Nucleic Acid Testing for West Nile Virus RNA in Plasma Enhances Rapid Diagnosis of Acute Infection in Symptomatic Patients

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Although nucleic acid amplification testing (NAAT) for West Nile virus (WNV) is useful in screening blood donors, such methods have not been studied in symptomatic patients. For diagnosis of WNV infection, 1.0 mL of plasma was tested by NAAT, and WNV-specific immunoglobulin M was assayed. Of 276 WNV cases, 191 were tested by both serology and NAAT. Of these, 86 (45.0%), 111 (58.1%), and 180 (94.2%) were detected by NAAT, serology, and combined NAAT and serology, respectively. NAAT-based screening was most useful within 8 days of the onset of symptoms. Viremia is common in early symptomatic WNV infection, and NAAT enhances diagnostic yield.

Nucleic acid amplification testing (NAAT) of blood donors for West Nile virus (WNV) RNA was introduced as a screening tool for the identification of viremic and potentially infectious donors in blood systems throughout North America in 2003. Despite the fact that low levels of viremia and testing of pooled serum samples limited the sensitivity of NAAT-based screening in this setting, large numbers of viremic donors were identified in geographic areas experiencing high levels of symptomatic WNV disease [1–3]. Although NAAT for WNV RNA has been reported in small studies using cerebrospinal fluid from patients with neurological diseases due to WNV, testing for viral RNA in blood from symptomatic persons has not been widely used, primarily because previous reports documented low-level and transient viremia in infected persons [4–6]. It is generally believed that viremia is no longer present at the time of onset of symptoms, and, as a result, serologic testing is the main diagnostic tool for WNV infection in symptomatic patients. Here, we describe the evaluation of NAAT of plasma for diagnosis of WNV infection and its correlation with WNV-specific IgM assays in population-based testing during the first year of WNV activity in Alberta, Canada.

Methods. During the inaugural WNV season (1 June–30 November 2003) in the province of Alberta (population, ∼3 million), all WNV diagnostic testing of symptomatic patients and follow-up testing of blood donors identified as positive for WNV RNA by Canadian Blood Services was performed at a central public health laboratory, the Provincial Laboratory for Public Health. Before the onset of the WNV season, physicians were instructed to collect both a serum sample for WNV serologic testing and a plasma sample for WNV RNA testing from all patients suspected of having WNV infection. Cerebrospinal fluid samples for NAAT were also collected when clinically indicated.

Serum samples were screened for WNV-specific IgM by 2 commercial EIA kits (West Nile Virus IgM Capture EIA; one by Focus Technologies and the other by PANBIO), in accordance with the manufacturers’ instructions. Samples positive by both IgM assays were retested using a second-generation IgM assay that incorporated a background subtraction step to rule out nonspecific reactivity (West Nile Virus IgM Capture EIA; PANBIO). Convalescent-phase serum samples were requested from all patients and were screened with an assay for WNV-specific IgG (West Nile Virus IgG EIA; Focus Technologies) in addition to the WNV-specific IgM assay. When the convalescent-phase IgG test yielded positive results, acute-phase and convalescent-phase serum samples were retested, using a hemagglutination inhibition assay and a plaque-reduction neutralization test (antigen for hemagglutination inhibition provided by and plaque-reduction neutralization test performed at the National Microbiology Laboratory, Winnipeg).

Nucleic acid was extracted from 1.0 mL of plasma, using a commercial kit (NucliSens Extraction Kit; bioMérieux), and was concentrated into an elution volume of 50 µL, using methods published and validated for hepatitis C virus [7]. Internal controls (specific for WNV) were spiked into each sample to monitor extraction efficiency and inhibitory effects.
Two different nucleic acid amplification methods were used to detect WNV-specific RNA, each targeting a different portion of the WNV genome. A sensitive method involving nucleic acid sequence–based amplification (NASBA) was used as described elsewhere [6], with sample preparation modified as detailed above to enable a larger volume of plasma to be analyzed. Amplified products were detected using a real-time fluorimeter (NucliSens EasyQ analyzer; bioMérieux). Samples giving a positive result were retested using reverse-transcriptase polymerase chain reaction (RT-PCR) assays to confirm WNV infection. RT-PCR for WNV was performed using a commercially available kit (RealArt WNV LC assay; Artus-Biotech) and the LightCycler (Roche Diagnostics). External positive and negative controls were included in each assay, and the sensitivity of the assay was assessed by dilution of titered virus into a background of negative plasma before extraction. WNV titer in specimens was estimated by reference to external controls.

