Clinical Utility of Commercial Enzyme Immunoassays during the Inaugural Season of West Nile Virus Activity, Alberta, Canada

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West Nile virus (WNV) has spread rapidly across North America, creating a need for rapid and accurate laboratory diagnosis on a large scale. Immunoglobulin M (IgM) capture enzyme immunoassays (EIA) became commercially available in the summer of 2003, but limited data are available on their clinical performance. Consolidated human WNV diagnostic testing for the province of Alberta, Canada, at the public health laboratory permitted a large-scale evaluation of the assays, covering a wide clinical spectrum. Two thousand nine hundred sixty-nine sera were tested, from 2,553 Alberta residents, and 266 cases were identified. Sensitivities of the Focus assay and first-generation Panbio IgM capture EIA were 79 and 80%, respectively. During the first week of illness only 53 to 58% of cases were positive, but sensitivity was 96 to 97% after day 8. Sensitivity for neurological cases was 92% overall. Specificity was high for the Focus kit at 98.9%, but only 82.9% for the first Panbio kit. A positive Focus WNV IgG result with a twofold rise in IgG index was a reliable indicator of acute flavivirus infection (67/67 WNV). Agreement between the IgG test and hemagglutinin inhibition titers in paired sera was at least 82%. Commercial IgM and IgG EIA proved useful for WNV diagnosis, provided follow-up sera were collected after 8 days of illness.

West Nile virus (WNV) has spread rapidly across North America since its introduction in 1999, creating a need for rapid and accurate laboratory diagnosis (3, 8). Historically, hemagglutination inhibition (HI) assays have been used for the serological diagnosis of arboviral infections (1). This technique is broadly cross-reactive among the flaviviruses and requires confirmatory testing for specific viral diagnosis. Plaque reduction neutralization titers (PRNT) are specific, but require containment level 3 viral culture and are laborious to perform. In-house immunoglobulin M (IgM) capture enzyme immunoassays (EIA) have been described and are more specific than the HI assay (7). Reagents for the IgM assay (IgM antibody capture enzyme-linked immunosorbent assay) were made available for a period to public health laboratories within the United States (6) but were not widely available in other countries. Commercial WNV IgM kits became available in the summer of 2003. Few reports have been published on their performance, and these provide limited data on comparison with the in-house Centers for Disease Control and Prevention assay (5). In Alberta, WNV serological and molecular testing was implemented at a single public health laboratory site to serve the province in 2003. The availability of the commercial assays during the first season of WNV activity provided an opportunity for the first large-scale, head-to-head evaluation of the kits in a previously naive population, where other flaviviruses are nonendemic. This evaluation was performed on all

human specimens submitted for WNV testing for the province, including West Nile fever (WNF) cases, using an expanded case definition incorporating molecular diagnosis to provide data on the fullest range of clinical presentations.

MATERIALS AND METHODS

West Nile virus diagnostic tests were conducted at one site of the Provincial Laboratory for Public Health. Consolidation at one site provided standardized testing of sufficient volume to evaluate test performance and provided comprehensive reporting to public health epidemiologists. A laboratory testing information bulletin was provided to all physicians and was posted on the laboratory website. This bulletin recommended testing patients with neurological presentations, patients with fever who had blood or organ donation or receipt, and immunocompromised patients with fever. Specimens recommended for testing were acute- and convalescent-phase sera, whole blood (EDTA), and cerebrospinal fluid (CSF), where appropriate. A clinical history form was circulated and placed on the website to assist with specimen triage and communication and to provide travel and vaccination history for interpretation of results. When specimens were received without history, the blank form was faxed to the physician for completion. Screening of asymptomatic blood or organ donors is not included in the study.

