by:			
		CDC MAC-ELISA		IgM IFA	
		+	−	+	−
Focus MAC-ELISA	+	35	0	175	3
	−	2	6	6	46
	+/−[b]	0	0	0	2
PANBIO MAC-ELISA	+	34	0	175	6
	−	2	6	6	140
	+/−	1	0	0	5

[a] MAC-ELISA, IgM antibody-capture ELISA.
[b] +/−, equivocal.

by Focus IgM-capture ELISA, and positive by PRNT (Table 2). Two additional samples were positive by CDC IgM-capture ELISA and negative by PANBIO IgM-capture ELISA, one of which was also IgM IFA positive.

We then compared commercial IgM-capture ELISA results to IgM IFA results for IgM panel 2, which contained 332 samples. The Focus assay had agreement, clinical sensitivity, and clinical specificity of 97.3, 96.7 (95% CI, 94 to 98%), and 98.0% (95% CI, 95 to 99%), respectively (Table 1). The PANBIO assay had agreement, clinical sensitivity, and clinical specificity of 96.3, 96.7 (95% CI, 94 to 98%), and 95.9% (95% CI, 93 to 98%), respectively (Table 1). A total of nine samples had discrepant results between Focus IgM-capture ELISA and IgM IFA. Six samples had positive results by IFA and negative results by Focus IgM-capture ELISA. Five of these six samples also had negative results by PANBIO IgM-capture ELISA. Three samples had negative results by IFA and positive results by Focus IgM-capture ELISA. One of these samples had a positive result, and the remaining two samples had negative results, by the PANBIO IgM-capture ELISA (Table 2).

A total of 12 samples had discrepant results between PANBIO IgM-capture ELISA and IgM IFA. Six of these 12 samples had positive results by IFA and negative results by PANBIO IgM-capture ELISA. Five of these six samples also had negative results by Focus IgM-capture ELISA. The sixth sample had positive Focus and CDC IgM-capture ELISA results. Six samples had negative results by IFA but positive results by PANBIO IgM-capture ELISA. Five out of six samples tested negative by Focus IgM-capture ELISA; the sixth sample had a positive result by Focus IgM-capture ELISA (Table 2).

Clinical information. Clinical data were obtained for 41 patients whose specimens were included in the evaluation of the Focus Technologies and the PANBIO IgM and IgG enzyme immunoassays. Patient ages ranged from 21 to 93 years, with an average age of 56 years. Sixty-eight percent (28 of 41 patients) experienced neurologic or musculoskeletal complications, including 9 patients (22%) with meningitis or meningoencephalitis and 3 patients with severe paralysis. Ages of patients with severe neurologic complications ranged from 26 to 76 years. Eighty-eight percent (36 of 41) reported headache and/or fever. Eight patients exhibited gastrointestinal symptoms, such as nausea and vomiting, including three patients with hematemesis. Two patients showed evidence of hypotension, and miscellaneous symptoms, such as rash, fatigue, chills, and weakness, were observed in nine patients. Correlations between the end point IFA titer, IgM-capture ELISA index values, and PRNT titers and patient symptoms were not observed; more-severe symptoms, such as meningitis and paralysis, did not necessarily equate to high levels of detectable IgM antibody. Individual symptoms, coupled with IgM serologic results, are presented in Table 3.

Comparison of commercial IgG enzyme immunoassays. We compared commercial WNV IgG ELISA and WNV IgG IFA results for 325 samples contained in the IgG panel. Initial agreement, clinical sensitivity, and clinical specificity of the Focus IgG assay were 85.3, 74.7, and 96.2%, respectively. Initial agreement, clinical sensitivity, and clinical specificity of the PANBIO IgG assay were 88.7, 88.7, and 88.8%, respectively (Table 4).

Samples with discrepant results in the Focus and PANBIO IgG ELISA evaluations were retested by both assays, and a second correlation determination comparing the individual ELISA result to a consensus of results was performed. A sample was interpreted as positive if two of the three assays (IFA and Focus and PANBIO IgG ELISAs) reported a positive result for the sample. Forty-seven samples had discrepant results in the Focus IgG assay evaluation compared to IgG IFA (Table 5). Forty-one samples had positive results by IgG IFA but negative results by Focus ELISA, and six samples had negative IgG IFA and positive Focus ELISA results. Thirty-five samples had discrepant results in the PANBIO IgG assay evaluation compared to IgG IFA. Eighteen samples had positive results by IgG IFA but negative results by PANBIO ELISA, and 17 samples had negative results by IFA but positive results by PANBIO ELISA. Discordant results were compared to a consensus of results for the sample in question, and the clinical sensitivity and clinical specificity were recalculated. In the comparison of the Focus IgG ELISA with the consensus results, clinical sensitivity increased to 86.2% (95% CI, 83 to 87%) and clinical specificity increased to 98.8% (95% CI, 96 to 100%) (Table 4). In the comparison of the PANBIO IgG ELISA with the consensus results, clinical sensitivity increased to 99.3% (95% CI, 96 to 100%) and clinical specificity increased to 92.6% (95% CI, 90 to 93%) (Table 4).

