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Evaluation of a New Commercial Enzyme Immunoassay for the Detection of IgM Antibodies to West Nile Virus Using a Ratio Method to Eliminate Nonspecific Reactivity

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As West Nile virus (WNV) has become endemic in the United States, following the first reported cases in New York during the summer of 1999, the demand for specific serology has increased. Several IgM capture ELISA assays for the detection of WNV-specific IgM have been approved by the Food and Drug Administration for in vitro diagnostic testing, including kits from Focus Diagnostics and InBios International, Inc. The Focus Diagnostics IgM capture ELISA has a background subtraction protocol and the InBios IgM capture ELISA implements a ratio method to detect nonspecific reactivity due to rheumatoid factor, heterophile antibodies, and other interfering substances. We compared the InBios IgM capture ELISA with the Focus Diagnostics capture ELISA. Agreement, sensitivity, and specificity of the InBios IgM capture ELISA were 99, 98, and 100%, respectively. Samples that originally tested positive on the Focus Diagnostics IgM capture ELISA without the subtraction protocol and were then determined negative following the subtraction protocol agreed 100% with the InBios IgM capture ELISA. We conclude that a method to eliminate background reactivity is a necessary portion of any anti-WNV IgM assay in order to eliminate false-positive results. J. Clin. Lab. Anal. 22:362–366, 2008.

Key words: West Nile virus; ELISA; serology; immunology

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne neuropathological flavivirus that is indigenous to Europe, Asia, Africa, Australia, and the Middle East (1,2). In 1937, the virus was first isolated from a febrile patient in the West Nile province of Uganda (3) and over 60 years later it was introduced in the United States. The first diagnosed case in the United States occurred during an epidemic in New York in which 59 patients were hospitalized and 7 died (4). In subsequent seasons the virus has spread across the country as birds migrate after being infected by mosquitoes of the *Culex* genus (5–7). The largest outbreak occurred during 2003 with 9,862 reported human cases across 46 states and the District of Columbia (data found on the Centers for Disease Control and Prevention's (CDC) website [http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount03.htm]). Although most people remain asymptomatic following infection, 20% develop flu-like symptoms and less than 1% (1 in 150) of infected individuals develop acute neurological disease with less than 1% of clinical cases resulting in death (2).

The detection of immunoglobulin M (IgM) has become the recommended method for determining acute WNV infection (8). IgM antibodies against WNV are detectable, in the majority of cases, 8 days following onset of the infection with detectable levels typically present for 1–2 months, whereas in some cases, IgM antibody levels remain detectable for 500 days or longer (9). Because of the cost and ease of use in the clinical laboratory, enzyme-linked immonosorbent

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assays (ELISAs) have become the primary serological test for the detection of antibodies against WNV. Focus Diagnostics (Cypress, CA) and InBios International, Inc. (Seattle, WA) both produce commercially available assays that are approved by the Food and Drug Administration (FDA) for in vitro diagnostic use to detect anti-WNV IgM antibodies.

The Focus Diagnostics WNV IgM Capture DxSelect ELISA uses recombinant WNV premembrane and envelope proteins (preM/E) as antigens (10) and mu-capture technology for the detection of anti-WNV antigen, as does the InBios West Nile Detect IgM Capture ELISA. Although the Focus assay uses a background subtraction protocol the InBios assay utilizes a ratio method to eliminate false-positive results due to interfering substances such as rheumatoid factor (RF), heterophile antibodies, and other interfering substances (11–14).

Both commercial IgM capture ELISA assays were evaluated using samples collected during the 2007 West Nile season. Agreement, sensitivity, and specificity were determined for the InBios IgM capture ELISA by comparing results to the Focus IgM capture ELISA. As RF and heterophile antibodies are common interfering substances (11–14) samples that contained high background reactivity underwent a test to determine RF levels and if they contained bovine heterophile antibodies.

MATERIALS AND METHODS

Human Sera

This study was approved by the Institutional Review Board of the University of Utah, IRB 7275. Ninety-nine serum samples were collected during the 2007 WNV season, de-identified, divided into three categories based on the Focus IgM capture ELISA result, which is currently the in-house method for WNV antibody testing in the clinical laboratory and stored at 2–8°C until completion of the study.

