CLINICAL AND VACCINE IMMUNOLOGY, June 2007, p. 665–668 Vol. 14, No. 6
1556-6811/07/$08.00+0 doi:10.1128/CVI.00480-06

Evaluation of an Enzyme Immunoassay for Detection of Immunoglobulin M Antibodies to West Nile Virus and the Importance of Background Subtraction in Detecting Nonspecific Reactivity▽

Mindy L. Rawlins,[1]* Erica M. Swenson,[1] Harry R. Hill,[1,2] and Christine M. Litwin[1,2]

Associated Regional and University Pathologists Institute for Clinical and Experimental Pathology[1] and Department of Pathology, Pediatrics and Medicine, University of Utah School of Medicine,[2] Salt Lake City, Utah

Received 19 December 2006/Returned for modification 31 January 2007/Accepted 27 March 2007

Since the introduction of West Nile virus (WNV) in the United States in 1999, several assays have become commercially available to detect antibodies against WNV. Capture enzyme-linked immunosorbent assays (ELISAs) for the detection of WNV-specific immunoglobulin M (IgM) have been approved by the Food and Drug Administration for clinical testing and are available from Focus Diagnostics and PanBio, Inc. The Focus Diagnostics IgM capture ELISA utilizes a background subtraction protocol in order to detect nonspecific reactivity due to rheumatoid factor, heterophile antibodies, or other interfering substances. A background subtraction procedure is not currently recommended for the PanBio IgM capture ELISA. In previous experiments, we determined the agreement, sensitivity, and specificity of the PanBio first-generation IgM capture ELISA compared to an immunofluorescence assay and the Centers for Disease Control and Prevention's IgM capture ELISA. The PanBio assay has since been reformulated to improve the specificity of the assay. We evaluated the reformulated PanBio assay with and without an antigen subtraction procedure and compared the results to the Focus IgM capture ELISA. Agreement, sensitivity, and specificity of the PanBio assay were, respectively, 85%, 95%, and 76% without the subtraction protocol and 94%, 95%, and 93% with the subtraction protocol. In general, when the subtraction protocol was applied to the PanBio IgM capture ELISA, there was a reduction in some, but not all, false-positive results. We suggest that all WNV IgM assays be standardized with a procedure such as background subtraction to eliminate nonspecific reactivity that may cause false-positive results.

West Nile virus (WNV), a mosquito-borne flavivirus, is an avian, equine, and human neuropathogen commonly found in Asia, Africa, Europe, and the Middle East (1, 3). The first appearance of WNV was in Uganda's West Nile province in 1937 (19). The virus was introduced into the United States in 1999 in New York City and resulted in an epidemic that caused 59 hospitalizations and seven deaths. The virus spread westward across the continental United States in four seasons (3). This rapid spread was most likely due to the migration of infected birds after contact with pools of *Culex* mosquitoes from geographic areas of infection (12, 15, 21). The largest outbreak thus far occurred in 2003 when 9,862 human cases of infection were reported in 46 states and the District of Columbia (data from the CDC website; http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount03.htm). Most people infected with the virus remain asymptomatic, 20% develop mild flu-like symptoms, and about 1 in 150 (<1%) develop acute neurologic disease which can result in stupor, paralysis, coma, and death (3).