Negative-control serum analysis. Negative-control sera were collected from 100 random healthy donors and were tested by both Focus and PANBIO's IgM and IgG enzyme immunoassays. All of the samples were collected in May and June 2002 in Salt Lake City. Two samples had positive results in the Focus WNV IgM-capture ELISA, and seven additional samples had positive results in the PANBIO WNV IgM-capture ELISA. Twelve samples had positive results in the Focus WNV IgG ELISA, and 27 samples had positive results in the PANBIO WNV IgG ELISA.

Reevaluation of the PANBIO IgM-capture ELISA. Based on the comparison data presented above, FDA clearance status, pricing considerations, and other factors, the Associated Regional and University Pathologists Laboratory of Immunology chose to use PANBIO's WNV IgM-capture and IgG ELISA for the 2003 WNV season. These assays replaced the serum IFA in May 2003.

TABLE 3. Clinical symptoms and IgM serologic results of patients in the IgM panels

Sample no./sex/age (yr)	IgM IFA titer[a]	Result[b] by:				Symptom(s)
		Focus (IV)	PANBIO (IV)	CDC	PRNT (titer)	
20/F/72	16	2.37	1.32	Pos	ND[c]	Headache, fever, meningitis, diarrhea
22/F/31	16	3.09	1.14	Pos	ND	Headache, fever, meningitis, encephalitis, diarrhea, coma
24/M/29	16	3.67	1.47	Pos	ND	Headache, fever, diarrhea, vomiting
25/M/67	256	3.29	1.49	Pos	ND	Confusion, weakness, renal insufficiency, myoglobinuria
26/M/76	64	2.65	1.48	Pos	ND	Fever, anorexia
30/M/26	32	2.03	1.16	Pos	640	Headache, fever, meningitis
33/F/36	256	2.84	1.25	Pos	ND	Headache, fever
36/F/44	64	3.01	1.36	Pos	ND	Headache, fever, myalgia
91/F/55	128	3.30	1.78	Pos	ND	Headache, fever, fatigue, joint pain
92/F/80	64	3.20	1.59	Pos	ND	Fever
95/M/70	128	3.07	1.29	Pos	ND	Headache, fever, meningitis, encephalitis
108/F/21	64	1.54	0.89	Pos	ND	Nausea, vomiting
124/M/67	128	3.35	1.42	Pos	5,120	Headache, fever
125/F/65	1,024	3.38	1.67	ND	ND	Fever, altered mental status, hematemesis
126/M/69	32	2.47	1.26	Pos	40	Headache, fever, meningitis, diplopia, ophthalmoplegia
127/F/62	<16	0.53	0.22	Neg	ND	Headache, anorexia
128/F/71	128	3.71	1.51	Pos	160	Fever
129/F/71	32	0.18	0.63	Neg	ND	Fever, confusion, tremor, hypotension
130/M/55	<16	0.48	0.23	Neg	ND	Pneumonia, septic shock
131/M/28	512	3.44	1.63	ND	ND	Headache, fever
132/F/34	256	3.21	1.79	Pos	320	Headache, fever
133/F/49	64	1.80	1.20	Pos	40	Headache, fever, confusion
134/M/69	<16	0.07	0.16	Neg	ND	Headache
135/F/75	256	3.52	1.60	Pos	320	Fever, facial weakness progressing to flaccid paralysis on left side
136/M/26	128	1.72	1.43	Pos	320	Headache, fever, meningitis, neck stiffness
137/F/unk[d]	64	3.80	2.09	Pos	ND	Fever, altered mental status, hematemesis
138/F/43	64	3.04	1.39	Pos	20	Headache
139/M/57	64	2.99	1.49	Pos	40	Fever, rash, myalgias
140/F/43	<16	0.90	1.02	Pos	40	Headache, encephalitis, nausea, vomiting, diarrhea, Guillain-Barré-type symptoms
141/F/93	1,024	3.21	1.56	Pos	80	Fever, fatigue
143/M/78	256	3.55	1.36	Pos	2,560	Altered mental status, respiratory failure
144/F/52	<16	0.19	0.39	Neg	20	Headache, fever
145/M/59	1,024	2.91	1.59	Pos	80	Headache, fever, meningitis, hypotension, chills
146/M/50	128	3.11	1.57	Pos	320	Fever, anorexia, myalgia
147/M/46	512	2.65	1.60	Pos	640	Fever, altered mental status, hematemesis
148/F/50	256	2.95	1.39	Pos	80	Headache, fever, quadraparesis
149/M/68	512	3.16	1.40	Pos	160	Fever, weakness, respiratory distress
150/F/75	<16	0.54	0.30	Pos	2,560	Headache, diplopia
152/F/53	64	3.47	1.58	Pos	40	Meningitis
177/F/42	512	3.61	1.62	Pos	ND	Headache, fever, anorexia, fatigue, maculopapular skin rash
188/F/75	1,024	4.09	1.46	Pos	160	Fever, seizures, coma

