

West Nile virus Surveillance: A Simple Method for Verifying the Integrity of RNA in Mosquito (Diptera: Culicidae) Pools

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ABSTRACT In a *West Nile virus* (WNV)-free ecosystem, it is essential to verify the integrity of RNA before concluding that RNA extracted from mosquito specimens is negative for WNV gene sequences. The primary objective of our study was to develop a rapid molecular assay to rapidly screen mosquitoes for the presence of 18S rRNA and WNV gene sequences. Mosquitoes, collected from multiple sites on the island of O'ahu, were pooled into groups of 1–50 mosquitoes according to capture site, date, and species. Using primer design software and the GenBank database, generic oligonucleotide primer pairs were designed to amplify mosquito 18S rRNA gene sequences from different species. RNA was extracted from mosquito pools, and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed for the presence of mosquito 18S rRNA and WNV gene sequences. Three of the seven primer pairs successfully detected 18S rRNA sequences for both *Aedes* and *Culex* by RT-PCR, and one primer pair successfully amplified 18S rRNA sequences for 15 different mosquito species. All 64 mosquito pools from 10 different sites on the island of O'ahu were negative for WNV nonstructural protein-5 gene sequences. This simple, one-step RT-PCR method for screening mosquito pools for arboviruses will become an increasingly valuable tool as WNV becomes endemic throughout the Americas.

KEY WORDS *West Nile virus*, reverse transcriptase-polymerase chain reaction, mosquito, arbovirus, *Aedes*, *Culex*

West Nile virus (WNV) is a mosquito-borne flavivirus widely distributed throughout Africa, the Middle East, and parts of Europe, Russia, India, and Indonesia. In 1999, an outbreak of WNV infection in the northeastern United States resulted in 62 laboratory-confirmed human cases (Nash et al. 2001), signaling the entry of WNV into the Americas. Since that time, WNV has spread rapidly across the United States. In 2003, nearly 9,000 human cases of WNV disease were reported from 45 states compared with only 149 cases during 1999–2001 (Centers for Disease Control and Prevention 2002). The impact of WNV disease in the Americas is likely to increase as WNV becomes endemic.

The transmission cycle of WNV involves mosquitoes feeding on infected birds and subsequently passing the virus to other birds and occasionally to incidental hosts, such as humans and horses. WNV has been detected in 43 mosquito species worldwide and in at least 33 species in North America (Hubalek and Halouzka 1999, Centers for Disease Control and Prevention 2002). Hawaii is home to *Aedes albopictus* (Skuse), *Aedes aegypti* (Linnaeus), *Aedes vexans nocturnus* (Theobald), and *Culex pipiens quinquefasciatus* (Say), all of which are potential vectors for WNV.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) has been effectively used for high-throughput screening of mosquito samples for the presence of WNV sequences. However, traditional RT-PCR remains a valuable surveillance tool because real-time RT-PCR technology has yet to reach all laboratories conducting surveillance studies, particularly in resource-poor countries. Furthermore, the amplicons generated using conventional RT-PCR technique can be used to obtain genetic information crucial for molecular epidemiological studies of WNV. Very little information is available on the amplification of mosquito “house-keeping” genes from different mosquito species using generic primer pairs. In a WNV-free ecosystem, such as Hawaii, it is critical to

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Table 1. Oligonucleotide primer pairs: locations, lengths, and sequences

Primer	Location	Genbank accession number	Length	Sequence
18S311	311–332	AB085210	21	5'-TTGCAACGGGTAACGGGGAATC-3'
18S723c	703–723	AB085210	20	5'-GCATCAGCGCCACACTACGAC-3'
18S417	417–440	AB085210	23	5'-ACGGGGAGGTATGTCAGCAGAAATA-3'
18S920c	897–920	AB085210	23	5'-TAATACTAATGCCCCCACTACTT-3'
18S693	693–714	AB085210	21	5'-GGTCGGCGCGTCTAGTGTGG-3'
18S1192c	1169–1192	AB085210	23	5'-TCCTGCTGGTCCCTTCCGTCAAT-3'
18S1544	1544–1564	AB085210	20	5'-GCTCCGTGATGCCCTTAGATG-3'
18S2064c	2064–2087	AB085210	23	5'-GCGGCAGCGTCCCTCGGAAAAA-3'
18S1744	1744–1764	AB085210	20	5'-CACACCGCCCGTCTACTAC-3'
18S2290c	2271–2290	AB085210	19	5'-GGACGGCCGCCACCACTGAC-3'
18S869	896–909	U48385	23	5'-ATTAGTATTACGGCCGAGAGGTG-3'
18S1388c	1368–1388	U48385	21	5'-CGGGCCGACGGTATCAACATC-3'
18S1269	1269–1292	U48385	24	5'-GTGCTGATGCCGCTTCTAGTTC-3'
18S1722c	1722–1743	U48385	22	5'-GCTAGTAGCCAGCGGGCTGTG-3'