The test algorithm is shown in Fig. 1. West Nile virus IgM enzyme immunoassays were performed using, on all sera, the commercial West Nile virus IgM capture kit EL0300M from Focus technologies (Cypress, California) and the first-generation West Nile virus IgM capture kit E-WNV01M (Panbio-1) from Panbio (Windsor, Australia) according to the manufacturers' instructions. The IgM index was calculated for each specimen by dividing the patient serum optical density reading by a standard cutoff reagent reading, performed with every run, as per the manufacturers' instructions. Specimens with an IgM index of ≥ 1.1 were considered positive. Discrepant results between the two kits were reported as indeterminate. Two-week follow-up sera were requested for all patients. IgM assays were repeated on all convalescent-phase sera using both kits. An IgG EIA (Focus) was performed on convalescent-phase sera according to the manufacturer's instructions. The IgG index was calculated by division of the patient serum optical density value by that of the cutoff reagent standard performed with each run, as per the manufacturer's instructions. An IgG index of >1.5 was

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FIG. 1. Test algorithm for WNV serology at Provincial Laboratory in 2003. Panbio-1 and Panbio-2 refer to the first- and second-generation WNV IgM assays from the company.

considered positive. For IgG-positive patients, the IgG test was repeated on the acute- and convalescent-phase specimens in the same run. Hemagglutination inhibition titers were performed against West Nile and St. Louis encephalitis viruses on acute- and convalescent-phase serum pairs using antigens prepared from suckling mice (1). WNV PRNT was performed on the first 38 serum pairs with rising or high HI titers as described previously (2). At the end of the season a second-generation WNV IgM kit became available (Panbio-2), which incorporated a control well for each serum specimen. The control well did not contain specific antigen, and the optical density for this well was subtracted from the WNV antigen well to control for nonspecific binding. This assay is referred to as the WNV IgM background subtraction assay.

CSF was extracted using the QIAamp viral RNA kit (QIAGEN Inc., Valencia, California). Extraction was from 225 μ l of CSF into an elution volume of 60 μ l. Plasma was extracted (from a 1-ml volume) and concentrated (to 50 μ l eluate) using a commercial kit (NucliSens extraction kit; bioMérieux, Durham, NC) according to the manufacturer's instructions.

Two different nucleic acid amplification methods were employed for detection of WNV-specific RNA in extracted nucleic acid, each targeting a different portion of the WNV genome. A sensitive method utilizing nucleic acid sequence-based amplification (NASBA) was used as a first-line assay for plasma samples. The method was as described previously (4) with modifications to enable a larger (1-ml) volume of plasma to be analyzed and with detection of amplified products using a real-time fluorimeter (NucliSens EasyQ analyzer; bioMérieux, Durham, NC). Reverse transcription-PCR (RT-PCR) for WNV in CSF was performed using a commercially available kit (RealArt WNV LC assay; artus-Biotech, San Francisco, CA) using the LightCycler (Roche Diagnostics, Quebec, Canada). Details on the clinical validation of the NASBA and RT-PCR assays have been submitted for publication (P. Tilley et al., submitted for publication).

As no test was likely to serve as a reliable "gold standard" when used alone, a case definition based on multiple test results was applied for analysis of the results. Patients were considered to be confirmed WNV cases if (i) PRNT was positive or (ii) both WNV NASBA and RT-PCR tests were positive. As these tests could not be performed on all patients, a probable case definition was also used. Patients were considered probable WNV cases if (i) the WNV IgM test was positive by both commercial kits and was subsequently positive by the second-generation Panbio kit incorporating a background subtraction step or (ii) the WNV HI test showed a fourfold rise in titer in the absence of recent travel.

RESULTS

West Nile virus was first reported in Alberta on 9 July 2003, having been found in a dead magpie, and the first human case was detected 9 August 2003 by PCR of a blood donor specimen. A preliminary validation was performed for the first 130 cases for which IgM was positive in both commercial assays. Of these, 108 showed a fourfold or greater rise in WNV HI titer. The remaining 22 serum pairs all showed a static HI titer of 320 or greater, (median, 1,280). Of 38 Focus- and Panbio-1 IgM-positive serum pairs subsequently tested by WNV PRNT, all were confirmed to be WNV infection.

