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Use of an Internal Positive Control in a Multiplex Reverse Transcription-PCR To Detect West Nile Virus RNA in Mosquito Pools

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We report on the use of West Nile virus Armored RNA as an internal positive control (IPC) for the extraction and reverse transcription-PCR (RT-PCR) of RNA extracted from field-collected mosquitoes and on a multiplex real-time Taqman RT-PCR to simultaneously detect the 3′ noncoding region of West Nile virus and the West Nile virus NS5-2 region comprising the IPC. Mosquito pools from the province of British Columbia, Canada ($n = 635$), were tested in duplicate and found to be negative for West Nile virus and positive for the IPC. Known West Nile virus-positive supernatants from mosquito pools from the provinces of Alberta and Manitoba were tested in duplicate and found to be positive for both regions of the West Nile virus genome. The mean cycle threshold (Ct) value for the IPC in batch extraction controls ± 2 standard deviations was found to be 36.43 ± 1.78 cycles. IPCs of 98.4% (624) of West Nile virus-negative pools fell within this range, indicating the reproducibility of RNA extraction and RT-PCR for pools varying in mosquito genus and number. A comparison of mosquito pool genera revealed no significant genus effect on the Ct value of the IPC. The incorporation of West Nile virus Armored RNA as an IPC allows monitoring of RNA extraction and RT-PCR and detection of false-negative results due to failures in these processes or to PCR inhibition, respectively.

West Nile virus (WNV) is a member of the family *Flaviviridae* generally transmitted to vertebrates by infected mosquitoes (5). Its genome consists of approximately 11 kb of single-stranded, positive-sense RNA encoding three structural (capsid, membrane, and envelope) and seven nonstructural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins flanked by 5′ and 3′ noncoding regions in a single open reading frame. Transmission involves birds and primarily *Culex* sp. mosquitoes, with humans as incidental hosts (2). Although human infection in areas where WNV is endemic is usually subclinical or mild, infection in some patients can result in severe disease (3).

WNV was originally isolated in Uganda in 1937 and has since been found in Africa, the Middle East, Australia, southern Europe, Russia, India, Indonesia, and, increasingly since 1999, in North America (2, 7). The Centers for Disease Control and Prevention website (cdc.gov/ncidod/dvbid/westnile) shows the spread of the virus from New York State in 1999 to a total of 44 states in 2002. Similarly in Canada, surveillance data on the Health Canada website tracks the spread of WNV from southern Ontario in 2001 westward to Saskatchewan and eastward to Nova Scotia in 2002 (www.hc-sc.gc.ca/english/westnile/). The value of mosquito surveillance for applying timely insect control is recognized as a means to predict and prevent future outbreaks (4, 6). The British Columbia Centre for Disease Control in Vancouver, Canada, is responsible for monitoring field-collected mosquitoes for WNV to deal with this public health threat in British Columbia.

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Testing of mosquito pools for WNV was performed using multiplex real-time reverse transcription-PCR (RT-PCR), which included an internal positive control (IPC). This method utilized a previously described Taqman RT-PCR approach, with the most notable addition being the incorporation of WNV Armored RNA (Ambion RNA Diagnostics), a pseudoviral particle containing the NS5-2 region of the WNV genome packaged inside bacteriophage coat proteins, as an IPC for the processes of RNA extraction, RT, and PCR (1, 4).

As previously described, mosquitoes were trapped in various locations in British Columbia, sorted according to genus and, if possible, species to a maximum of 50 per pool, and stored at −70°C in 1.5-ml Biopur Safe-Lock tubes (Eppendorf, Hamburg, Germany) until tested (5). Pools were homogenized in an MM300 mixer mill (Qiagen, Valencia, Calif.) at 25 Hz for 30 s in the presence of 1 ml of cold BA-1 diluent (4) and one 3-mm sterilized tungsten-carbide bead and centrifuged at 10,000 × *g* for 2 min at 4°C in a refrigerated benchtop centrifuge. An extraction control consisting of 1 ml of cold BA-1 diluent was processed in parallel with each batch of samples. WNV Armored RNA (approximately 1,400 copies in 1 μl) was added to 140 μl of supernatant from each homogenized sample and extraction control prior to RNA extraction with a QIAamp viral RNA kit (Qiagen). RNA was eluted in 60 μl of elution buffer in accordance with the manufacturer's protocol and stored at −20°C for RT-PCR testing the same day. Supernatants of known positive mosquito pools received in lysis buffer from Manitoba ($n = 26$) and Alberta ($n = 10$) were similarly processed.