establish the quality and integrity of RNA extracted from mosquitoes before concluding that mosquito specimens are negative for WNV gene sequences. Our goal was to include a control amplification reaction for each mosquito pool to be analyzed for the presence of WNV. If RNA from a particular mosquito pool was to amplify successfully for a target mosquito “house-keeping” gene sequence, negative results for amplification of WNV gene sequences could be attributed to the absence of WNV rather than failure to extract good quality RNA from the mosquito pool. In this report, we describe development of molecular tools to rapidly screen mosquitoes for the presence of mosquito 18S ribosomal RNA (rRNA) gene sequences and WNV gene sequences.

Materials and Methods

Mosquito Collection and Nucleic Acid Extraction. Mosquitoes, collected using carbon dioxide-baited traps from 10 different sites throughout O’ahu, were sorted by date, location, and species. In addition, mosquito pools containing species not found in Hawaii were kindly provided by Dr. Duane J. Gubler and Dr. Roger Nasci (Division of Vector-Borne Infectious Diseases, CDC, Ft. Collins, CO) (Table 3). The mosquitoes were placed in 12 by 75-mm 6-ml disposable tubes; each tube contained 1–50 mosquitoes. Homogenizing buffer, consisting of 50 mM Tris-base in Hank’s balanced salt solution, 1% bovine serum albumin, 0.35 g/l NaHCO₃, 0.25 μg/ml amphotericin B, 0.25 μg/ml sodium desoxycholate, 10 U/ml penicillin G, and 10 μg/ml streptomycin (all from GIBCO, Rockville, MD), was added to each tube at a volume of 1 (for ≤10 mosquitoes/tube) or 2 ml (for 11–50 mosquitoes/tube). After the addition of four steel-clad beads, the tubes were capped and vortexed for 1 min. The mosquito homogenates were removed from the steel beads using transfer pipettes and placed into 1.5-ml microcentrifuge tubes. After centrifugation at 14,000 rpm for 1 min to pellet solid debris, the supernatants were transferred to new 1.5-ml microcentrifuge tubes. Total RNA was extracted from 160 μl of either the fresh or stored (–70°C) supernatant using the QIAamp Viral RNA mini-kit (QIAGEN, Valencia, CA)

according to the manufacturer’s instructions. RT-PCR was performed immediately on the extracted RNA or after storage of RNA at –70°C.

Oligonucleotide Primer Design. Several RT-PCR-based protocols have been employed to successfully amplify WNV sequences from human and avian specimens (such as blood, serum, and tissue) and from mosquitoes (Briese et al. 2000, Lanciotti et al. 2000, Nasci et al. 2002). As mentioned earlier, however, no assay has been developed to screen different mosquito species for WNV gene sequences with proper controls in place to ensure the validity of negative results. The 18S rRNA gene was selected as a “house-keeping” gene because of its conserved sequence and high copy number. An extensive literature and GenBank search revealed limited information about 18S rRNA sequences from *Aedes* and *Culex* mosquitoes. We analyzed the 18S rRNA gene sequences for *Ae. albopictus* (GenBank accession number AB085210) and *Cx. tritaeniorhynchus* (GenBank accession number U48385) using Primer Select software (DNA Star, Madison, WI) to generate seven different oligonucleotide primer pairs, which were then synthesized (Table 1; Invitrogen, Carlsbad, CA) for use in RT-PCR.

Nucleic Acid Amplification. To determine the ability of each 18S rRNA primer pair to amplify target



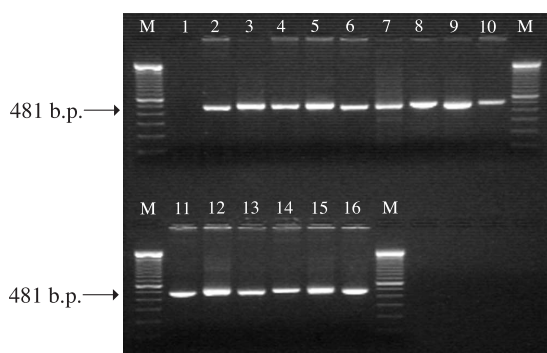
Fig. 1. RT-PCR amplification of mosquito 18S rRNA sequences using different primer pairs. Lanes 1–7 represent reactions in which water was included in place of template RNA (negative controls), lanes 8–14 represent reactions in which *Ae. albopictus* RNA was used as a template, and lanes 15–21 represent reactions in which *Cx. P. quinquefasciatus* RNA was used as a template. Primer pairs used in each reaction include the following: 18S311 and 18S723c for lanes 1, 8, and 15; 18S417 and 18S920c for lanes 2, 9, and 16; 18S693 and 18S1192c for lanes 3, 10, and 17; 18S1544 and 18S2064c for lanes 4, 11, and 18; 18S1744 and 18S2290c for lanes 5, 12, and 19; 18S869 and 18S1388c for lanes 6, 13, and 20; and 18S1269 and 18S1722c for lanes 7, 14, and 21 (Table 1).