We used the \( \chi^2 \) test to examine significant differences in specimens found to be positive by NAAT from patients with West Nile fever versus West Nile neurological syndrome. Because no test was likely to serve as a reliable standard when used alone, a case definition based on multiple test results was applied for analysis of the results. Patients were considered to have confirmed WNV infection if the results of a plaque-reduction neutralization test were positive or the results of both NASBA and RT-PCR tests for WNV RNA were positive. Because these tests could not be performed for all patients, a probable case definition was also used. Patients were considered to have probable WNV infection if they had positive results of WNV-specific IgM tests with both commercial kits and subsequently had positive results by the second-generation assay incorporating a background subtraction step or if the WNV hemagglutination inhibition test showed a 4-fold increase in titer in the absence of recent travel. Other flaviviruses that could cause cross-reactivity in the hemagglutination inhibition test are not endemic in Alberta. Because 2003 was the first year of WNV activity in Alberta, long-term persistence of IgM antibody to the virus did not complicate interpretation [8].

**Results.** Both WNV NAAT assays were sensitive (<0.01 TCID\(_50\)/mL of plasma) and had comparable in vitro performance (data not shown). In preliminary validation testing, 65 of 66 patients who were positive for WNV in the 2 independent NAAT assays were positive for WNV-specific IgM initially or on follow-up. A single patient, lost to further follow-up, was still negative for WNV-specific IgM when last tested.

During the 2003 WNV season, 2553 patients were tested for WNV infection at the Provincial Laboratory for Public Health. Only 2 patients were asymptomatic, identified during blood donor screening and referred to the Provincial Laboratory for Public Health for confirmation. A summary of the specimens submitted for testing and results is shown in figure 1.

According to the case definition above, 276 (10.8%) of the persons tested in Alberta during 2003 had laboratory evidence of WNV infection. Of these, as determined using Alberta WNV case definitions (available at: http://www.health.gov.ab.ca/public/wnv_casedefinitions.pdf), 228 (82.6%) had West Nile fever, 46 (16.7%) had West Nile neurological syndrome, and 2 (0.7%) were asymptomatic.
A total of 1202 plasma samples from 1067 patients were tested for WNV RNA. WNV RNA was detected by NASBA in 92 samples and was confirmed by RT-PCR in 91. The single NASBA-positive PCR-negative case was IgM positive and was confirmed by plaque-reduction neutralization serologic testing. By use of NAAT, 83 (35.9%) of 231 plasma samples from patients with West Nile fever were found to be positive, whereas 7 (9.5%) of 74 plasma specimens from patients with West Nile neurological syndrome were found to be positive (P < .0001, χ² test).

For 191 of the 284 patients with documented WNV infection, both serum and plasma from the first blood collection were submitted for WNV testing, enabling a direct comparison between WNV-specific IgM and NAAT methods. Sixty-nine WNV infections (36.1%) were detected by NAAT alone, 94 (49.2%) by serologic testing alone, 17 (8.9%) by both types of testing, and 11 (5.8%) by neither type of testing on the first blood sample. When both assay types were used together, 94.2% of our patients with WNV infection could be identified on the basis of the initial blood specimens submitted.

The relative utility of NAAT and IgM assays was highly dependent on the time since the onset of symptoms. The sensitivity of NAAT of plasma collected from patients with WNV infection during the first 8 days of illness was 56.2% (figure 2), compared with 54.1% for IgM assays. After the first week of illness, IgM testing yielded positive results in 98.4% of cases, and WNV RNA was rarely detected (4.3% of cases).

