A few years ago, most people living in the United States would not have known of the existence of a flavivirus known as West Nile virus (WNV). Indeed, most clinical microbiologists and medical laboratory scientists would only have had a limited knowledge of the virus. All that has changed in the last three to four years.

In 1999, WNV emerged in New York and from there has spread to most of the continental United States, with successive seasons seeing the migration of the infection north, south, and west from the New York epicenter. WNV has a broad host range, including birds (the primary host), humans, and other animals. Indeed, WNV has had a significant health and economic impact on horses and industries dependent upon horses, such as horse racing. That birds are the primary host of WNV is important to the understanding of the importation and spread of the virus. As based on available evidence, the New York outbreak appears to have resulted from the importation of WNV via viremic migratory birds, with an isolate from a dead bird in New York being closely related to a 1998 isolate of WNV from a goose in Israel.

Basic science

West Nile virus is a member of the flaviviridae, which includes agents that cause such important diseases as yellow fever, Japanese encephalitis, dengue fever, St. Louis encephalitis (SLE), and Central European encephalitis. The flaviviruses are some of the medically most important of the arthropod-borne viruses (arboviruses), with the mosquito being the arthropod vector for a number of these viruses, including WNV. Flaviviruses are char-

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**Serological diagnosis of WEST NILE VIRUS**

By Stuart L. Hazell, PhD

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**LEARNING OBJECTIVES**

Upon completion of this article the reader will be able to:

1. Recognize the history, hosts, vector, epidemiology, and probable origin of West Nile virus (WNV) in the United States.
2. Recognize the family of viruses and the nucleic acid for WNV.
3. Recognize the clinical presentations of WNV disease and who is at greatest risk of life-threatening disease.
5. Recognize the tests that are performed for early and late diagnosis and their advantages and disadvantages.
6. Recognize the most specific and most sensitive tests for WNV and how this relates to positive and negative predictive values.
characterized as having a genome consisting of single-stranded RNA enclosed by a nucleocapsid, which, in turn, is enveloped in a lipid membrane.

The spread of the virus may be attributed to the primary reservoir for the virus: birds. Certainly, the introduction of WNV into North America has had an overwhelming impact on selected immunologically naïve native species. Birds are the primary host, with humans and animals (such as horses) being secondary and usually “dead-end” hosts.

Mosquitoes belonging to the *Culex pipiens* complex are the primary vector for WNV. A recent publication in *Science* has suggested differences in the behavior and physiology of members of the *Culex pipiens* complex in the United States may account for the rapid spread of the infection. Briefly, the report suggests that mosquitoes belonging to *Culex pipiens* complex in North America may bridge bird and human hosts, being both “bird-biters” and “human-biters,” whereas European counterparts appear to specialize as either “bird-biters” or “human-biters.”

**Clinical presentation**

In the United States, WNV infection is most commonly seen over the summer months. Cases begin to appear in June, peak in August and September, then disappear in the fall. This pattern of disease relates to climate and the activity of mosquitoes.

Human infection with WNV may be either silent or present as frank disease with a range of signs and symptoms. Symptoms may include a general malaise accompanied by fever and chills, headache, backache, and muscle and joint pain (myalgia and arthralgia). One of the signs that accompanies these symptoms is the occurrence of a maculopapular rash in up to half the patients presenting with symptoms. While such disease may be mild and self-limiting, in a subset of infected patients more severe disease is observed.

Over the course of the spread of WNV across the United States, reported mortality rates have run at up to 10% of the diagnosed population. The mortality rate appeared to decrease in the 2003 season to approximately 3%, with over 9,000 cases reported to the Centers for Disease Control and Prevention (CDC). The apparent reduction in mortality may have been due to any one of three factors. First, the awareness of the infection and associated disease within the medical community may have resulted in earlier intervention in at-risk patients. Second, more asymptomatic individuals may have presented for testing, thus overstating the apparent disease burden. Third, the virus may be becoming less virulent. The first two are possible; the third awaits further data.

Mortality is more commonly associated with the elderly presenting with severe neurological manifestations, including meningitis, encephalitis, and acute flaccid paralysis syndrome. In children, severe manifestations of infection are associated with raised intracranial pressure and seizures. In New York City during August and September of 1999, 59 patients aged from five to 90 years (median age 71 years) were hospitalized with WNV infection. Sixty-three percent presented with clinical signs of encephalitis, 27% with muscle weakness, and 10% with flaccid paralysis. Seven of the patients died. Independent risk factors for severe disease included age greater than 75 years and the presence of diabetes mellitus.

While the mosquito represents the main vector for transmission, other pathways to infection are known to exist. Because of the prevalence of silent infections, there is a risk from contaminated blood supplies or transmission via infected organs in transplant patients.