Serology, particularly the detection of WNV immunoglobulin M (IgM) in serum, has become the primary method for determining acute WNV infection (2). The majority of infected persons have detectable IgM antibodies 8 days following onset of infection, and, in most cases, IgM antibodies remain detectable for 1 to 2 months. In some cases, IgM antibodies have been detected for 500 days or longer following disease onset (16). Commercial assays, including immunofluorescence assays (IFAs) and enzyme-linked immunosorbent assays (ELISAs) for the detection of IgG- and IgM-specific antibodies to WNV are commercially available for diagnostic use. While IFA has high sensitivity and specificity, with 4 to 10% cross-reactivity with other flaviviruses (7, 10, 11), this method is relatively labor intensive. Both Focus Diagnostics (Cypress, CA) and PanBio, Inc. (Columbia, MD) commercially distribute ELISAs that are approved by the Food and Drug Administration for diagnostic use. The Focus Diagnostics WNV IgM capture DxSelect ELISA uses a WNV preM/E recombinant protein antigen (4) for the detection of WNV-specific IgG and IgM. The IgM assay is a mu-capture assay that utilizes a background subtraction protocol to identify false-positive reactions due to nonspecific reactivity from interfering substances such as rheumatoid factor (RF), heterophile antibodies, and other interfering substances (5, 6, 9, 14). The PanBio WNV IgM capture ELISA uses inactivated purified native WNV antigen for the detection of WNV-specific IgG and IgM antibodies. Although the PanBio IgM assay is also a capture assay, no background subtraction protocol is recommended by the manufacturer.

We evaluated both of these commercial IgM capture ELISA systems using samples collected during the 2006 West Nile season. We also used samples from a previous study that had been collected during the 2002 West Nile season and that had

* Corresponding author. Mailing address: ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108. Phone: (801) 583-2787, ext. 2840. Fax: (801) 584-5109. E-mail: mindy.rawlins@aruplab.com.

▽ Published ahead of print on 11 April 2007.

been tested by both IgM IFA and the CDC IgM capture ELISA. Although the agreement, sensitivity, and specificity of the PanBio IgM capture WNV assay were determined in this previously published study (10), the PanBio assay has recently been reformulated to reduce false-positive results. In the present study, the performance characteristics of the reformulated PanBio IgM assay were evaluated and compared to the Focus IgM assay. Although PanBio does not recommend a background subtraction protocol with its IgM assay, we added a background subtraction step to the PanBio procedure to evaluate whether this protocol could improve specificity. Agreement, sensitivity, and specificity were determined both with and without the use of the background subtraction method for the PanBio assay.

MATERIALS AND METHODS

Human sera. This study was approved by the Institutional Review Board of the University of Utah (IRB 7275 and IRB 10972). A total of 127 serum samples were used in this study. The sera were subdivided into three groups.

Group I consisted of 40 serum samples that were collected for a previous study during the 2002 WNV season. Thirty-three of these samples had tested positive by IgM IFA and the Focus IgM capture ELISA. Eighteen of these 33 IgM-positive samples had been sent for confirmation by a plaque reduction neutralization test (PRNT) and were confirmed positive by PRNT. Seven samples that had tested negative by the Focus IgM capture ELISA were also included in this group. Six of the seven samples had negative results by IgM IFA, with the one positive having a low titer of 1:32. Clinical history and symptoms had previously been obtained for all samples in this group (10).

Group II consisted of 56 serum samples that were submitted to the clinical laboratory for WNV IgM testing during the 2006 WNV season. Samples were collected based on results from the Focus IgM capture assay, which is currently the in-house method for WNV antibody testing in the clinical laboratory. Twenty-four of these samples had tested positive, and 32 samples had tested negative for IgM antibodies to WNV.

Group III consisted of 31 serum samples that initially had positive or equivocal results but were subsequently considered equivocal or negative following the background subtraction procedure using the Focus IgM capture ELISA during the 2006 WNV season. Twenty-one of these samples had initially tested positive, and 10 had initially tested equivocal by the Focus IgM capture ELISA in the clinical laboratory. Testing of these samples was repeated using the manufacturer's recommended background subtraction protocol; 14 of 21 positive samples had negative results, and 7 of 21 samples had equivocal results following background subtraction in the clinical laboratory. Eight of 10 equivocal samples had negative results, and 2 of 10 had equivocal results following background subtraction by the Focus IgM capture assay in the clinical laboratory.

Direct identifiers had been removed from all samples in group I prior to testing, and samples had been stored at −20°C following completion of a previous study (10). Direct identifiers had been removed from all samples from groups II and III after collection from clinical frozen storage. All samples were thawed and stored at 2 to 8°C until testing was completed.