Table 2. Amplification of mosquito 18S rRNA gene sequences from *Aedes* and *Culex* spp. using different primer pairs

Primer pair	Product size	RT-PCR results	
		<i>Ae. albopictus</i>	<i>C. quinquefasciatus</i>
18S311 and 18S723c	413	Positive	Negative
18S417 and 18S920c	504	Positive	Positive
18S693 and 18S1192c	500	Positive	Negative
18S1544 and 18S2064c	521	Positive	Positive
18S1744 and 18S2290c	547	Positive	Positive
18S869 and 18S1388c	544	Positive	Negative
18S1269 and 18S1722c	547	Positive	Negative

sequences in total RNA extracted from *Aedes* and *Culex* samples collected on O'ahu, a standard two-step RT-PCR protocol was developed. First, cDNA was synthesized from each *Ae. albopictus* and *Cx. p. quinquefasciatus* RNA sample using 16 μ l RNA in a 40- μ l reaction mixture, which contained 1 \times cDNA synthesis buffer, 200 U Superscript II RT, 0.01 M dithiothreitol (DTT) (all from Invitrogen), 20 U RNasin ribonuclease inhibitor, 0.5 μ g random hexamer primers (both from Promega, Madison, WI), and 0.5 mM each dNTP (Applied Biosystems, Foster City, CA). The RT reactions were carried out on a Gene Amp PCR System 9700 (Applied Biosystems) at 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min. For the second step of the RT-PCR, 5 μ l of each of the above cDNA was included in a 25- μ l reaction mixture containing 1 \times PCR buffer, 2 mM MgCl₂, 0.625 U *Taq* polymerase, 0.2 mM each dNTP (all from Applied Biosystems), and 10 pmol each of the forward and reverse primers (Table 1). Negative controls containing deionized water instead of an RNA sample were included. The cycling conditions were as follows: 45°C for 1 h, 94°C for 3 min (one cycle), and 94°C for 30 s, 60°C for 1 min, and 68°C for 3 min (45 cycles), followed by 72°C for 7 min (one cycle). Ten microliters of each RT-PCR product was size-fractionated electrophoretically on a 2% agarose gel and stained with ethidium bromide. This procedure was performed for pools of mosquitoes containing various mosquito species (Table 3).

For the detection of WNV RNA from mosquito RNA, we modified a previously described protocol

**Fig. 2.** RT-PCR amplification of mosquito 18S rRNA sequences from 15 different species using 18S417 and 18S920c primers. Lane 1 represents a reaction in which water was included in place of template RNA (negative control). Lanes 2–16 represent reactions using RNA extracted from 15 different mosquito pools, each containing different species as described in Table 3.

(Lanciotti et al. 1999). RT-PCR was performed using the TITAN RT-PCR kit (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer's instructions. For each 50- μ l reaction, 10 picomoles of each oligonucleotide primer for the 18S rRNA gene (18S417 and 18S920c) or WNV (WN9483 and WN9794c) (Lanciotti et al. 2002) as well as 10 μ l of extracted RNA were added in separate tubes. Deionized water was used as a negative control instead of RNA sample, and RNA extracted from tissue culture supernatant (WNV-infected Vero cells) was used as a positive control. The cycling conditions were as follows: 45°C for 1 h, 94°C for 3 min (one cycle), and 94°C for 30 s, 60°C for 1 min, and 68°C for 3 min (45 cycles), followed by 72°C for 7 min (one cycle). RT-PCR products were analyzed by agarose gel electrophoresis as described above.