Ultimately, 2,969 sera were received from a total of 2,553 Alberta residents, of whom 118 were considered to have confirmed WNV infections and 148 were probable cases. Eight patients had insufficient testing for categorization. Two WNV cases were asymptomatic blood donors, while 46 were subsequently determined to have West Nile virus neurological syndromes (WNNS), according to the Alberta WNV case definitions (http://www.health.gov.ab.ca/public/WNV/pdf/case_definitions.pdf). Mean ages of cases and noncases were 45.7 and 38.7 years, respectively ($P < 0.0001$, analysis of variance). WNNS patients were significantly older than WNF patients (50.7 versus 44.2 years, $P < 0.0001$) and noncases (50.7 versus 38.7, $P < 0.0001$). Gender was not significantly different between cases

TABLE 1. Performance of the Focus and Panbio-1 WNV IgM capture enzyme immunoassay kits compared to the case definitions

Test result	No. of positive sera/no. tested (%) for:					
	Confirmed cases	Probable cases	All cases	Noncases	Cases <8 days ^a	Cases $\geq$ 8 days
Focus IgM positive	138/220 (63)	215/228 (94)	353/448 (79)	25/2,335 (1.1)	89/169 (53)	231/238 (97)
Panbio-1 IgM positive	146/220 (66)	213/228 (93)	359/448 (80)	261/2,336 (11)	97/167 (58)	229/239 (96)
WNV HI positive	115/165 (70)	120/136 (88)	225/301 (75)	22/252 ^b (8.7)	43/98 (44)	169/175 (97)

^a Sera collected within 8 days of symptom onset, as reported by the physician on the history form.

^b HI was used during the early season prior to EIA availability. These are not the same group of noncases tested by EIA above.

FIG. 2. Kinetics of Focus WNV IgM results for WNV cases, by day of illness when serum was collected. neg, negative; POS, positive.

and noncases (49.1% female versus 53.0%, respectively, $P = 0.09$).

The performance of the IgM kits is summarized in Table 1. Sensitivity of the Focus kit was 79.3% for all cases, and specificity was 98.9%. The Panbio-1 kit had a similar sensitivity, at 81.0%, but considerable lower specificity than the Focus kit, at 82.9%. The positive predictive values in our population were 93% for the Focus kit and 60% for the Panbio-1 kit. In 89 cases, the initial IgM tests were negative, and WNV infection was identified by concomitant NASBA and PCR ($n = 71$) or by follow-up serology ($n = 72$). The kinetics of the IgM response in WNV cases measured using the Focus IgM kit are shown in Fig. 2. The kinetics of the Panbio-1 IgM kit are nearly identical (data not shown). Sensitivity of the IgM assays during the first week of illness was only 53 to 58%, but was 96 to 97% after that point. For the 46 WNNs patients, the sensitivity of the WNV IgM test was 92% for both commercial kits.

All 208 convalescent-phase IgM-negative sera were screened for IgG, and 10 were positive. Of these, no additional WNV

cases were identified by rising HI titers; all had stable low flavivirus titers, and the results were considered to reflect past flavivirus infections.

Acute- and convalescent-phase IgG index values, calculated relative to the kit cutoff control as per the manufacturers' instructions, were compared to HI titers to determine whether IgG could be used quantitatively and serve as a replacement for HI. The IgG index data are summarized in Fig. 3 and Table 2. A positive IgG result, with a twofold rise in IgG index, was 100% specific for acute flavivirus infection, as all such patients were WNV cases ($n = 67$). One acute dengue case had a rising index but remained just below the IgG-positive cutoff point. Twenty-three WNV cases were IgG positive but did not have a twofold IgG index rise. Of these, all had high (≥ 320) HI titers and five showed a fourfold rise in HI titer. Of the seven patients who did not show an IgG response, three had convalescent-phase sera collected less than 14 days after the onset of illness. Overall, the agreement between paired IgG and HI titers was 82%.

DISCUSSION

In-house IgM tests have been described previously for diagnosis of arboviral infections in the United States and Europe (11, 13). Commercial kits for WNV IgM have only recently become available and offer the possibility of rapid and larger-scale testing but have not been well evaluated in the literature (5). In this study, the first two available WNV IgM EIA kits were employed prospectively on a population basis and evaluated in light of both previously available HI titers and new molecular assays.