**Laboratory-based diagnosis**

The diagnosis of WNV infection conforms to standard conventions of diagnostic virology. The “gold standard” for the diagnosis of infectious disease is isolation of the agent of disease. In practice, however, viral isolation has limited utility. The selection of the most appropriate laboratory diagnostic tool(s) should relate to the natural history of infection (Figure 1). In the case of WNV, onset of symptoms is preceded by a period of viremia. This viremia persists for a short period after the onset of symptoms. Thus, if the patient presents early, viral culture — or more appropriately real-time PCR — can be used to detect virus. Viral culture is laborious and relatively expensive, thus real-time PCR offers an effective alternative. Real-time PCR also offers sensitivity and a level of viral quantification.

In the case of blood banking and organ donation, the security of supply correlates to the risk of the presence of infectious units of virus. Real-time PCR offers a sensitive and efficient tool for the assessment of the risk of transmission of WNV to recipients of blood or tissue. For the detection of virus in the blood supply, PCR is usually conducted on pooled samples, facilitating a cost-effective method of screening the blood supply.

**Serology**

When WNV first appeared in the United States in 1999, the CDC responded by developing an IgM capture enzyme-linked immunosorbent assay (MAC ELISA) and conducting plaque reduction neutralization tests (PRNT) as a confirmatory assay on samples positive by the MAC ELISA.


**Table 1: Summary of the utility and application of common diagnostic assays for the detection of WNV infection.**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Serology</th>
<th>Viral culture / PCR</th>
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<tbody>
<tr>
<td>Serum</td>
<td>IgM antibody</td>
<td>Viral culture</td>
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<td></td>
<td>IgM is an indicator of acute primary infection.</td>
<td>Viral culture is of limited utility in the detection of virus from serum samples. Even in acute infection the capacity to isolate virus is limited, with few samples yielding a positive result.</td>
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<td></td>
<td>Current FDA-cleared assays for WNV are IgM capture ELISAs, giving enhanced sensitivity. There is evidence that in WNV infection, IgM may persist and could provide misleading clinical laboratory data.</td>
<td>Real-time PCR: Like viral culture, PCR is of limited utility in the detection of virus from serum samples. In acute infection, the capacity to detect virus is limited, with as few as 1/10 samples yielding a positive result.</td>
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<tr>
<td></td>
<td>IgG antibody</td>
<td></td>
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<tr>
<td></td>
<td>IgG has limited utility in the detection of acute WNV infection. IgG can be detected approximately six to eight days following the onset of symptoms. Paired sera may provide a retrospective diagnosis.</td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>IgM antibody</td>
<td>Viral culture</td>
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<tr>
<td></td>
<td>Detection of IgM in CSF is an indicator of acute primary infection. It should be noted that only a limited dilution of CSF (~1/5) should be used for testing with a capture IgM assay. No commercial assays have yet been cleared by the FDA for use with CSF.</td>
<td>Viral culture is of some utility in the detection of virus from acute CSF samples. Real-time PCR: Real-time PCR is of utility in the detection of virus from CSF samples. In acute infection, up to 60% of samples may yield a positive result.</td>
</tr>
<tr>
<td>Tissue</td>
<td>Not applicable. Sera from donor may be tested, but is of limited utility if the presence of virus is the primary concern.</td>
<td>Real-time PCR: Real-time PCR is of utility in the detection of virus from tissue samples. The primary objective is the assessment of the risk of transmission of infection to a transplant patient. Also of utility when examining tissues from persons who have died of suspected WNV infection. Viral culture: Viral culture is of some utility in the detection of virus from tissue. PCR is the method of choice.</td>
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**PRNT**

While being a valuable tool in the diagnosis of WNV infection and being the most specific of any of the flavivirus serological assays, PRNT exhibits a number of limitations, which restrict the test's capacity to be exploited for high-volume measurement of antibody against WNV. First, PRNT does not differentiate between IgM and IgG; thus, without prior measurement of both IgM and IgG by another method, it is difficult to determine if the assay is detecting current or past infection. PRNT also requires cell culture, viable (infectious) virus, and careful titration of the test virus.

**CDC MAC ELISA**

The CDC developed the MAC ELISA to facilitate the diagnosis of acute infection. To support wider testing, the CDC provided antigen to state health laboratories for in-house testing in their own laboratories. Key points about the CDC MAC ELISA were that the assay was sensitive (>95%), but suffered from a level of cross-reactive with other flaviviruses. The positive-to-negative ratio of a sample tested against both WNV and SLE was required to differentiate these infections. The MAC ELISA was also time-consuming, requiring up to two days to perform the assay. The MAC ELISA became part of the standard, however, required by the FDA to gain clearance for commercial IgM assays.