[a] IFA titers are shown as reciprocal values. IFA reference interval, <16.
[b] Focus, PANBIO, and CDC, Focus, PANBIO, and CDC IgM antibody-capture ELISAs, respectively. ELISA and PRNT reference intervals, <0.90 and <10, respectively. IV, index value.
[c] ND, not done.
[d] unk, unknown.

Near the middle of July 2003, it was decided that a revalidation of the PANBIO IgM assay would be performed. The Focus Technologies IgM-capture ELISA was also reevaluated by using the same panel of clinical samples. Results from these evaluations showed that the false-positivity rate of the PANBIO IgM-capture ELISA had increased significantly from the initial validation. With the original validation panel, the false-positivity rate was 1.9%, or six false positives out of 321 samples. Between June 20 and July 23, the false-positivity rate

TABLE 4. Comparison of agreement, sensitivity, and specificity between Focus and PANBIO IgG ELISAs

Test	% Agreement		% Sensitivity (95% CI)		% Specificity (95% CI)	
	Initial	Final	Initial	Final	Initial	Final
Focus IgG ELISA	85.3	93.0	74.7	86.2 (83–87)	96.2	98.8 (96–100)
PANBIO IgG ELISA	88.7	95.8	88.7	99.3 (96–100)	88.8	92.6 (90–93)

TABLE 5. Summary of IgG results comparing commercial WNV IgG ELISA to WNV IgG IFA

Test	Result	No. of samples with indicated result by:				
		IgG IFA		Consensus		
		+	−	+	−	+/−[a]
Focus IgG ELISA	+	121	6	125	2	0
	−	41	151	20	167	5
	+/−	4	2	2	1	3
PANBIO IgG ELISA	+	141	17	145	12	1
	−	18	135	1	151	1
	+/−	7	7	1	7	6

[a] +/−, equivocal.

TABLE 6. Summary of IgM revalidation results comparing PANBIO and Focus WNV MAC-ELISAs[b] corrected by UDOH

Test	Result	No. of samples with corrected MAC-ELISA result of:		
		+	−	+/−[a]
Focus MAC-ELISA	+	49	10	1
	−	0	332	0
	+/−	0	0	8
PANBIO MAC-ELISA	+	48	25	1
	−	1	304	7
	+/−	0	13	1

[a] +/−, equivocal.
[b] MAC-ELISA, IgM antibody-capture ELISA.

increased to 6.6%, (47 false positives out of 712 total clinical samples). These 47 samples had negative results on the Focus WNV IgM-capture ELISA and the CDC's WNV IgM-capture ELISA, as performed by the UDOH. While the specificity of the PANBIO assay apparently dropped from what was seen in the initial validation, the specificity of the Focus assay remained unchanged. This rate of false positives was still within the specificity ranges indicated by PANBIO, but it was apparent that modification of our testing algorithm would be necessary in order to limit the number of false positives that were reported to our clients. Therefore, we initiated the procedure of retesting all samples that initially screened positive on the PANBIO WNV IgM assay using the Focus WNV IgM assay. If the result was positive by both methods, a positive result was reported; if the result was positive by PANBIO and negative by Focus, a negative result was reported.

Results from between July 31 and August 25 were also analyzed. Out of a total of 1,924 samples, 117 samples, or 6.1%, were classified as false positives with the PANBIO WNV IgM assay. Specificity of the PANBIO IgM assay was only 93.0%.

