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West Nile Disease in New Mexico: The Quest for Nucleic Acid

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ABSTRACT

West Nile virus (WNV) was first detected in New Mexico in 2002, with the first human cases appearing in 2003. Since that time it has become endemic in the region, and as of year-end 2005, 330 New Mexicans had been diagnosed with West Nile Fever or the more severe neuroinvasive disease as reported by the New Mexico Department of Health. An ongoing study at the University of New Mexico has collected interview and physical exam data for these individuals as well as collecting cerebrospinal fluid (CSF) and serum from their period of acute and convalescent illness. While all of these samples have been tested to determine WNV IgM seropositivity, none of them have been characterized by the use of nucleic acid amplification test (NAT). The purpose of this study is to characterize this sample set using Real-Time reverse transcriptase polymerase chain-reaction (RT-PCR), an extremely sensitive NAT. The serum and CSF archived collection at UNM represents one of the most comprehensive and best-characterized sample sets available in the United States. A total of 115 samples, 111 serum and 4 CSF, were analyzed. None of the 115 samples had detectable West Nile nucleic acid.

INTRODUCTION

West Nile virus (WNV) is a member of the genus *Flavivirus*. Significant *Flavivirus* species include yellow fever, dengue, and a complex of encephalitis viruses among which West Nile is included. WNV is an enveloped single-stranded positive-sense RNA virus. The genome is approximately 11,000 nucleotides and contains a 5' non-coding region, followed by capsid, envelope, and membrane protein coding regions, and seven non-structural protein coding regions (Campbell, et al). Differences in WNV pathogenicity may be attributable to nucleotide mutations in the membrane, envelope, or non-structural protein coding regions of the WNV genome (Beasley, et al).

British virologists first characterized WNV from the blood of a febrile woman in the West Nile region of Uganda in 1937 (Smithburn, et al). The same virus was later isolated from 3 children experiencing a mild febrile disease, thus WNV was established as a human pathogen (Melnick, et al). WNV was found to cycle between *Culicine* mosquitoes and native birds, with birds being the natural amplifying host and humans secondary hosts. For decades WNV was felt to produce only a fairly mild dengue-like

human illness with fever, malaise, lymphadenopathy and rash. A 1951 Israeli outbreak of WNV typified these characterizations of WNV as a self-limited mild febrile illness without sequelae (Goldblum, et al). The cases of encephalitis and death which now appear in cases of human WNV infection were rarely seen until the mid 1990s when an apparent change in virulence occurred demonstrated by outbreaks of severe encephalitis in Romania, Russia, Israel, and Algeria. Up to 60% of the patients in these outbreaks presented with neuroinvasive disease. The mortality rate was 4-7% resulting from neurologic damage.

WNV was first detected in the Americas in New York City in 1999. Genetic sequencing showed that the virus showed almost total homology with an Israeli strain of the virus (Lanciotti, Roehrig, et al). It is presumed the virus came from an infected bird illegally transported into the United States. Since that date over 16,000 cases of WNV and 660 deaths due to WNV have been reported in the United States as of 2004. The CDC reported that neuroinvasive disease was present in over 30% of the reported cases since that date. WNV has spread from coast to coast since the New York outbreak, and human cases have now been reported across the United States and Canada. The WNV epidemic has become the largest outbreak of human arboviral encephalitis in North America (Soloman, et al). The state of New Mexico experienced an initial outbreak of 209 reported cases of WNV in 2003 and the disease has been endemic since that time— with 88 cases confirmed by the New Mexico Department of Health in 2004 and 33 cases in 2005.

The clinical picture of WNV infection in the United States today shows that approximately 80% of infected individuals experience asymptomatic infection with WNV (Mostashari, et al). The majority of symptomatic individuals experience a self-limited illness characterized by acute fever, malaise, headache, fatigue, muscle pain, and weakness. These symptoms known as ‘West Nile fever,’ are severe, but self limited. The disease was originally felt to last less than one week, however new data, including the comprehensive New Mexico study, clearly indicate patients may experience symptoms such as fever for several weeks, and fatigue for up to one year post-infection. In less than 1% of individuals, neuroinvasive disease occurs leading to meningitis, encephalitis, and myelitis as possible outcomes (Mostashari, et al). Patients diagnosed with neuroinvasive

disease often present with focal neurologic deficits for months to years (Sejvar, et al). Neuroinvasive WNV is also associated with a 9% mortality rate (O'Leary, et al).