Commercial IgM capture ELISAs. All specimens from groups I, II, and III were tested for IgM antibodies against WNV using the Focus Diagnostics WNV IgM capture DxSelect ELISA and the PanBio WNV IgM capture ELISA. Testing was performed according to the manufacturers' instructions. For the Focus assay, serum samples, positive and negative controls, and the cutoff calibrator were diluted 1:101 in sample diluent and added to microwells coated with rabbit anti-human IgM antibodies. Following a 1-h incubation at room temperature, the wells were washed, and reconstituted recombinant WNV antigen was added. The wells were incubated for 2 h at room temperature, followed by a second wash. Horseradish peroxidase-conjugated mouse anti-flavivirus conjugate was added and incubated for 30 min. A third wash step was employed, and tetramethylbenzidine and hydrogen peroxide substrate were added to each well. After 10 min, 1 M sulfuric acid was added to each well to stop the reaction. The absorbance of each well was determined spectrophotometrically at 450 nm using a microplate reader (Spectramax M5; Molecular Devices Corp., Sunnyvale, CA). An index value result was calculated for each specimen by dividing the optical density (OD) of the specimen well with the mean OD of the cutoff calibrator tested in triplicate. Samples with an index value of ≥1.11 were considered reactive (positive), values of 0.90 to 1.10 were indeterminate (equivocal), and values of ≤0.89 were considered nonreactive (negative).

Testing of all positive samples was repeated using the manufacturer's recommended background subtraction procedure to check for false positives due to nonspecific reactivity (14). All serum samples were incubated in two separate duplicate wells and washed as described above. WNV antigen was added to the first well (antigen well) and sample diluent (phosphate-buffered saline [PBS]) was added to the second well (background well). The assay was completed according to manufacturer's specifications. For each sample, the OD of the background well was subtracted from the OD of the antigen well. An index value result was calculated for each specimen using the reduced OD divided by the OD of the cutoff calibrator tested in triplicate.

For the PanBio IgM capture assay, serum samples, positive and negative controls, and the cutoff calibrator were diluted 1:101 in sample diluent and added to wells of a microtiter plate coated with polyclonal sheep anti-human IgM antibodies. Following a 1-h incubation at 37°C, wells were washed, and a horseradish peroxidase-conjugated mouse anti-WNV monoclonal antibody-WNV antigen complex solution was added to each well and incubated for 1 h at 37°C. Following a wash step, tetramethylbenzidine substrate was added. After 10 min, 1 M phosphoric acid was added and the absorbance of each well was determined spectrophotometrically at 450 nm. An index value result was calculated for each specimen by dividing the OD of the specimen well by the mean OD of the calibrator tested in triplicate. Samples with an index value of ≥1.11 were considered positive, values of 0.90 to 1.10 were equivocal, and values of ≤0.89 were considered negative.

Background subtraction of PanBio IgM capture ELISA. All samples from group III initially had positive or equivocal results but were subsequently considered equivocal or negative following the background subtraction procedure using the Focus IgM capture ELISA in the clinical laboratory. PanBio does not recommend utilizing a background subtraction procedure with its IgM capture ELISA to test reactive specimens. Testing of the samples from group III was repeated on the PanBio IgM capture ELISA with a modification of the manufacturer's protocol to include a background subtraction procedure. Samples were tested in two identical wells of a microtiter plate; following the first wash step, antigen was added to the first well (antigen well), and sample diluent was substituted for the antigen in the second well (background well). Testing was completed according to manufacturer's specifications, and the OD of the background well was subtracted from the OD of the antigen well at 450 nm to determine the result. An index value result was calculated using the reduced OD of the sample divided by the mean OD of the cutoff calibrator tested in triplicate.