In this study, using multiple testing formats, the overall sensitivity of the IgM tests was 79 to 80%, while the sensitivity during the first 7 days of illness was only 53 to 58%. The sensitivity after the first week of illness was 96 to 97%. This delayed antibody response is more common than in most earlier reports (9, 10, 13). This difference may be due to the

FIG. 3. WNV IgG indices (Focus) for WNV cases and noncases. Acute- and convalescent-phase sera were run concurrently. Indices were calculated by division of patient serum optical density by kit control optical density, as per the manufacturers' instructions. An index of ≥ 1.5 is considered a positive test. ♦ (right), patient in the noncase group determined to have acute dengue acquired outside Alberta.

TABLE 2. Performance of the Focus WNV IgG enzyme immunoassay kit compared to the case definitions and WNV HI assay

IgG test result	No. of patients						
	Confirmed cases	Probable cases	All cases	Noncases	WNV HI negative	WNV HI positive, stable titer	WNV HI positive, fourfold rise in titer
IgG negative or indeterminate in both sera	3	4	7	212 ^a	16	0	8 ^a
IgG positive, stable level	6	17	23	13	5	23	6
IgG positive, twofold rise in optical density index	66	37	103	0	1	8	90

^a One patient with acute dengue showed a rise in HI titer but remained IgG indeterminate.

comprehensive population-based format of this study, which included a high percentage of patients with WNF. The sensitivity of the IgM assays for WNNS patients was higher, 92%, although our numbers were small. Thus, if serology is used as the sole method for WNV testing, it is important to stress to ordering physicians that a follow-up specimen, collected after the first week of illness, is required for reliable serological diagnosis.

The specificity of the IgM assays in initial validation was acceptable, when two kits were used together, and compared with HI and PRNT. Individually, a positive test using the Focus kit alone proved to be reliable in our population, with a positive predictive value of 93%, but the Panbio kit in its original format was problematic (positive predictive value, 60%). A subsequent version of the kit, with a subtraction control well for each specimen, corrected this problem, without compromising the test sensitivity. These assays have therefore proven clinically useful for timely West Nile diagnosis in a region where St. Louis encephalitis, Japanese encephalitis, and dengue are nonendemic. The specificity in the presence of other acute flavivirus infections cannot be determined from our population.

In some patients with WNV infection and previous exposure to flaviviruses, the IgM response is suppressed and only an IgG response is seen (3). In this study, no patients were identified who had an HI and IgG titer rise in the absence of an IgM response. Anamnestic responses therefore appear to be a rare problem for the IgM tests in our population.

The performance of IgM assays in upcoming years remains to be seen, given the recent report of persistent IgM positivity in many patients using in-house tests (12). It is likely that changing titers will need to be demonstrated to confirm an acute diagnosis in future years. At present this is cumbersome with HI and PRNT methods, but data reported here indicate that the IgG EIA can be of use for demonstration of rising antibody levels in most patients and compares favorably with the HI assay. A twofold increase in IgG index proved to be due to acute flavivirus infection in all cases. As seen in the HI assay, stable high IgG indices did not differentiate between recent and past flavivirus infections. The IgG assay also missed 5% of our WNV cases, particularly when follow-up sera are collected early, within 2 weeks of disease onset.

As convalescent-phase sera were submitted in a minority of cases, a limitation of this study is that some cases with delayed seroconversion may have been missed. The use of nucleic acid testing identified many cases on the first blood collection and partially compensated for the lack of follow-up sera. It remains

possible, however, that the sensitivities of the IgM kits may be lower than reported here if there were undetected cases.

In summary, new commercially available enzyme immunoassays proved to be useful clinical tools in the inaugural WNV season in the province of Alberta, a region nonendemic for other flaviviruses. The sensitivity of the IgM assays is high, especially for neurological cases, provided that follow-up sera are collected after the first week of illness. Specificity is high for the Focus kit, and for the Panbio kit when background subtraction is employed. Rises in WNV IgG index are highly predictive of current flavivirus infection, but the use of assays that detect IgG alone may give rise to false negatives in convalescent-phase sera collected from approximately 5% of cases.

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