Comparison of Extraction Volumes for the Detection of West Nile Virus (WNV) by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

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Abstract

West Nile Virus (WNV) is an arbovirus of public health concern in Minnesota since it was first identified in 1999. The Minnesota Department of Health (MDH) primarily uses serological testing for detection of the virus, which has limited specificity due to IgM cross-reactivity with other arboviruses. Currently under investigation at MDH is an alternative platform using RNA extraction followed by reverse transcription-polymerase chain reaction (RT-PCR) to detect WNV in urine specimens. This project seeks to establish whether increasing the sample volume for RNA extraction prior to RT-PCR will improve the sensitivity of the testing. To address this question, thirty known WNV-negative urine specimens archived from 2016 at MDH were thawed, and two extraction volumes (200 uL and 1 mL) were performed to compare RNA yield from each. The sensitivity of WNV targets by RT-PCR for the larger volume extraction was evaluated by comparing cycle threshold values (Ct values) from samples inoculated with WNV for both volumes. RNA yield was increased overall using the large volume compared to the smaller volume extraction. However, Ct values were not statistically decreased, suggesting a need for further testing. These results indicate that though RNA yield is improved, it does not improve the sensitivity of WNV detection for RT-PCR. Regardless, these findings could prove to be a useful starting point for alternative testing methods that could help to reduce uncertainty and timely confirmatory testing.

Intro

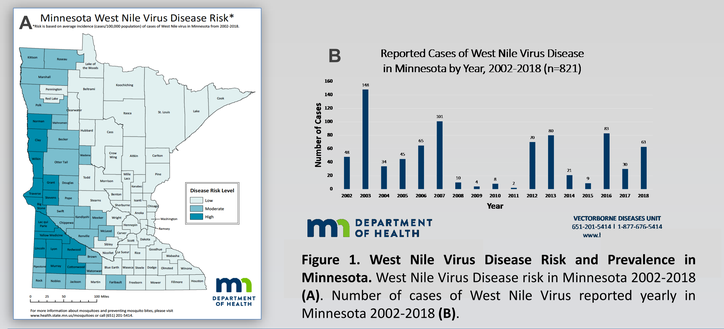

Figure 1. West Nile Virus Disease Risk and Prevalence in Minnesota. West Nile Virus Disease risk in Minnesota 2002-2018 **(A)**. Number of cases of West Nile Virus reported yearly in Minnesota 2002-2018 **(B)**.

West Nile Virus (WNV) is the most common arboviral illness reported in the United States and is associated with mosquito populations in Western Minnesota **(Figure 1A)**. Since it was first identified in the US in 1999, WNV has infected thousands of individuals, with mortality as high as 7% (Gorchakov et al., 2019). Most cases of WNV are not associated with any clinical manifestations, though a subset of patients present with febrile symptoms comparable to seasonal flu. An even smaller proportion of patients (<1%) develop severe neuroinvasive disease, which can present as encephalitis, acute flaccid paralysis, and meningitis (Landry et al., 2019).

The Minnesota Department of Health (MDH) is currently evaluating the utility of performing Reverse Transcription PCR (RT-PCR) on clinical urine specimens as an improved protocol for detection of WNV. MDH currently uses an extraction volume of 200 uL, which is subsequently analyzed using RT-PCR.

This study attempts to demonstrate whether increasing the urine volume for extraction to 1 mL will reduce the cycle threshold (Ct) value and therefore increase the sensitivity of the WNV RT-PCR. The first level of testing performed seeks to compare RNA yield between the two extraction volumes in archived WNV-negative urine samples in order to determine the effectiveness of the increased volume. The second layer of testing compares the Ct values from RT-PCR performed on a WNV-inoculated set of samples in order to establish whether the larger volume increases the sensitivity for WNV targets. Comparison of the two extraction volumes will help optimize existing MDH protocol for WNV detection in urine and determine the necessity of implementing a larger volume extraction technique

Materials & Methods

Overview:
- Nucleic acid extraction was performed on 30 frozen urine specimens archived from 2016 at Minnesota Department of Health (MDH). Extracts were amplified using reverse-transcription polymerase chain reaction (RT-PCR).
- Two extraction volumes were used: 200 uL and 1 mL.

RNA Extraction and Quantitation:
- Extractions were performed using the MagnaPure LC automated instrument and glass bead methodology.
- RNA from both extraction volumes quantified and compared

Inoculation by WNV Isolate & Mosquito Pools
- Seven additional urine specimens were inoculated with either 1:1000 WNV isolate or 1:1 positive mosquito pool.
- Regular and large volume extractions were performed on all inoculated specimens using same methodology as previously described

RT-PCR for Sensitivity Analysis of WNV Targets:
- RNA target amplification of both extraction volumes performed by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and analysis with TaqMan technology (qPCR).
- Two well-characterized West Nile Virus targets (3' and ENV') used for analysis of samples inoculated with WNV isolate & mosquito pools
- Cycle threshold (Ct) values obtained were used to compare sensitivities between regular and large volume extracts.