The first category consisted of 42 samples that tested positive on the Focus IgM capture ELISA. As per the Focus assay procedure, a subtraction protocol was performed but yielded no interfering substance (background) reactivity. The second category consisted of 15 samples that tested positive on the Focus assay and subsequently tested negative, when the subtraction protocol was applied, owing to high background reactivity. The third group was 42 samples that tested negative on the Focus assay and required no subtraction procedure.

Commercial IgM Capture ELISA Assays

All 99 samples were tested for anti-WNV IgM antibodies using both the Focus Diagnostics West Nile Virus IgM Capture DxSelect ELISA and the InBios West Nile Detect IgM Capture ELISA.

For the Focus IgM capture ELISA testing was performed according to the manufacturer's protocol. Cutoff calibrator, positive and negative controls, and serum samples were all diluted 1:101 in sample diluent and added to microwells coated with rabbit antihuman IgM antibodies. Following a 1 hr incubation of the plate at room temperature, the wells were washed and reconstituted WNV preM/E antigen was added. The wells were then incubated for 2 hr at room temperature and a second wash step was performed. Horseradish peroxidase (HRP)-conjugated mouse antiflavivirus conjugates were added to the wells and incubated at room temperature for 30 min. After a third wash step, liquid tetramethylbenzidine (TMB) was added to each well and incubated for 10 min. To stop the reaction, 1 M sulfuric acid was added to each well. A spectrophotometer utilizing a 450 nm filter (Spectramax M5; Molecular Devices Corp., Sunnyvale, CA) was used to determine the absorbance of each well. The index value (IV) result of each specimen was then determined by dividing the optical density (OD) of the corresponding well by the mean (OD) of the cutoff calibrator. Samples with an $IV < 0.90$ were considered negative for anti-WNV IgM antibodies, samples with an $IV > 1.10$ were considered positive for anti-WNV IgM antibodies whereas samples with an IV ranging from 0.90 to 1.10 were indeterminate (equivocal). For the purpose of this study, equivocal samples were not used. Samples that tested positive were repeated using the manufacturer's protocol to subtract out nonspecific reactivity (14). Serum samples were incubated in two separate duplicate wells and washed, as previously described. Reconstituted WNV preM/E antigen was added to the first well (antigen well), whereas sample diluent was added to the second well (background well). The assay was then completed as described above. To determine the IV result for the sample the OD of the background well was subtracted from the OD of the antigen well and divided by the mean OD of the cutoff calibrator.

The InBios assay was run according to the manufacturer's instructions. Serum samples and positive and negative controls were diluted 1:100 in sample diluent and added, in duplicate, to wells of a microtiter plate coated with goat antihuman IgM antibody. Following a 1 hr incubation of the microtiter plate at 37°C the wells were washed. To the first duplicate wells WN antigen (WNRA) was added, whereas to the second duplicate wells normal cell antigen (NCA) was added. The NCA is a culture supernatant of the COS-1 cell line and is used to detect nonspecific reactivity. The wells were incubated at 37°C for 1 hr. After the plate was washed a second time, conjugate containing monoclonal antibody against

WNV E protein antigen (6B6C-1 mAb) conjugated with HRP was added to each well. After the final 1 hr incubation at 37°C a third wash was performed. A 5 min soak at room temperature with *En*Wash was employed and the wells were washed a fourth time. After a 10 min incubation of the plate at room temperature with liquid TMB, 1 N sulfuric acid was used to stop the reaction. Each microtiter plate was immediately read on a spectrophotometer with a 450 nm filter and the OD of each well was determined. For each control and sample the immune status ratio (ISR) was calculated by dividing the OD of the well to which WNRA was added by the OD of the well to which NCA was added. Samples with an ISR < 4.47 were considered negative for anti-WNV IgM antibodies and samples with an ISR > 5.66 were considered positive for anti-WNV IgM antibodies. Samples that fell within the range 4.47–5.66 were considered equivocal.