Following these analyses, 400 sera that were consecutively submitted for WNV testing were run in parallel on both the Focus and PANBIO WNV IgM-capture assays. With Focus as the "gold standard," agreement, clinical sensitivity, and clinical specificity of the PANBIO IgM assay were 90.2, 80.0, and 92.2%, respectively. Thirty-seven samples with discrepant results were retested by the UDOH using the CDC-approved testing protocol. Twelve samples had positive results by Focus but negative results by PANBIO. UDOH reported 1 sample as positive, 1 sample as equivocal, and 10 samples as negative for WNV IgM antibodies. This demonstrated a false positivity rate of 2.5% for the Focus IgM assay (Table 6). Twenty-five samples were reported positive by PANBIO and negative by Focus. UDOH reported all 25 samples as negative for WNV IgM antibodies, demonstrating a false positivity rate of 6.3% for the PANBIO IgM assay. Resolved agreement, clinical sensitivity, and clinical specificity were 97.4, 100.0, and 97.1%, respectively, for the Focus IgM-capture assay and were 93.1, 98.0, and 92.4%, respectively, for the PANBIO IgM-capture assay (Table 7).

Although the overall specificity of the PANBIO assay has remained roughly the same, the positive predictive value has shifted as the seroprevalance of WNV infection increases. For the initial validation panel, which contained samples that were selected based on their IFA results, the positive predictive value for the PANBIO IgM assay was 0.967. However, when the results from clinical samples tested between July 31 and August 25 were included, the PANBIO WNV IgM-capture ELISA positive predictive value was found to be 0.681. Then, during the second evaluation, the positive predictive value was 0.658, demonstrating that 34.2% of positive samples were falsely positive. For comparison, the positive predictive value of the Focus WNV IgM-capture ELISA during the second evaluation was 0.831, i.e., 16.9% of samples that were positive by the Focus IgM assay were falsely positive.

DISCUSSION

The performance of several different WNV antibody detection assays has been evaluated during the past several years (2, 4, 6, 9, 13), but this study describes both the initial validation and the follow-up revalidation of commercially available WNV enzyme immunoassays. One of the many benefits of commercially prepared ELISAs is the standardization of a testing procedure, allowing for increased reproducibility of results among individuals and reference laboratories. In the initial evaluation, both the Focus Technologies and the PANBIO IgM-capture ELISAs accurately detected cases of acute WNV infection with sensitivities and specificities of 94% and above, compared to the CDC's IgM-capture ELISA and IgM IFA (Table 1). The 95% CI for sensitivity and specificity showed that the differences between the assays were not statistically significant for either of the two IgM panels tested.

Both WNV IgG enzyme immunoassays also performed relatively well. After resolution of discordant samples, the agreement, clinical sensitivity, and clinical specificity of both assays indicated that they detected evidence of current or past expo-

TABLE 7. Comparison of agreement, sensitivity, and specificity, as corrected by UDOH, between Focus and PANBIO MAC-ELISAs[a]

Test	% Agreement	% Sensitivity (95% CI)	% Specificity (95% CI)
Focus MAC-ELISA	97.4	100.0 (92–100)	97.1 (96–97)
PANBIO MAC-ELISA	93.1	98.0 (89–100)	92.4 (91–93)

[a] MAC-ELISA, IgM antibody-capture ELISA.

sure to a member of the flavivirus family in the samples tested, although the sensitivities of the two assays do not appear to match that of the IFA. A total of 62 out of 325 samples were found to have discrepant results between the two commercial IgG enzyme immunoassays and IgG IFA (data not shown). Of the samples detected as negative by one or both of the commercial ELISAs, 39 out of 62 samples had low end point titers by IFA (1:16 to 1:64) and four samples had titers of 1:128 to 1:256. Seventeen of these samples were also positive for IgM antibodies against WNV and were drawn from patients with evidence of WNV fever and/or meningoencephalitis. It is possible that positive IgG samples are more likely to be identified at an IFA screening dilution of 1:16 than at an ELISA screening dilution of 1:100, but an attempt to raise the specificity of the commercial IgG ELISAs caused some low-titer IFA samples to be falsely negative by the commercial IgG enzyme immunoassays. Since IgG testing on single serum samples is not recommended for the detection of active WNV infection, these results serve as a reminder of the importance of testing paired sera for IgG antibodies. Data analysis also showed that, when ELISA results were compared to a consensus of IgG results, rather than the IFA alone, the PANBIO IgG assay demonstrated greater sensitivity than the Focus IgG assay and the Focus IgG assay demonstrated greater specificity than the PANBIO IgG assay. The 95% CI ranges for both assays showed no overlap between sensitivities and specificities; therefore, the differences between the assays were statistically significant.