Primers targeting the NS5-2 region of WN-NY99 (GenBank accession no. AF196835) were designed in our laboratory with Primer Express, version 2.0.0 (Applied Biosystems, Foster City, Calif.). The primers NS5-2F (5′ GAA GAG ACC TGC

GGC TCA TG 3′) and NS5-2R (5′ CGG TAG GGA CCC AAT TCA CA 3′) and the VIC reporter–6-carboxy tetramethylrhodamine (TAMRA) quencher-labeled NS5-2 probe (5′ VIC-CCA ACG CCA TTT GCT CCG CTG-TAMRA 3′) were derived from genome bp 10001 to 10020, 10047 to 10066, and 10022 to 10042, respectively. Taqman RT-PCR was performed as described previously with the exception that the 3′ noncoding region (3′NCR) and NS5-2 region were detected in multiplex (4). In addition to 7.5 pmol each of the 6-carboxyfluorescein (FAM)-labeled WN3′NC probe and the VIC-labeled NS5-2 probe and 50 pmol each of the 3′NCR primers (WN3′NC-forward and WN3′NC-reverse), 10 pmol each of the NS5-2 primers was added to a 50-μl reaction mixture. The NS5-2 primer concentrations were optimized to not interfere with the rate of the primary 3′NCR reaction. Five microliters of RNA extract from samples and extraction controls was subjected in duplicate to 40 amplification cycles in an ABI Prism 7900HT sequence detection system (Applied Biosystems) using an initial 30-min incubation at 48.0°C, followed by a 10-min 95.0°C heating stage and 40 cycles of 95.0°C for 15 s alternating with 60.0°C for 1 min. To monitor the PCR, each run included two replicates of the following controls: (i) a no-template control (NTC) consisting of diethyl pyrocarbonate-water in place of the template and (ii) positive PCR controls consisting of approximately 2.5×10^4 (strong positive) and 10^3 copies (weak positive) of a lineage 1 strain of WNV.

The data were analyzed with SDS, version 2.1, software (Applied Biosystems). The IPC was detected in the Taqman RT-PCR as an increase in fluorescence of the VIC-labeled NS5-2 probe, while WNV was detected as an increase in the fluorescence of the FAM-labeled WN3′NC probe. Pools with cycle threshold (Ct) values of less than 40 cycles for both probes were confirmed for WNV by using previously described reaction conditions and a primer-probe set targeting the WNV *env* gene on a Taqman 7900HT with cycling conditions described above (4). Mosquito pools were considered positive for WNV if they had Cts <40 cycles for all three WNV targets (NS5-2, 3′NCR, and *env*).

Although the Ct values of the IPC in the actual mosquito samples were used to detect enzyme inhibition in the samples, these values were not used to calculate statistics for quality control purposes because every sample matrix was different in terms of mosquito species, number, or both. In contrast, because the sample matrix in the extraction controls was well defined and uniform across batches, these IPC Cts were used to calculate the quality control ranges employed to monitor RNA extraction and RT-PCR. The calculated means ± 2 standard deviations from the extraction control IPC Cts were used to construct a Levey-Jennings chart onto which the Ct values for the spiked-in IPC in the actual samples could be plotted and from which the data could be examined over time for trends.

Of 635 British Columbia mosquito pools tested, all were positive for the NS5-2 region and negative for the 3′NCR, indicating the presence of the IPC and the absence of WNV in the samples. This confirmed successful RNA extraction and RT-PCR and the absence of inhibition. The calculated mean (±2 standard deviations) of the extraction control IPC Cts was 36.43 ± 1.78 cycles. The IPC Cts of 98.4% (625 of 635) of the WNV-negative pools fell within this range, indicating reproducible RNA extraction and RT-PCR in pools varying in mosquito species and number. The outlying 1.6% of Cts (10 of 635) were retested. In most cases, mosquito extracts had a slightly higher (<1 cycle) IPC Ct than the corresponding extraction control, suggesting slight inhibition from the mosquito tissue. Samples with IPC Cts >40 cycles (there were none in this study) would have been considered falsely negative due to failure in RNA extraction, RT, or PCR inhibition.