Results and Discussion

Of the 972 mosquitoes collected during December 2002 and January 2003, 874 (90%) were identified morphologically as *Cx. p. quinquefasciatus*, and the

Table 3. Amplification of mosquito 18S rRNA gene sequences from various mosquito species using 18S417/920c primer pair

Lane number	Genus	Species	Authority	Number of mosquitoes in pool	RT-PCR results
2	<i>Culex</i>	<i>nigripalpus</i>	Theobald	5	Positive
3	<i>Anopheles</i>	<i>crucians</i>	Wiedemann	38	Positive
4	<i>Ochlerotatus</i>	<i>taeniorhynchus</i>	Wiedemann	4	Positive
5	<i>Anopheles</i>	<i>punctipennis</i>	Say	1	Positive
6	<i>Culex</i>	<i>restuans</i>	Theobald	1	Positive
7	<i>Culex</i>	<i>salinarius</i>	Coquillett	50	Positive
8	<i>Aedes</i>	<i>vexans nocturnus</i>	Theobald	24	Positive
9	<i>Mansonia</i>	<i>titillans</i>	Walker	9	Positive
10	<i>Uranotaenia</i>	<i>sapphirina</i>	Osten Sacken	1	Positive
11	<i>Coquillettia</i>	<i>perturbans</i>	Walker	1	Positive
12	<i>Anopheles</i>	<i>quadrimaculatus</i>	Say	3	Positive
13	<i>Ochlerotatus</i>	<i>infirmatus</i>	Dyar and Knab	2	Positive
14	<i>Aedes</i>	<i>albopictus</i>	Skuse	1	Positive
15	<i>Culex</i>	<i>pipiens quinquefasciatus</i>	Say	2	Positive
16	<i>Toxorhynchites</i>	<i>alboinensis</i>	Doleschall	1	Positive