Interfering Substance Testing

To determine if RF or bovine heterophile antibodies were responsible for generating the 15 samples with high background reactivity, an RF panel ELISA [EL-RF/3 (IgM-IgG-IgA); TheraTest Labs, Lombard, IL] was performed on the samples followed by a bovine heterophile antibody latex agglutination (Mono-Latex; Wampole Laboratories, Princeton, NJ) on all negative RF samples. Testing was performed according to the manufacturers' protocol.

For the RF panel samples were each split into three aliquots. The first aliquot was diluted 1:101 in a pepsin digestion solution. The second and third aliquots were diluted 1:201 in sample diluent. Following a 2–3 hr incubation at 37°C of the samples diluted in the pepsin digestion solution, all of the sample dilutions, cutoff calibrators, and positive and negative controls were added to a microtiter plate coated with purified rabbit IgG antigen. Following a 1 hr incubation at room temperature, the wells were washed. To the appropriate wells either anti-IgA, anti-IgM, or anti-F(ab')$_2$ (anti-IgG) was added and incubated for 30 min at room temperature. Chromogen was added into all wells following a final wash procedure. Fifteen minutes after chromogen was added, stopping reagent was dispensed into each well. The absorbance of each well was determined using a spectrophotometer with a 450 nm filter and international unit (IU) results were calculated by dividing the OD of the calibrator by the calibrator concentration (provided by manufacturer) and multiplying this result by the absorbance of each sample. For the RF IgG samples were positive if their IU > 21; for the RF IgM samples were positive if their IU > 26; for the RF IgA samples were positive if their IU > 36.

Following the RF panel testing, negative RF samples were tested on the heterophile antibody latex agglutination. One drop of sample and positive and negative controls were placed on a glass slide and one drop of mono-latex reagent (bovine antigen sensitized latex particles) was dispensed on the initial drop. Each slide is rocked for 2 min. A positive result is indicated by agglutination of the solution.

Statistical Analysis

To determine the overall agreement, clinical sensitivity, clinical specificity, and the 95% confidence intervals (CI) for sensitivity and specificity, two-by-two contingency table analysis was used (15). As previously stated, samples that were equivocal on the Focus Diagnostics WNV IgM capture ELISA were excluded from the study. Results of the InBios WNV IgM capture ELISA were compared with the Focus results and results that disagreed were repeated in duplicate on both assays.

RESULTS

Agreement, sensitivity, and specificity were determined by comparing results from the InBios IgM capture ELISA to the Focus IgM capture ELISA for all 99 samples. Agreement, sensitivity and specificity were 99, 98, (95% confidence interval [CI], 95–99%) and 100% (95% CI, 96–100%), respectively (Table 1).

One out of 42 samples that tested positive on the Focus assay tested negative on InBios whereas all 15 negative samples that had high background reactivity on the Focus assay tested negative on the InBios assay (Table 2) and all 42 samples that were negative on the Focus assay tested negative on the InBios assay.

To determine if the samples that originally tested positive on the Focus without the antigen subtraction protocol and subsequently tested negative following the antigen subtraction protocol had high background as a result of RF or heterophile antibody interference; a RF

TABLE 1. Comparison of the Focus IgM Capture ELISA With the InBios IgM Capture ELISA

	Focus IgM capture ELISA	
InBios IgM capture ELISA result	Positive	Negative
Positive	41	0
Negative	1	57
	InBios IgM capture ELISA vs. Focus IgM capture ELISA (95% CI)[a]	
% Agreement	99.0	
% Sensitivity	97.6 (93–98%)	
% Specificity	100.0 (96–100%)	

[a]CI, confidence interval.

panel and a heterophile latex agglutination assay were performed. Of the 15 high background samples, 5 tested positive for RF IgM (Table 2), 2 of those 5 tested positive for RF IgA and 1 of the 5 tested positive for RF IgG. The ten samples that were negative for RF were then tested for bovine heterophile antibodies using a latex agglutination assay. Of the ten samples, two tested positive for bovine heterophile antibodies.