The Focus IgM assay showed significant improvement in specificity over the Focus in-house assay evaluated by Prince and Hogrefe (9), which demonstrated overall agreement, sensitivity, and specificity of 80.6, 94.4 (95% CI, 86 to 99%), and 70.0% (95% CI, 63 to 73%), compared to testing by the CDC and state public health laboratories. As emphasized in the study by Prince and Hogrefe (9), the specificity of the in-house assay was lower than desired, resulting in a high number of false positives. In a screening assay, however, false positives are preferred over missing true positives. Ideally a screening assay also shows characteristics of a confirmatory assay, demonstrating both high sensitivity and high specificity. This reduces the number of samples that must be confirmed by alternative methods.

Our evaluation of the PANBIO IgM-capture ELISA immunoassay initially indicated only slightly lower clinical sensitivity and clinical specificity than those reported by Gould et al. (S. Gould, A. Valks, A. M. Baldwin, S. Hazell, and B. Hanson, Abstr. 19th Annu. Clin. Virol. Symp. Annu. Meet. Pan Am. Soc. Clin. Virol., abstr. T49, 2003). The statistics from the second evaluation of the PANBIO IgM assay, however, showed a specificity statistically different from that noted by Gould et al., who reported specificity of 98.1% (95% CI, 95 to 100%).

Following our second evaluation of the PANBIO WNV IgM-capture assay, significant differences in its performance in comparison to the Focus assay were noted. The specificity of the PANBIO IgM assay dropped from 96 to 93%, while that of the Focus IgM assay remained essentially the same, dropping from 98 to 97%. This apparent change in specificity was due to the unbiased nature of the second evaluation panel. Since the second evaluation panel consisted of all samples submitted for WNV testing, the panel more accurately represented the testing population and included samples that contain antibodies that interfere with the enzyme immunoassay, thereby causing false-positive reactions. These problematic samples, which occur in the general population, were screened out of the first validation panel by the selection of samples with negative IFA results. Forty-seven samples that were falsely positive on the PANBIO IgM assay were retested by IFA and had negative results, showing how this subgroup was inadvertently excluded from the first validation panel.

To correct the problem with false-positive results, both Focus Technologies and PANBIO have made available supplementary procedures similar to the antigen subtraction step performed by the CDC. In these procedures, positive samples are retested in two separate wells, with one well acting as the test well and the second well acting as a control well. Antigen is added to the test well but not to the control well. At the conclusion of the assay, the absorbance of the control well is subtracted from the absorbance of the test well, providing a "corrected" absorbance. If the control well shows high background in the absence of WNV antigen, this interference will be subtracted out of the actual result, allowing the true absorbance of each sample to be calculated. In tests performed by both Focus and PANBIO, these background subtraction procedures allowed the detection of false-positive results. These supplementary procedures allow a laboratory to screen out false-positive results, thus lowering the chances of reporting false-positive results to the client or the appropriate state health facility.

False positives were also observed in the panel of 100 negative-control sera. The Focus IgM-capture assay performed the best in this analysis and showed only a 2% false-positivity rate, while the Focus IgG assay reported 12% of the samples as false positives. The PANBIO IgM-capture assay showed a 9% false-positivity rate, while 27% of the samples were reported as false positives in the PANBIO IgG assay. These samples were collected from healthy donors who lacked prior exposure to WNV, providing an accurate representation of a preepidemic population. Note, however, that some IgG positives in this panel might represent prior exposure to the cross-reacting St. Louis encephalitis virus. Because of the spread of WNV, future panels of negative-control sera will require correlation with travel history and clinical symptoms before the assumption that the samples are lacking WNV IgG or IgM antibodies can be made.

As the fifth WNV season comes to a close, it becomes increasingly evident that WNV will continue to be a public health concern. Fortunately, serologic methods of WNV detection have improved and are readily available. These assays are comparable to IFA but require much less time and labor. While the initial validation of the Focus Technologies and PANBIO WNV IgG and IgM assays showed that both of the Focus and PANBIO IgM assays performed without any significant difference in assay performance, our second validation shows that the Focus WNV IgM-capture ELISA outperformed the PANBIO WNV IgM-capture ELISA. Our experience demonstrates the importance of the continuous validation of immunologic assays (3), even after a successful primary validation and/or FDA clearance. Revalidation allows assay performance to be evaluated as the test population shifts through various

phases and permits modifications of testing algorithms to be made without compromising patient care.

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