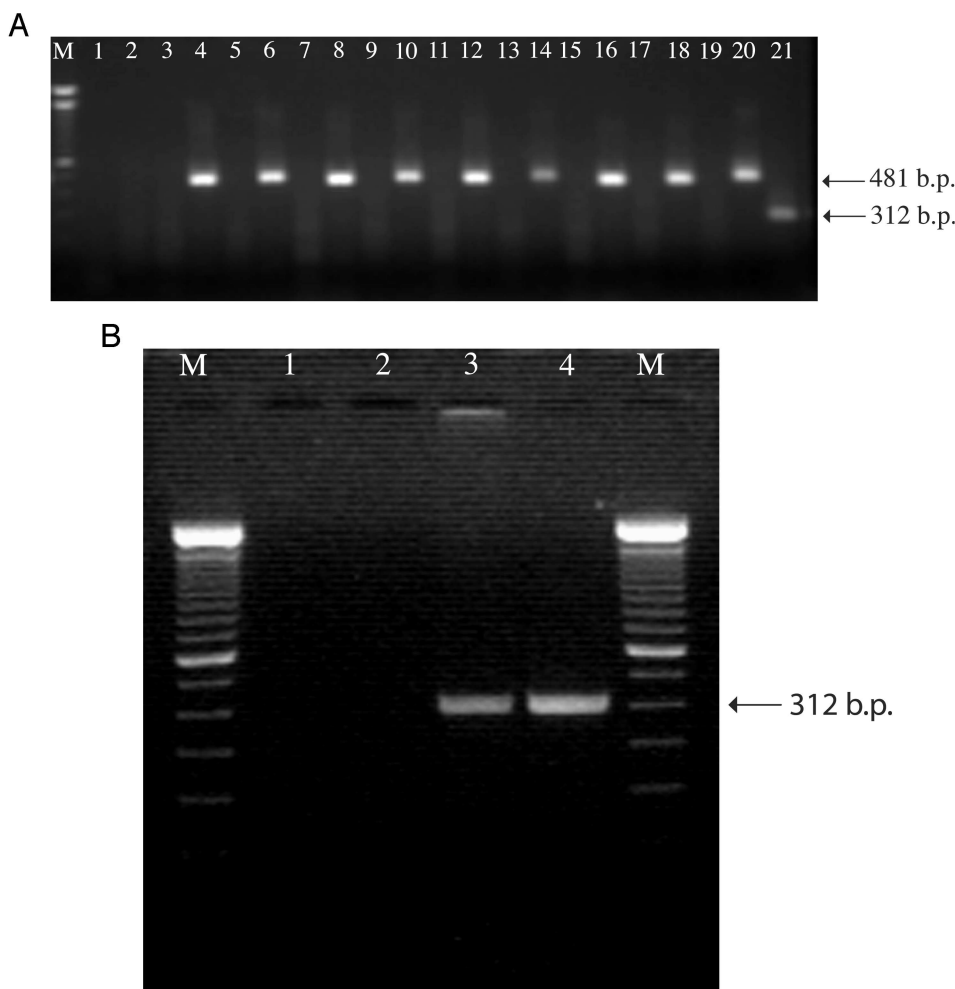


Fig. 3. (A) RT-PCR amplification of WNV and 18S rRNA sequences from nine independent mosquito RNA samples. Lanes 1 (primers = WN9483 and WN9794c) and 2 (primers = 18S417 and 18S920c) represent reactions in which water was substituted in place of template RNA (negative controls). Primers for amplifying WNV (WN9483 and WN9794c) were used for lanes 3, 5, 7, 9, 11, 13, 15, 17, and 19. Primers for amplifying 18S rRNA (18S417 and 18S920c) were used for lanes 4, 6, 8, 10, 12, 14, 16, 18, and 20. RNA was extracted from mosquito pools containing either *Cx. p. quinquefasciatus* (lanes 3–16) or *Ae. albopictus* (lanes 17–19). Lane 21 represents the reaction in which WNV RNA extracted from infected cell culture supernatant was used as a template and primers included WN9483 and WN9794c (positive control). (B) The RT-PCR protocol is effective in amplifying WNV RNA from mosquito pools provided by the CDC. Lane 1 represents a reaction in which water was substituted in place of template RNA (negative control). Lanes 2 and 3 represent reactions using RNA extracted from a WNV-negative mosquito pool and WNV-positive mosquito pool, respectively. Lane 4 represents a reaction using RNA extracted from WNV tissue culture supernatant as described in the Materials and Methods section (positive control).

remaining 98 (10%) were *Ae. albopictus*. As shown in Fig. 1, all oligonucleotide primer pairs for the 18S rRNA gene were successful in amplifying target sequences from the *Ae. albopictus*-extracted RNA, whereas only three primer pairs were successful in amplifying target sequences from the *Cx. p. quinquefasciatus* extracted RNA (Table 2). Despite the high degree of sequence homology for the 18S rRNA gene for different species, only three of seven primer pairs successfully amplified the 18S rRNA gene region for both species. These results were repeated in three independent experiments using mosquito-derived

RNA samples as templates for RT-PCR (data not shown). Interestingly, the ethidium bromide-stained DNA band fluorescence was similar between RNA extracted from 1 mosquito or from 50 mosquitoes. One of the amplicons (*Ae. albopictus* RNA amplified with 18S417/920c) was selected, and its identity was confirmed as an 18S rRNA sequence using an ABI 3100 sequencer.

Once we identified which oligonucleotide primer pairs were effective in amplifying the 18S rRNA genes for both species, we wanted to determine how effective these primer pairs were for detecting RNA ex-

F1

T2

tracted from a wide range of mosquito species. Therefore, we chose one primer pair (18S417/920c) and performed two-step RT-PCR on RNA extracted from a total of 15 different mosquito species (Table 3). As shown in Fig. 2, 18S rRNA gene sequences were detected in all 15 species using this primer pair. These results suggest that this primer pair is quite effective in its ability to detect 18S rRNA gene sequences in a wide range of mosquito species, including several species involved in transmission of arboviruses (Table 3).

We then used these primers along with WNV primers to screen all mosquito RNA samples. A representative gel is shown in Fig. 3A. To date, a total of 64 mosquito pools collected on O'ahu have been screened using this assay system. All mosquito pools were negative for WNV nonstructural protein-5 (NS5) gene sequences but positive for the 18S rRNA gene. To ensure that the extraction and amplification protocol could effectively detect WNV RNA from WNV-positive mosquito pools, RT-PCR was performed on a WNV-positive mosquito pool received from the CDC. As shown in Fig. 3B, the protocol was successful in detecting WNV RNA from this WNV-positive mosquito pool.

Because we anticipated that WNV sequences would not be present in the mosquitoes captured in Hawaii, it was necessary to first develop controls to confirm that any absence of WNV sequences was because of a lack of WNV rather than poor-quality RNA extracted from the mosquitoes. Specifically, amplification and detection of a "house-keeping" gene was needed for every RNA sample during the RT-PCR screening assay. Our results demonstrate a simple, one-step RT-PCR method for screening pools of mosquitoes from a wide range of species for WNV. Importantly, clear interpretation of the results from this assay are made possible by including generic oligonucleotide primers for mosquito 18S rRNA gene, although the added costs of including this control must be considered. The use of primers specific for 28S rRNA has been used in previous studies to separate mosquito specimens (Nasci et al. 2002). However, our study is the first to describe primer pairs capable of detecting 18S rRNA gene sequences in RNA extracted from a wide range of mosquito species. Such molecular surveillance techniques will become increasingly valuable as WNV becomes endemic throughout the United States. Furthermore, this method may be modified to screen for other medically important mosquito-borne viruses, such as St. Louis encephalitis and dengue viruses.

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