**Development of 2003 commercial IgM assay**

In 2002, in response to the FDA call for companies that develop diagnostic tests to make available an FDA-cleared commercial assay for the 2003 season, our company had an FDA-cleared IgM ELISA on the market in June 2003, one day prior to the first case of the season.

The PanBio WNV IgM ELISA assay was cleared for the testing of sera from patients exhibiting neurological manifestations of WNV infection, and was also used in a number of laboratories during the 2003 WNV season for testing all samples submitted for evaluation of exposure to WNV.

In clinical trials conducted for the FDA, this WNV IgM ELISA exhibited an overall sensitivity of 98.3% and a specificity of 93.1% (cumulative data from three test sites). From a clinical laboratory standpoint, sensitivity and specificity are important performance characteristics. These two measures are intrinsic to the assay and allow assays to be broadly compared one to another. In an ideal setting, assays would all have a sensitivity and specificity of 100%. Practically, this is difficult to achieve. Yet, in a clinical application, it is not sensitivity and specificity per se that provide the best understanding of the use and interpretation of the results of any assay, but the positive and negative predictive values (PPV and NPV) of an assay. These latter two measures depend upon both the intrinsic performance of the assay (sensitivity and specificity) and also on the prevalence of infection in the population being tested.

Why is this important? It is important, as failure to understand the performance of an assay in a specific clinical setting may lead to erroneous findings or the misapplication of a test. In the case of our assay used according to the label in a patient population where 25% of those presenting have symptoms consistent with serious WNV infection were infected, the PPV would be of the order of 82.6% and the NPV would be of the order of 99.4%. Thus, the laboratory and clinician would have a high degree of certainty that a negative result was truly negative and reasonable certainty that a positive result was truly positive.

*Continues on page 16*
Now, applying the same assay off label in a population having a low prevalence of infection, such as the general population during the early stage of the WNV season, we would see a dramatic reduction in the PPV of the same assay (12.6%) with a good NPV of 99.98%. The NPV and PPV in this situation indicate that we can have a high level of confidence in a negative finding, yet need to be cautious in attributing a positive result to a sample giving a positive reading. Informed practitioners, the manufacturer, and the FDA understand this relationship between NPV, PPV, and disease prevalence. Consequently, the assay specifications indicate that positive results should be confirmed by PRNT or other appropriate secondary assessment.

We have developed a next-generation WNV IgM assay, which has been submitted to the FDA. This assay has improved sensitivity and specificity. It should be noted that an assay having 99% sensitivity and specificity is still subject to the dictates of the relationship between disease prevalence and PPV and NPV. Thus, in a 25%-prevalence environment, an assay that is 99% sensitivity and specificity would have a PPV of 97% and a NPV of 99.7%. In a 1%-prevalence environment, the same test would have a PPV of 50% and an NPV of 99.9%.

Antibody persistence

An issue with the diagnosis of acute infection with WNV is the reported persistence of IgM over months. In most cases, this is not a concern. That is, if a person presents with signs and symptoms consistent with WNV infection and the laboratory reports a positive IgM titer specific for WNV, then the conclusion may be drawn that the person has an acute case of WNV infection. This situation can be difficult when a person has had a silent WNV infection and later presents with symptoms consistent with fever of unknown origin. If this patient tests positive for WNV, then there is a risk of the true origin of the symptoms being missed with concomitant consequences.

Effectively differentiating acute infection from persistent antibody requires testing of paired serum samples, which are not always available, and the demonstration of a significant rise in antibody titer.

Conclusion

WNV has had a marked impact on the people of the United States over the last four years. The available evidence suggests that the virus may continue to circulate in the population for some time and represents an ongoing health hazard of concern to the public. Serological testing provides important data that can be used to assist clinicians in the treatment and management of infected individuals. New versions of WNV assays are being developed and released for clinical laboratory use. Even tests with high sensitivity and specificity, however, cannot overcome the impact of low prevalence on disease in test populations. It is important for health professionals to understand the strengths and limitations of the assays they use to allow for appropriate interpretation of test data.

References


Clarification:

Several readers of our May 2004 cover story, “Saving laboratory records: what, how, how long?” [p.10] have brought to our attention errors in Table 2. Drs. Dan Baer and Robert Nakamura based the CAP recommendations on an older publication used in their laboratories and failed to make changes reflected in the 2001 revision of CAP’s recommendations currently on its website, www.cap.org/apps/docs/laboratory_accreditation/retention_1101.pdf. We regret these errors.

Keep in mind, however, that CAP’s recommendations exceed CLIA requirements, are only recommendations, and would not lead to a deficiency in inspection by CAP if CLIA requirements were followed.

The following updates should be made: (1) Blood bank records, donor and recipient records, records of employee signatures, initials, and identification should be retained for 10 years rather than “forever,” as published in the article, and (2) specimen paraffin blocks should be retained 10 years instead of “five years,” as published in our article.