Commercial IFA. Samples from group III, excluding three samples of insufficient volume, were tested by IgM IFA using slides with wells containing WNV-infected cells (PanBio, Inc.). Serum samples were diluted 1:16 with goat anti-human IgG absorbent diluent (11, 13) in microtiter tubes. The samples were centrifuged for 2 min, and 25 μl of supernatant was added to the wells of the IFA slides. Slides were incubated for 90 min at 37°C in a moist chamber and washed for 10 min in PBS. Following the wash, 25 μl of anti-mouse and anti-human IgM dual-species fluorescein-labeled conjugate (Focus Diagnostics) was added to the wells, and the slides were incubated for 30 min in a moist chamber at 37°C. The slides were then washed with PBS for 10 min, a coverslip was applied, and the slides were examined at a magnification of ×400 using an Olympus (Tokyo, Japan) BH-2 transmitting fluorescence microscope with a 100-W mercury lamp. Results were determined based on the cytoplasmic fluorescence observed in each sample well. Fluorescence was assigned using the following scale: moderate to intense apple-green cytoplasmic fluorescence received a score of 2 to 4+; low intensity or dim, but definite, cytoplasmic fluorescence received a score of 1+. Samples showing 1+ or greater cytoplasmic fluorescence over 5% or more of the well were considered positive for IgM antibodies to WNV. The titers of samples with positive results were determined by diluting each sample 1:16 in goat anti-human IgG diluent and serially diluting to 1:256 in PBS. The titer at which cytoplasmic fluorescence of 1+ or greater was observed was reported as the result for each sample.

Statistical analysis. Two-by-two contingency table analysis was used to determine agreement, clinical sensitivity, and clinical specificity for the PanBio WNV IgM capture ELISA assay. The 95% confidence intervals (CI) for the clinical sensitivity and clinical specificity were also determined. Equivocal results were not included in the calculations, and the Focus WNV IgM capture ELISA assay was used as the reference method. Results from the PanBio WNV IgM capture ELISA were compared to results from the Focus WNV IgM capture ELISA, and discordant samples were retested in duplicate for each assay. Results from the PanBio IgM capture ELISA using the background subtraction protocol were also compared to the Focus WNV IgM capture ELISA results, and testing of discordant samples was repeated in duplicate with each assay.

TABLE 1. Summary of IgM results comparing the PanBio WNV IgM capture ELISA to the Focus Diagnostics WNV IgM capture ELISA[a]

PanBio IgM capture ELISA result	No. of samples with Focus IgM capture ELISA result that was:		
	Positive	Negative	Equivocal
Positive	53	14	4
Negative	3	44	5
Equivocal	1	3	

[a] In the comparison of the PanBio IgM capture ELISA against the Focus IgM capture ELISA, the agreement was 85.1%, the sensitivity was 94.6% (95% CI, 88 to 98%), and the specificity was 75.9% (95% CI, 69 to 79%).

RESULTS

Clinical information was available for each sample in group I, and the results for each sample in this group were in agreement with the results from our previous studies (10) for both the Focus IgM capture assay and the PanBio IgM capture assay, resulting in 100% agreement, 100% sensitivity, and 100% specificity for both assays. Agreement, sensitivity, and specificity of the PanBio IgM capture ELISA were also determined by comparing the PanBio IgM antibody results to the Focus IgM antibody results for all of the samples from groups I, II, and III. The agreement, sensitivity, and specificity were 85%, 95% (95% CI, 88 to 98%), and 76% (95% CI, 69 to 79%), respectively (Table 1).

Three out of 57 samples that tested positive for IgM antibodies by the Focus IgM capture ELISA had negative results by the PanBio IgM capture ELISA. Fourteen of 61 samples that had negative results by the Focus assay tested positive by the PanBio assay. All 14 of the discrepant samples positive by the PanBio assay had initially tested positive by the Focus assay but were considered negative following background subtraction according to the Focus protocol in the clinical laboratory. Four samples that initially had positive results by the Focus IgM capture ELISA and were considered equivocal after background subtraction were also positive by the PanBio IgM capture ELISA.