TABLE 2. Results of Samples That Tested Negative on the Focus IgM Capture ELISA Following the Antigen Subtraction Protocol

Sample number	Focus IgM ELISA+Ag subtraction[a]	InBios IgM ELISA+Ag Ratio[b]	Rheumatoid factor IgM[c]	Bovine heterophile antibody
3	0.25	0.969	7	Negative
8	0.33	1.455	7	Negative
14	0.00	0.938	**164**	ND[d]
17	0.00	1.061	11	Negative
18	0.00	0.164	**106**	ND
24	0.21	1.547	**203**	ND
27	0.00	1.500	**204**	ND
32	0.03	0.996	22	Negative
33	0.00	0.886	1	Negative
35	0.00	1.067	19	Negative
36	0.00	1.161	**97**	ND
37	0.02	2.244	2	Negative
38	0.00	2.006	5	**Positive**
39	0.00	3.070	9	**Positive**
40	0.00	1.262	2	Negative

[a]Focus IgM capture ELISA positive above 1.10.
[b]InBios IgM capture ELISA positive above 5.66.
[c]Rheumatoid factor IgM positive above 27.
[d]ND, not done.

DISCUSSION

It has been previously reported that substances such as RF and heterophile antibodies can cause interference in immunoassays (13,16,17) and false-positive results in WNV assays (10). Several lines of evidence support the need for all anti-WNV IgM assays to eliminate this background reactivity. To eliminate false-positive results owing to interfering substances, the CDC screening ELISA (CDC/SPHL WNV-specific IgM ELISA) utilizes a procedure that identifies if background reactivity is present by performing an antigen subtraction procedure (10). During the 2001 WNV season Focus Technologies conducted a study that compared results of the Focus WNV IgM capture ELISA to the CDC screening ELISA and showed that, without a background subtraction protocol, a 56% false-positivity rate was observed, leading Focus to implement the antigen subtraction protocol following any sample that screens positive (without antigen subtraction) to their commercial assay procedure (14). A Nebraska Public Health Laboratory (NPHL) study showed that 6.5% of low positive (IV range 1.1–3.5) samples assayed using the Focus IgM capture ELISA contained interfering substances at levels high enough to qualitatively change the results from positive to equivocal (18).

Despite the different calculation of units between the Focus WNV IgM capture ELISA and the InBios WNV IgM capture ELISA (see Materials and Methods) a strong correlation exists between the results (Fig. 1). This strong correlation is likely owing to the use of the same antigen (recombinant preM/E) in both assays and

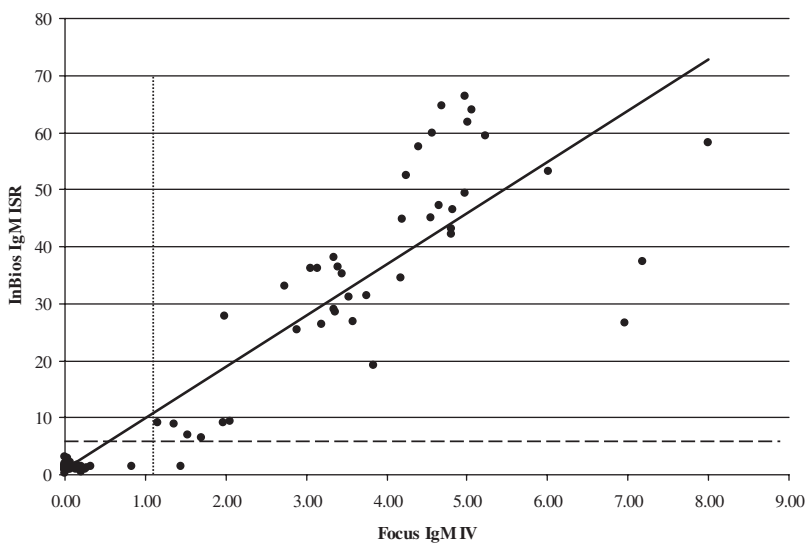

Fig. 1. Linear regression analysis of the Focus IgM capture ELISA index value (IV) results compared with the InBios IgM capture ELISA immune status ratio (ISR) results. The correlation coefficient of the linear regression line (solid) is $R^2 = 0.8566$ ($P < 0.0001$). The dotted line represents Focus cutoff value of 1.10 and the dashed line represents the InBios cutoff value of 5.66.

the use of an antigen subtraction protocol to eliminate background reactivity in both procedures.