Virus loads were estimated for 92 plasma samples found to contain detectable WNV RNA by RT-PCR. The mean plasma virus load was 7.5 × 10⁴ copies/mL (SD, 2.1 × 10⁴ copies/mL; range, 5.0–1.4 × 10⁵ copies/mL) or 21.0 TCID₅₀/mL (SD, 56.8 TCID₅₀/mL; range, 0.2–372.0 TCID₅₀/mL). No association was noted between plasma virus load and number of days after onset of symptoms, concomitant WNV-specific IgM detection, or clinical presentation (West Nile fever vs. West Nile neurological syndrome).

Discussion. Alberta is located just west of Saskatchewan, the province in Canada that was most severely affected by the WNV epidemic in 2003. On the basis of our case identification and midyear population data (April 2004; available at: http://www.statscan.ca/), the WNV infection rate documented in Alberta in 2003 was ∼9/100,000 population. However, this rate is likely an underestimate of the true prevalence of WNV infection, because of the presence of asymptomatic cases.

Our study demonstrates the improved sensitivity and rapidity of WNV diagnosis that is possible when NAAT is added to serologic testing. The use of this technology as a diagnostic tool in Alberta in 2003 led to reporting of WNV cases that was increased over that obtained by use of serologic testing alone. However, because less than half of our symptomatic population was tested by NAAT, it is probable that cases of WNV infection were missed among those patients tested early by serologic testing alone [9].

Previous studies using culture or NASBA and RT-PCR NAAT methods suggested that plasma viremia was of a very low titer and short duration [4–6, 10] and had usually cleared by the time the patient presented with symptoms. In this report, we document viremia in more than half of our patients presenting during their first week of illness. One possible explanation for this finding is that our modified extraction procedure enabled testing of a larger volume of plasma with a higher concentration at elution than did previous methods (1 mL of plasma eluted into 0.05 mL vs. 0.2 mL eluted into 0.1 mL). The virus loads found in positive plasma specimens were variable, but some were extremely low (0.2 TCID₅₀ or 50 copies/mL of plasma). Estimated virus loads in plasma from our study overlapped with those recently reported for blood donors [2, 11] and those observed in experimental infection of horses [12] and rhesus macaques [13]. In the blood donor population, dilution of the specimen in mini-pools results in false-negative results for ∼33% of viremic donors, because of the low titers of circulating virus [1].

A second possible explanation for the large number of viremic patients is that our population-based study included all patients tested in the province, and most WNV-infected patients had West Nile fever. Previous studies have been restricted to WNV-infected patients with neurological diseases who presented to tertiary care centers, and these patients are more likely to be antibody-positive. Blood donor screening has found that the presence of IgM antibody is associated with lower levels of virus. We found very little overlap between WNV viremia and IgM detection, confirming observations in blood donor populations and primate experimental systems [13]. Of note, NAAT detected WNV-infected patients missed by IgM serologic testing and vice versa, and the 2 technologies complement one another.
missing only 5%–6% of patients when both tests are used together on initial samples submitted.

The high rates of viremia we documented in patients with WNV infection early after the onset of symptoms is consistent with the large number of “asymptomatic” blood donors identified by blood services through their NAAT–based screening programs. It is noteworthy that a significant proportion of blood donors identified as viremic or implicated as sources of transfusion-acquired WNV infection in 2002 and 2003 had either mild or recent symptoms at the time of or immediately after donation [2, 6, 14].

Although WNV-specific IgM tests are now in common use in North America and have approval from the US Food and Drug Administration and from Medical Devices (Health Canada), NAAT of blood is performed on a large scale only for blood donation screening. Because the laboratory infrastructure for NAAT is widely available in provincial and state laboratories, implementation of NAAT for WNV is achievable. Costs could be contained by consolidation at reference laboratories, as in this study, and algorithms targeting early infection could be developed. More rapid and sensitive diagnosis, with reduced need for follow-up convalescent-phase serologic testing, could also yield clinical savings and efficiency.

In summary, because low-level WNV viremia is common in patients early after the onset of symptoms, NAAT of plasma is a useful supplement to serologic testing for diagnosis of WNV infection. Using these combined diagnostic tools on a population basis, we observed that the vast majority (94%) of cases could be detected on the first blood sample.

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References