West Nile diagnostics have focused on serological tests of WNV-specific IgM correlated with a clinical history. For most patients, WNV IgM antibody persists in serum for 3-6 months and is replaced by WNV IgG antibody, which is very long lasting. As a consequence of the short duration of WNV IgM antibody, its presence in serum or CSF has been used to establish a diagnosis of an acute or recent infection. Because of long-term IgM persistence in WNV infected humans, IgM alone cannot be used as a marker of acute disease. IgM is present in 36% of patients 12 months after clinical disease (Roehrig, et al). Diagnosis can also be made by detection of virus in CSF, serum, or tissue using nucleic acid amplification tests (NAT). The most effective of these requires inoculation of cell culture or mice and subsequent reverse transcriptase amplification of the virus from these tissues (Hayes et al). Direct amplification of viral RNA from patient CSF and serum has proven to be less reliable. Real-Time reverse transcriptase PCR (RT-PCR) appears to be the best NAT for direct amplification of viral RNA showing, in one trial, detection of WNV at quantities less than 1 plaque-forming unit (PFU) (Lanciotti, et al).

Dr. Diane Goade's lab at the University of New Mexico currently houses the largest collection of acute West Nile CSF, blood and serum in the United States. Our study will consist of screening these acute samples with RT-PCR. All of the samples have been previously assessed by serologic methods, confirming West Nile IgM antibody. With a sample size of 77 acute specimens and 38 convalescent specimens, this will be among the largest Real-Time PCR surveys of acute West Nile CSF and serum done to date. While previous data suggests that finding viral nucleic acid in human specimens is unlikely, this is the most sensitive test for such analysis. We feel pursuit of this screening is important to help us understand if the long-term elevation of IgM and IgG seen in many of these samples is related to persistent or relapsing viremia.

METHODS

Sample Acquisition:

Samples have been collected as a part of an ongoing longitudinal WNV study underway at the University of New Mexico. Specimens were collected between 2003 and 2007.

Samples from both the acute phase (day 1-30) and convalescent (day 31 and up) of patient illness were available for analysis. Patient samples have been approved under HRRC Approval form 03-445. The HRRC has approved the samples to be used for nucleic acid analysis.

RNA extracted from serums of WNV-negative volunteers was used for negative controls. These samples were obtained with volunteer consent under HRRC Approval.

Sample Processing:

The specimens were processed and stored under pre-PCR conditions in a dedicated -80°C freezer. WNV infection was confirmed on all samples by identification of WNV-specific IgM and IgG using ELISA (Focus Technologies). RNA was isolated directly from patient CSF and serum using the QIAamp viral RNA mini-kit (Qiagen). Nucleic acid yield from the isolation was verified on all samples using the NanoDrop 1000 UV/Vis Spectrophotometer (Thermo Scientific).

Real-Time Quantitative PCR assay:

The Real-Time Quantitative PCR (RT-PCR) assay was performed using the ABI Prism 7000 (PE Applied Biosystems). Primers and probes recognizing WNV-specific RNA sequences were used as described by Lanciotti, et al. Samples were amplified using TaqMan Gold RT-PCR reagents (Applied Biosystems) per the manufacturer's specifications. All samples were run in duplicate.

Controls:

The positive control RNA was provided by Dr. Greg Ebel. Stock virus in the positive control had 4.0×10^8 PFU per 0.1mL. A standard curve was produced from serial tenfold dilutions of RNA extracted from this stock (*Figure 1*).

Negative control RNA was extracted from serums of WNV-negative volunteers.

RESULTS

A total of 115 acute and convalescent samples were analyzed. Samples defined as 'acute' were obtained between day #1 and day #30 of the patients' illness, day #1 being the

earliest blood sample taken on a patient's day of presentation. This designation is consistent with clinical determinants of acute disease—where manifestations of measurable acute disease are present until day #30. We analyzed 77 acute patient samples (4 CSF and 73 serum samples) and 38 convalescent (all serum) patient samples.

None of the samples, acute or convalescent, were positive upon analysis with RT-PCR. All samples were run in duplicate to assure accurate interpretation. Amplification results from a sample set of acute samples are shown in *Figure 1*. This figure demonstrates successful amplification of the positive control dilutions (in duplicate) at 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 PFU equivalents, respectively, from left to right. The positive controls are the only samples in *Figure 1* that pass the green threshold line. In a positive RT-PCR reaction a sample will pass the green threshold line demonstrating presence of West Nile virus nucleic acid. The patient samples in this figure are each a different color and present as either jagged lines (negative samples) or as lines with a gradual slope that do not reach the threshold (negative samples with fluorescence artifact).

Figure 2 shows a Standard Curve based on the samples shown in *Figure 1*, with an R-value of 0.998079. The data retrieved from assays of the other samples, both acute and convalescent, were similar to these findings.