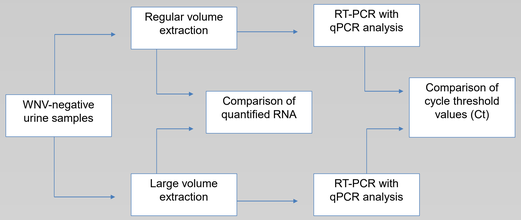

Figure 2. First Tier Testing Schematic. Flowchart depicting chronology of testing for comparison of quantitated RNA for both regular and large volume extraction kits.

Discussion

Increasing the extraction volume size did not result in a notable difference in the detection of the WNV **(Figure 4)**. It does not appear that the addition of a large volume extraction to the methods currently in place at the Minnesota Department of Health (MDH) will directly contribute to an overall improvement in the detection of West Nile Virus (WNV). It was important to first consider three factors regarding implementation of this method before arriving at this conclusion: cost, additional time spent testing, and overall value added to result interpretation.

The large volume extraction was performed using the same extraction platform and reagents previously indicated for use at MDH. Additional cost of this method would be due to the purchase of the large volume kit, which is necessary in order to use a sample size of 1 mL. Because the large volume kit requires automated extraction exclusive from the regular volume extraction, implementing this testing would require an additional 2-3 hours to the existing workflow. Extra time spent by laboratory personnel would not significantly alter the existing RT-PCR workflow as the samples would be run simultaneously on a single 96-well plate. Lastly, it does not appear as though performing the large volume extraction would strengthen results interpretation, since the sensitivity of the molecular methods used was not improved.

The amount of RNA in the large volume extracts was consistently doubled in comparison to the small extracts, which correlates with the five-fold increase in volume **(Figure 3,4)**. The basis of this study relied upon the proposition that a larger volume of urine would correspond with a higher yield of RNA in the extract. The lack of a statistically significant reduction in Ct values overall suggests that there may be an inhibitory component presented by using a larger urine volume. The higher concentration of RNA in the larger volume extraction may interfere with the WNV RT-PCR target amplification.

Results

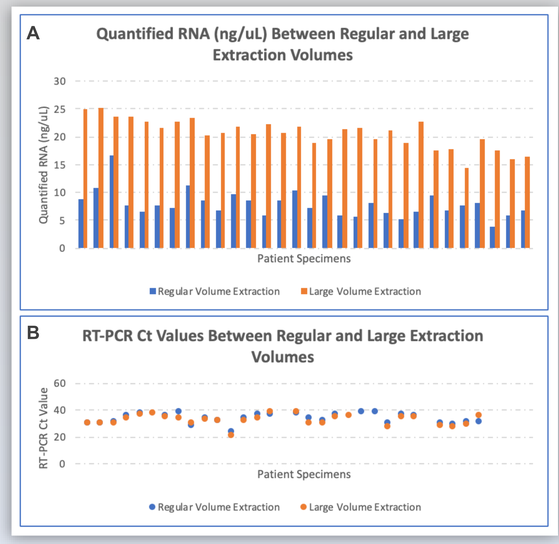

Figure 3. Comparison of Regular and Large Extraction Methods by RNA Quantification and RT-PCR. The RNA yield of two extraction volumes (200 uL and 1 mL) from clinical urine specimens were compared **(A)**. Reverse-transcription PCR (RT-PCR) was performed on each extract using Rnase P as a target. Threshold cycle values (Ct values) obtained from RT-PCR are compared for both extraction volumes **(B)**.

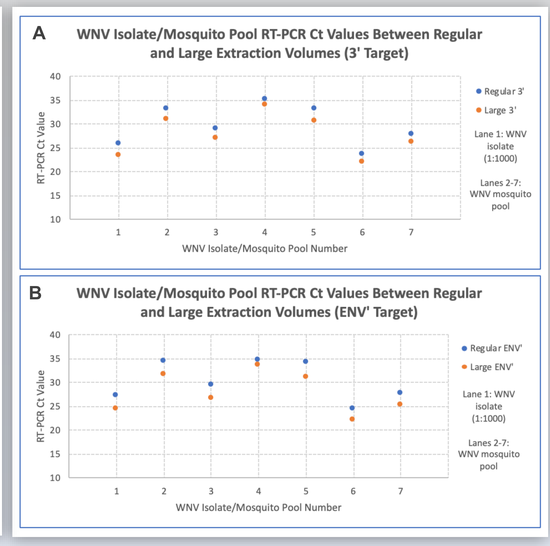

Figure 4. Comparison of WNV RT-PCR target Ct values between regular and large extraction volumes. Reverse Transcription PCR (RT-PCR) was performed on extracts from WNV-inoculated samples (lane 1 is 1:1000 WNV isolate, lanes 2-7 are 1:1 mosquito pool). Threshold cycle values (Ct values) were obtained for the 3' WNV target **(A)** and ENV' WNV target **(B)** for each sample.

Future Testing

It does not appear that this study can independently improve WNV detection. However, it provides a Segway into other potential avenues of future testing that may ultimately improve the sensitivity of WNV detection. Repeat testing with a more sensitive amplification method could prove useful considering the findings of this study. For instance, RT-PCR has a lower limit of detection of approximately 200 copies/mL, while transcription-mediated amplification (TMA) can detect as few as 9 copies/mL (Baty et al., 2012). Given these parameters, it may be theorized that employing TMA for molecular testing of WNV could help improve the sensitivity of the current standard of testing at MDH.

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