The 36 WNV-positive mosquito pools from Alberta and Manitoba had WN3′NC Cts ranging from 18.5 to 34.6 cycles and NS5-2 Cts ranging from 21.2 to 36.2 cycles. Although the IPC Cts of some WNV-positive pools were more than 2 standard deviations below the mean, this was not a cause for concern; since the purpose of the IPC was to rule out false negatives, these low Cts negated this concern by confirming successful RNA extraction and RT-PCR. For any WNV-positive sample, the WN3′NC Cts were consistently 2 to 3 cycles lower than the NS5-2 Cts, probably due to the limiting quantities of NS5-2 primers in the reaction, further indicating that the NS5-2 reaction targeted against the IPC did not adversely affect the 3′NCR WNV detection reaction.

In all cases, negative findings were obtained with the NTCs, indicating lack of reagent contamination with WNV RNA. The extraction controls included with each batch gave Cts >40 cycles for the WN3′NC probe, indicating absence of WNV RNA in the diluent and tubes in which the mosquitoes were processed. The PCR-positive controls yielded similar Cts for the NS5-2 and 3′NCR reactions (i.e., 32 to 33 cycles for both probes in the strong positive PCR control and 37 to 38 cycles for both probes in the weak positive PCR control), again suggesting that the 3′NCR reaction was not adversely affected by the NS5-2 reaction.

Pools from different mosquito genera were compared to see if there were genus-specific inhibition effects. The mean IPC Cts for the genus groupings were as follows: *Anopheles* spp. ($n = 54$), 34.95 cycles; *Coquilletidia* spp. ($n = 64$), 36.49 cycles; *Culex* spp. ($n = 227$), 36.58 cycles; A*edes* and *Ochlerotatus* spp. ($n = 145$), 36.61 cycles; *Culiseta* spp. ($n = 103$), 36.99 cycles. As the difference between the most disparate groups was only 5.6%, mosquito genus was not considered to have a significant impact on NS5-2 PCR results.

WNV Armored RNA was used as an IPC for the processes of RNA extraction, RT, and PCR, while the positive (strong and weak) and negative (NTC) PCR controls were included to monitor the PCR and reagents. The presence of a fluorescent VIC signal in the Taqman RT-PCR assay indicated the success of the above processes and the absence of PCR inhibition in the extraction controls and samples, while the absence of such would have suggested a possible failure in any of these or the presence of enzyme inhibitors. The absence of a FAM signal for the 3′NCR probe in the extraction controls suggested a lack of cross-contamination among samples in the respective batches, whereas the presence of such a signal at less than 40 cycles would have indicated the opposite.

Routine mosquito pool surveillance necessitates quality control measures to ensure the success of the method as well as the detection of false-negative results due to RNA extraction failure or enzyme inhibitors. The incorporation of WNV Armored RNA into Taqman RT-PCR for West Nile virus in mosquito pools enables the laboratory to simultaneously detect West

Nile virus and monitor the processes of RNA extraction and RT-PCR in multiplex.

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REFERENCES

1. **Briese, T., X.-Y. Jia, C. Huang, L. J. Grandy, and W. I. Lipkin.** 1999. Identification of a Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis. Lancet **354:**1261–1262.
2. **Campbell, G. L., A. A. Marfin, R. S. Lanciotti, and D. J. Gubler.** 2002. West Nile virus. Lancet Infect. Dis. **2:**519–529.
3. **Hayes, C. G.** 1989. West Nile fever, p. 59–88. *In* T. P. Monath (ed.), The arboviruses: epidemiology and ecology, vol. V. CRC Press, Inc., Boca Raton, Fla.
4. **Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, and J. T. Roehrig.** 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a Taqman reverse-transcriptase PCR assay. J. Clin. Microbiol. **38:**4066–4071.
5. **Monath, T. P., and F. X. Heinz.** 1996. Flaviviruses, p. 961–1035. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology. Raven Press, New York, N.Y.
6. **Shi, P.-Y., E. B. Kauffman, P. Ren, A. Felton, J. H. Tai, A. P. Depuis, S. A. Jones, K. A. Ngo, D. C. Nicholas, J. Maffei, G. D. Ebel, K. A. Bernard, and L. D. Kramer.** 2001. High-throughput detection of West Nile virus RNA. J. Clin. Microbiol. **39:**1264–1271.
7. **Smithburn, K. C., T. P. Hughes, A. W. Burke, and J. H. Paul.** 1940. A neurotropic virus isolated from the blood of a native of Uganda. Am. J. Trop. Med. **20:**471–492.