DISCUSSION

Our analysis showed a total of 115 samples, both acute and convalescent, serum and CSF, negative upon attempted amplification with RT-PCR. All of these samples had been previously tested with ELISA. All samples acute and convalescent, had IgM response and many had IgG as well. Because of this, there was reason to believe finding virus in any of these samples would be rare. However, we pursued this research to help us understand if the long-term elevation of IgM and IgG seen in many of these samples was related to persistent or relapsing viremia. Because our sample size was significantly larger than similar studies, we felt that we would have additional power to observe such a phenomenon should it exist. Whether such a phenomenon would be observable or not remains in question. Because we were not able to detect WNV nucleic acid in even our

most acute samples, casts doubt that any reappearance of virus in a convalescent sample would be observable with this technique.

We feel that the assay was robust and performed properly. It should be noted that our positive controls were positive and negative controls negative consistently as was expected. We diluted our positive control to 1×10^1 with successful amplification showing our ability to amplify WNV from low viral load samples. It has been reported that a minimum of 40 viral copies/mL are necessary for Real-Time PCR amplification (Lanciotti). We also 'spiked' negative control RNA with dilute positive control RNA to assure that the amount of non-viral RNA in a sample did not inhibit the reaction in these 'spiked' samples. Thus, we believe that if there was detectable WNV nucleic acid in the samples, our assay would have been able to amplify it. In addition, sample processing and storage was performed according to commonly accepted protocol.

Table 1 shows confidence intervals for both our acute and convalescent samples. For the acute specimens (N=77) we would not expect more than 4.68% difference in results if repeated (CI=0-4.68%). For the convalescent specimens (N=38) we would not expect more than 9.25% difference in results if repeated (CI=0-9.25%).

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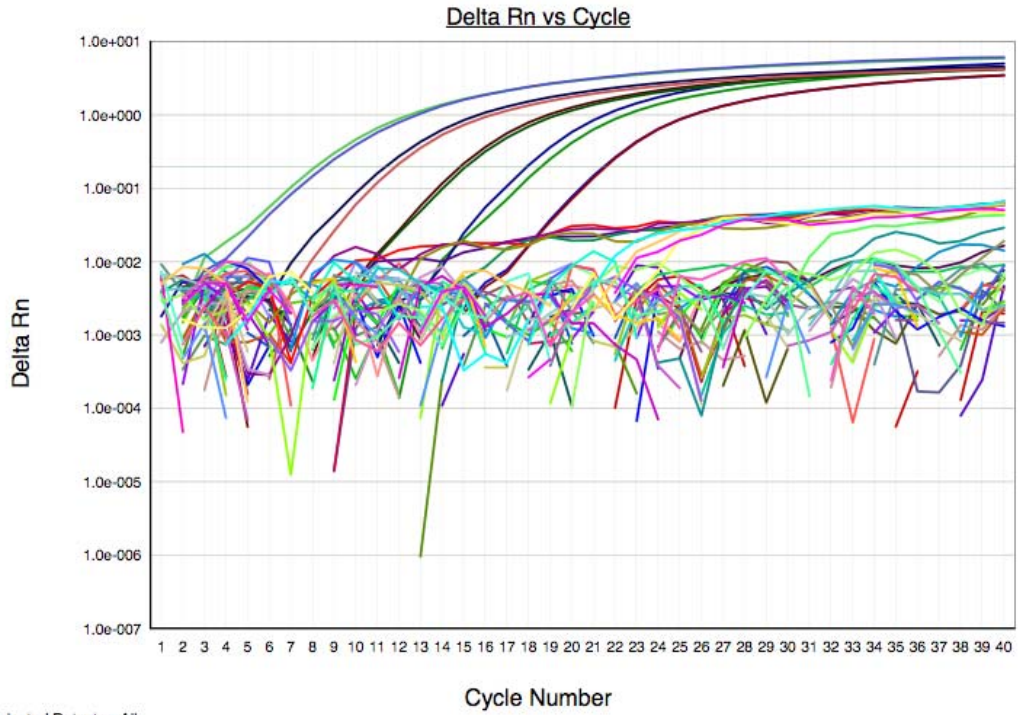
TABLES

Table 1. Confidence Intervals based on Number of Samples

	# Samples Tested	% Positive (Confidence Interval)
Acute Samples	77	0 (0-4.68)
Convalescent Samples	38	0 (0-9.25)

FIGURES

Figure 1. RT-PCR Sample Fluorescence by Cycle



Selected Detector: All
Well(s): A1-H3,H6-H12
Document: WNVnewACUTEplate (Absolute Quantification)

Figure 2. RT-PCR Standard Curve for Samples in Figure 1.

