

Persistent Infection with West Nile Virus Years after Initial Infection

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(See the editorial commentary by Gould, on page 1.)

West Nile virus (WNV) RNA was demonstrated in 5 (20%) of 25 urine samples collected from convalescent patients 573–2452 days (1.6–6.7 years) after WNV infection. Four of the 5 amplicons sequenced showed >99% homology to the WNV NY99 strain. These findings show that individuals with chronic symptoms after WNV infection may have persistent renal infection over several years.

West Nile virus (WNV) is an important flavivirus in North America [1]. Since WNV was first detected in the United States in 1999 [2], ~25,000 human clinically evident infections have been reported, with >1000 deaths [3, 4]. Less than 1% of WNV-infected people develop acute neuroinvasive disease (including meningitis, encephalitis, and flaccid paralysis) or death [5].

Evidence of persistent WNV infection has been demonstrated in experimentally infected monkeys and hamsters with virus or viral RNA found in brain tissue [6, 7]. In addition, hamsters experimentally infected with WNV developed chronic renal infection and shed virus in the urine for up to 8 months [8, 9]. Serial urine specimens from these animals contained 10²–10⁴ plaque-forming units of infectious virus per milliliter. Immunohistochemical staining of tissues showed no evidence of

WNV antigen in brain, liver, spleen, lungs, or bladder, but kidney tissue showed moderate to strong antigen staining. Infectious virus was recovered by cocultivation of trypsinized fresh kidney cells on Vero cells [9]. These experimental data raise the possibility that persistent renal infection may occur in humans. To date, however, WNV has been demonstrated by reverse-transcription polymerase chain reaction (RT-PCR) only in the urine of a patient with acute WNV infection [10].

When WNV reached Houston in 2002, we established a longitudinal study to follow hospitalized WNV-infected patients to determine risk factors for encephalitis and to understand long-term clinical sequelae. Methods for WNV confirmation and study inclusion criteria have been described elsewhere [11]. Written informed consent was obtained upon recruitment. This cohort now numbers 112 patients who are evaluated every 6 months, at which time blood is collected and a questionnaire ascertaining subjective and objective measurements of clinical sequelae is completed. At 1 year after infection, ~60% of patients remain symptomatic, particularly those who were encephalitic. Resolution of symptoms plateaued ~2 years after infection, and after 5 years 60% of patients who presented with encephalitis continued to report clinical symptoms. Chronic symptoms were significantly associated with the persistence of detectable anti-WNV serum immunoglobulin M ($P = .026$) and also with a history of hypertension. Cytokine studies showed that many of the chronically symptomatic patients also had significantly elevated plasma levels of interferon γ -inducing protein, a marker of active viral infection (K.M., unpublished data). There was also evidence of suppression of the type 2 T helper pathway, which might be an indicator of immunosilencing processes facilitating viral persistence. Finally, the deaths of 5 participants were attributed to chronic renal failure. Collectively, these observations led us to hypothesize that some members of the cohort were persistently infected with WNV and that the kidney could be a preferred site of continued replication and source of shedding. Accordingly, we developed protocols for the collection and processing of urine for the detection of viral nucleic acid by RT-PCR.

Fresh urine was collected from a group of cohort participants into RNase-free tubes that contained 3.3 U/ μ L of Protector RNase inhibitor (Roche Diagnostics). An aliquot was immediately extracted, and RNA was isolated from convalescent urine according to the manufacturer's protocol for the Qiagen MinElute Virus Spin kit (Qiagen). The urine was not concentrated or pretreated before testing. In addition to standard positive, negative, and reagent controls, we included urine from

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known WNV antibody-negative healthy volunteers to exclude the possibility that amplicon or crossover contamination occurred during the extraction procedure. Extraction procedures, reactions, and electrophoresis were performed in separate laboratories for the same reason.