To determine whether the low specificity of the PanBio assay compared to the Focus assay was due to high background, testing of the 18 samples that were positive by the PanBio assay but negative or equivocal by the Focus assay was repeated in duplicate using the background subtraction procedure for both assays (Table 2). After background subtraction, 9 of the 18 discrepant samples were considered negative, 4 samples were equivocal, and 5 remained positive by the PanBio IgM capture assay. These samples were also tested by IgM IFA, excluding one of the positives due to insufficient sample volume. Sixteen of the 18 samples had negative results by IFA (Table 2). One sample was IFA positive with a titer of 1:32 but had negative IgM results for the Focus and PanBio IgM capture ELISAs following background subtraction. This sample was positive by the PanBio assay, however, prior to the use of the subtraction protocol. Agreement, sensitivity, and specificity of the PanBio IgM capture assay were recalculated using the antigen subtraction data for the 14 samples that were positive by the PanBio assay but negative by the Focus assay (Table 3). Agreement, sensitivity, and specificity were 93.8%, 94.6% (95% CI, 89 to 98%), and 92.9% (95% CI, 87 to 93%), respectively.

TABLE 2. Antigen subtraction results from 18 discrepant sera as tested by the Focus WNV IgM capture ELISA, PanBio WNV IgM capture ELISA, and WNV IgM IFA

Sample no.	Focus IgM ELISA index value[a]		PanBio IgM ELISA index value[a]		IgM IFA titer[b]
	Without antigen subtraction	With antigen subtraction	Without antigen subtraction	With antigen subtraction	
1	1.64	0.02	2.10	0.22	<16
6	1.22	1.10	3.32	3.02	<16
7	1.47	0.00	2.65	0.36	<16
8	1.13	0.96	1.14	1.02	<16
10	0.96	0.04	1.88	0.08	<16
11	1.36	1.10	1.24	1.08	<16
12	1.17	0.34	6.59	1.85	<16
13	2.28	0.00	4.32	1.82	ND
16	1.06	0.84	1.31	0.97	<16
17	3.13	1.00	2.13	0.00	<16
18	1.62	0.00	3.42	1.65	<16
19	1.35	0.00	3.39	1.62	<16
84	0.92	0.00	1.55	0.42	<16
87	1.00	0.00	1.47	0.52	<16
88	8.78	0.00	8.05	0.00	32
89	1.59	0.00	3.96	0.85	<16
90	1.30	0.00	1.45	0.49	<16
91	1.86	0.00	1.74	1.04	<16

[a] See Materials and Methods for calculation of the index value. The cutoff value for the Focus and PanBio IgM capture ELISAs was <0.90.
[b] IFA titers are shown as reciprocal values. The cutoff value for the IgM IFA was <16. ND, not done.

DISCUSSION

Numerous studies have been published demonstrating interference from RF and heterophile antibodies in immunoassays (8, 9, 17). Recent studies have shown the importance of including a background subtraction procedure to eliminate false-positive reactions from interference by these substances when testing for WNV IgM antibodies. The CDC screening ELISA procedure incorporates control wells without antigen to measure background signal for each patient specimen tested. The wells containing antigen must have an absorbance that is at least twice the absorbance of the background wells for the results to be interpretable. This allows each specimen to be

TABLE 3. Summary of agreement, clinical sensitivity, and clinical specificity of the PanBio WNV IgM capture ELISA compared to the Focus Diagnostics WNV IgM capture ELISA using antigen subtraction[a]

Result of PanBio IgM capture ELISA with antigen subtraction	No. of samples Focus IgM capture ELISA with the indicated result after antigen subtraction		
	Positive	Negative	Equivocal
Positive	53	4	1
Negative	3	52	6
Equivocal	1	5	2

[a] In the comparison of the PanBio IgM capture ELISA with antigen subtraction and the Focus IgM ELISA with antigen subtraction, the agreement was 93.8%, the sensitivity was 94.6% (95% CI, 89 to 98%), and the specificity was 92.9% (95% CI, 87 to 93%).