Based on the high sensitivity and specificity of the Focus WNV IgM capture ELISA (10) and the strong correlation with the InBios WNV IgM capture ELISA, the InBios assay is a highly sensitive and specific alternative to the Focus assay. An important measure of performance of any WNV assay is the ability of the assay to eliminate false-positive results that contain high background reactivity. Because of the InBios assay's 100% agreement with the Focus assay on samples with high background reactivity the InBios assay is highly effective at eliminating false-positive reactions that might occur as a result of background reactivity. Additionally, it was shown that 7 of the 15 samples with high background reactivity contained either RF or bovine heterophile antibodies (Table 2), neither of which produced any interference in the InBios assay. Ultimately, methods to eliminate background reactivity should be incorporated into any anti-WNV IgM assay because they are inexpensive and highly effective at increasing specificity of an assay by eliminating false-positive results.

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REFERENCES

1. Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ. West Nile virus. Lancet Infect Dis 2002;2:519–529.
2. Guharoy R, Gilroy SA, Noviasky JA, Ference J. West Nile virus infection. Am J Health Syst Pharm 2004;61:1235–1241.
3. Smithburn KC, Hughes TP, Burke AW, Paul JH. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med Hyg 1940;20:471–492.
4. Nash D, Mostashari F, Fine A et al. The outbreak of West Nile virus infection in the New York City area in 1999. N Engl J Med 2001;344:1807–1814.
5. Malkinson M, Banet C, Weisman Y et al. Introduction of West Nile virus in the Middle East by migrating white storks. Emerg Infect Dis 2002;8:392–397.
6. Rappole JH, Derrickson SR, Hubalek Z. Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerg Infect Dis 2000;6:319–328.
7. Turell MJ, Sardelis MR, O'Guinn ML, Dohm DJ. Potential vectors of West Nile virus in North America. Curr Top Microbiol Immunol 2002;267:241–252.
8. CDC. 2003. Epidemic/epizootic West Nile virus in the United States: Guidelines for surveillance. Prevention and Control. p 25.
9. Roehrig JT, Nash D, Maldin B et al. Persistence of virus-reactive serum immunoglobulin m antibody in confirmed West Nile virus encephalitis cases. Emerg Infect Dis 2003;9:376–379.
10. Hogrefe WR, Moore R, Lape-Nixon M, Wagner M, Prince HE. 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. J Clin Microbiol 2004;42:4641–4648.
11. Huebner J. 2004. Antibody–antigen interactions and measurements of immunologic reactions. In: GB Pier, L Wetzler, editors. Immunology, infection and immunity. Washington, DC: ASM Press. p 207–232.
12. Kim M, Wadke M. Comparative evaluation of two test methods (enzyme immunoassay and latex fixation) for the detection of heterophil antibodies in infectious mononucleosis. J Clin Microbiol 1990;28:2511–2513.
13. Levinson SS, Miller JJ. Towards a better understanding of heterophile (and the like) antibody interference with modern immunoassays. Clin Chim Acta 2002;325:1–15.
14. Prince HE, Hogrefe WR. 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 2003;10:177–179.
15. Fleiss JL. 1981. Statistical Methods for Rates and Proportions. New York: Wiley.
16. Kricka LJ. Human anti-animal antibody interferences in immunological assays. Clin Chem 1999;45:942–956.
17. Salonen EM, Vaheri A, Suni J, Wager O. Rheumatoid factor in acute viral infections: Interference with determination of IgM, IgG, and IgA antibodies in an enzyme immunoassay. J Infect Dis 1980;142:250–255.
18. Sambol AR, Hinrichs SH, Hogrefe WR, Schweitzer BK. Performance of a commercial flavivirus (West Nile) IgM capture analyte specific reagents assay using a screening test for interfering factors (IF) during a West Nile virus epidemic season in Nebraska. Clin Vaccine Immunol 2006;14:87–89.