Oligonucleotides used for RT-PCR have been described elsewhere [12–14]. First-stage primers were 1401 (5'-ACCAACTA-CTGTGGAGTC-3') and 1845 (5'-TTCCATCTTCACTCTACT-3'), which amplify a 445-bp region of the WNV envelope protein [14]. The amplification reaction was performed according to manufacturer's protocol for the One-Step RT-PCR kit (Qiagen). An aliquot from the first round of PCR was used for the nested PCR with the Taq PCR Core kit. Nested primers were 1485 (5'-GCCTTCATACACACTAAAG-3') and 1732 (5'-CCAATGCTATCACAGACT-3'), which amplify a 248-bp region [14]. For both the first-round and nested PCR, 10 µL of each reaction was resolved on an agarose gel (1% wt/vol), stained with ethidium bromide, and visualized with the Gel Bio-Doc-It system for the presence of amplicons. PCR cleanup (Qiagen Quickspin) was performed on the first round of PCR product for those specimens that had a positive band detected, and the amplicons were sent to Lone Star Laboratories (Houston) for nucleic acid sequencing.

We tested urine specimens from 25 convalescent patients. RT-PCR results were positive in both the first round and nested reactions for 5 (20%) of 25 urine specimens collected between 573 and 2452 days after the onset of acute clinical disease (Table 1). Four of the 5 amplicons from the PCR products from the primary RT-PCR could be sequenced and were found to be >99% homologous to the WNV NY99 strain.

Of the 5 positive patients, 4 reported chronic symptoms, including weakness, fatigue, memory loss, and ataxia. All had a clinical presentation of encephalitis, were male, and had a history of hypertension. One patient developed kidney failure following his illness. Three patients were >6 years past their initial infection.

Although we suspected viral persistence in some patients with

chronic symptoms, finding 1 in 5 urine specimens to be positive was unanticipated. For additional assurance, we repeated the entire RT-PCR procedure in a different location to further exclude the possibility of amplicon contamination. Urine specimens that had been flash-frozen in dry ice and ethanol at the time of collection were sent to the University of Texas Medical Branch in Galveston. RNA was extracted using the QIAamp Viral RNA Mini kit (Qiagen) and then screened by nonnested RT-PCR using oligonucleotide primers targeting a different region of the WNV E gene. Nucleotide sequencing of amplified products confirmed the presence of WNV RNA in samples from 2 of the patients (patients 2002–42 and 2002–43; GenBank accession numbers GQ495619 and GQ495620, respectively). We are attempting to isolate virus from urine but to date have not succeeded. We expected to have difficulty obtaining an isolate because the previous study that identified WNV RNA in urine from an acutely infected human patient was unable to do so [10]. We have also found that the viral RNA degrades quickly in urine, with RT-PCR results becoming negative after 2 or more freeze/thaw cycles. This is a concern; therefore, we are working toward optimizing the testing of urine for the presence of viral RNA.

We report here for the first time, to our knowledge, that WNV is capable of long-term persistence in patients, particularly in the presence of chronic clinical symptoms. The finding of viral RNA in the urine of these patients is suggestive of ongoing viral replication in renal tissue, which is consistent with the hamster model. The public health impact of these findings is considerable. It will be important to explore and understand the underlying mechanisms related to the shedding of viral RNA in the urine, whether shedding is constant or intermittent, and whether this represents true infection resulting in clinically evident disease. Additionally, all 5 of our positive individuals were older men, and we are unsure at this point whether this is a significant finding or simply related to chance. We are currently establishing means to clinically evaluate all cohort participants, particularly with regard to renal

Table 1. Description of the Subset of West Nile Virus (WNV) Cohort Participants Whose Urine Tested Positive by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) for WNV Nucleic Acid

Patient	Clinical presentation of acute WNV	Sex	Age at onset, years	Days (years) between onset and urine collection	Hypertension ^a	Chronic renal disease	Chronic symptoms	Detectable IgM	
								At time of urine collection	At 1 year after onset
2002-015	Encephalitis	Male	45	2422 (6.6)	Yes	No	Yes	No	No
2002-042	Encephalitis	Male	74	2442 (6.7)	Yes	Yes	Yes	No	Yes
2002-043	Encephalitis	Male	74	2452 (6.7)	Yes	No	Yes	No	Yes
2004-016	Encephalitis	Male	78	1581 (4.3)	Yes	No	No	No	No
2007-003	Encephalitis	Male	68	573 (1.6)	Yes	No	Yes	No	No

NOTE. IgM, immunoglobulin M.

^a Patient had a history of hypertension prior to WNV infection.

function and other abnormalities possibly related to persistent infection, with a focus on developing treatment options.

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