screened for false positivity due to high background (4). In a study during the 2001 WNV season, weakly reactive samples as tested by the Focus assay were nonreactive by the CDC IgM screening assay when the background subtraction protocol was not utilized. A background subtraction procedure was incorporated into the Focus assay protocol as a result of this study (14). In a separate study, the background subtraction procedure resulted in a sensitivity and a specificity of 99.3% when non-WNV flavivirus sera were excluded from the analysis. However, it is important to note that the subtraction procedure does not reduce cross-reactivity with non-WNV flavivirus sera. The overall cross-reactivity for the Focus IgM ELISA to flavivirus sera was 12% despite subtraction, with 31% of St. Louis encephalitis patients found to be WNV IgM positive (4). Currently, the PanBio IgM capture assay does not have a background subtraction protocol to screen for interfering substances that can cause false-positive and false-negative results.

Studies comparing the PanBio first-generation WNV IgM capture assay to the Focus WNV IgM capture assay showed a specificity of only 82.9% for the PanBio assay (20). Although the PanBio assay has been reformulated since the aforementioned study was conducted, our present study actually showed a lower specificity of 75.9% for the current reformulated assay compared to the Focus assay. Eighteen of 31 samples that had high background and were considered either negative or equivocal after subtraction using the Focus assay were positive by the PanBio assay, for a false-positive rate of 58% for these 31 samples. Applying a background subtraction procedure to the PanBio protocol increased the specificity from 75.9% to 92.9%.

A study by Sambol et al. at the Nebraska Public Health Laboratory showed that specimens in the low-positive index value range of 1.2 to 3.5 by the Focus assay had a false-positive rate of 6.5%, in which 52 of 794 positive specimens had interfering factors such as RF and heterophile antibodies at high enough levels that the results were equivocal following antigen subtraction. The Nebraska Public Health Laboratory currently sends all WNV-positive samples for reflex testing for RF, heterophile antibodies, and PRNT as a result of the study (18). Similarly, all 31 samples that contained high background in our study, except for one with an index value of 8.78, had index values in the equivocal to low-positive range between 0.92 and 3.13 by the Focus assay before background subtraction. Of the 18 false positives by the PanBio assay, 12 samples had index values in the low-positive range between 1.22 and 2.76, and the remaining 6 samples had index values of >3.5. These data indicate the importance of using a background subtraction procedure, particularly for samples that have initial results that fall in the low-positive range. Five of the 18 samples remained positive following subtraction using the PanBio method; all but one sample were negative by IFA, showing that a background procedure may help to eliminate some, but not all, false-positive results for the PanBio assay.

Assays used to detect WNV IgM-specific antibodies must be highly sensitive and specific to avoid false-positive and -negative reporting of results. Laboratories must ensure that testing and result interpretation are performed properly (16). Our study, as well as the studies of others (14, 18), indicates that interfering substances can produce erroneous results and should be considered in assay protocols for WNV IgM antibody testing. We recommend that all WNV IgM assays be standardized with respect to the evaluation of samples with high background that may cause false-positive results.

ACKNOWLEDGMENTS

This work was supported by the ARUP Institute for Clinical and Experimental Pathology.

We thank PanBio, Inc., for supplying reagents used in this study.

REFERENCES

1. **Campbell, G. L., A. A. Marfin, R. S. Lanciotti, and D. J. Gubler.** 2002. West Nile virus. Lancet Infect. Dis. **2:**519–529.
2. **Centers for Disease Control and Prevention.** 2003. Epidemic/epizootic West Nile virus in the United States: guidelines for surveillance, prevention, and control, 3rd ed. Centers for Disease Control and Prevention, Atlanta, GA.
3. **Guharoy, R., S. A. Gilroy, J. A. Noviasky, and J. Ference.** 2004. West Nile virus infection. Am. J. Health Syst. Pharm. **61:**1235–1241.
4. **Hogrefe, W. R., R. Moore, M. Lape-Nixon, M. Wagner, and H. E. Prince.** 2004. 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. **42:**4641–4648.
5. **Huebner, J.** 2004. Antibody-antigen interactions and measurements of immunologic reactions, p. 207–232. *In* G. Pier, J. Lyczak, and L. Wetzler (ed.), Immunology, infection, and immunity. ASM Press, Washington, DC.
6. **Kim, M., and M. Wadke.** 1990. Comparative evaluation of two test methods (enzyme immunoassay and latex fixation) for the detection of heterophil antibodies in infectious mononucleosis. J. Clin. Microbiol. **28:**2511–2513.
7. **Koraka, P., H. Zeller, M. Niedrig, A. D. Osterhaus, and J. Groen.** 2002. Reactivity of serum samples from patients with a flavivirus infection measured by immunofluorescence assay and ELISA. Microbes Infect. **4:**1209–1215.
8. **Kricka, L. J.** 1999. Human anti-animal antibody interferences in immunological assays. Clin. Chem. **45:**942–956.
9. **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.
10. **Malan, A. K., T. B. Martins, H. R. Hill, and C. M. Litwin.** 2004. Evaluations of commercial West Nile virus immunoglobulin G (IgG) and IgM enzyme immunoassays show the value of continuous validation. J. Clin. Microbiol. **42:**727–733.
11. **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. Am. J. Clin. Pathol. **119:**508–515.
12. **Malkinson, M., C. Banet, Y. Weisman, S. Pokamunski, R. King, M. T. Drouet, and V. Deubel.** 2002. Introduction of West Nile virus in the Middle East by migrating white storks. Emerg. Infect. Dis. **8:**392–397.
13. **Martins, T. B., T. D. Jaskowski, C. L. Mouritsen, and H. R. Hill.** 1995. An evaluation of the effectiveness of three immunoglobulin G (IgG) removal procedures for routine IgM serological testing. Clin. Diagn. Lab. Immunol. **2:**98–103.
14. **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.
15. **Rappole, J. H., S. R. Derrickson, and Z. Hubalek.** 2000. Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerg. Infect. Dis. **6:**319–328.
16. **Roehrig, J. T., D. Nash, B. Maldin, A. Labowitz, D. A. Martin, R. S. Lanciotti, and G. L. Campbell.** 2003. Persistence of virus-reactive serum immunoglobulin M antibody in confirmed West Nile virus encephalitis cases. Emerg. Infect. Dis. **9:**376–379.
17. **Salonen, E. M., A. Vaheri, J. Suni, and O. Wager.** 1980. Rheumatoid factor in acute viral infections: interference with determination of IgM, IgG, and IgA antibodies in an enzyme immunoassay. J. Infect. Dis. **142:**250–255.
18. **Sambol, A. R., S. H. Hinrichs, W. R. Hogrefe, and B. K. Schweitzer.** 2007. Performance of a commercial immunoglobulin M antibody capture assay using analyte-specific reagents to screen for interfering factors during a West Nile virus epidemic season in Nebraska. Clin. Vaccine Immunol. **14:**87–89.
19. **Smithburn, K. C., T. P. Hughes, A. W. Burke, and J. H. Paul.** 1940. A neurotropic virus isolated from the blood of a native of Uganda. Am. J. Trop. Med. Hyg. **20:**471–492.
20. **Tilley, P. A., R. Walle, A. Chow, G. C. Jayaraman, K. Fonseca, M. A. Drebot, J. Preiksaitis, and J. Fox.** 2005. Clinical utility of commercial enzyme immunoassays during the inaugural season of West Nile virus activity, Alberta, Canada. J. Clin. Microbiol. **43:**4691–4695.
21. **Turell, M. J., M. R. Sardelis, M. L. O'Guinn, and D. J. Dohm.** 2002. Potential vectors of West Nile virus in North America. Curr. Top. Microbiol. Immunol. **267:**241–252.