West Nile Virus Infection Alters Midgut Gene Expression in *Culex pipiens quinquefasciatus* Say (Diptera: Culicidae)

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**Abstract.** Alterations in gene expression in the midgut of female *Culex pipiens quinquefasciatus* exposed to blood meals containing 6.8 logs plaque-forming units/mL of West Nile virus (WNV) were studied by fluorescent differential display. Twenty-six different cDNAs exhibited reproducible differences after feeding on infected blood. Of these, 21 cDNAs showed an increase in expression, and 5 showed a decrease in expression as a result of WNV presence in the blood meal. GenBank database searches showed that one clone with increased expression, CQ G12A2, shares 94% identity with a leucine-rich repeat-containing protein from *Cx. p. quinquefasciatus* and 32% identity to Toll-like receptors from *Aedes aegypti*. We present the first cDNA clone isolated from female *Cx. p. quinquefasciatus* midgut tissue whose expression changes on exposure to WNV. This cDNA represents a mosquito gene that is an excellent candidate for interacting with WNV in *Cx. p. quinquefasciatus* and may play a role in disease transmission.

**INTRODUCTION**

Arboviruses are responsible for emerging and re-emerging infectious diseases throughout the world, thereby contributing to an increase in disease burden and impacting public health.1–3 West Nile virus (WNV; family *Flaviviridae*, genus *Flavirus*) is the most widely spread arbovirus, occurring on all continents except for Antarctica.4 Since its introduction into North America in 1999, WNV has established itself across the region because of its ability to infect a variety of *Culex* spp. mosquitoes capable of virus transmission to local and migratory bird species.5–8 *Culex pipiens pipiens* L., *Cx. tarsalis* Coquillett, *Cx. p. quinquefasciatus* Say, and *Cx. nigripalpus* Theobald are the primary enzootic vectors contributing to human WNV epidemics in the United States.9,10

The above-mentioned *Culex* spp. mosquitoes are all competent laboratory vectors of WNV. Competence of a mosquito to transmit an arbovirus is determined by internal and external factors.11,12 One of the important factors influencing the ability of a mosquito to transmit a virus is establishment of an infected midgut. The female mosquito midgut is involved in blood digestion, and thus, viruses ingested with the blood must enter and exit the midgut epithelial cells to infect salivary glands from which they can be transmitted to a naïve host.13,14 Elements of the midgut can act as a barrier to pathogens ingested with the blood that must be circumvented for an infection to be established.15–17 The barrier can either be physical or caused by interference by enzymes that function in blood digestion processes. Changes in mosquito midgut gene expression after blood ingestion has been documented.18–20 Blood ingestion up-regulates the expression of numerous genes involved in nutrient uptake, metabolism, stress responses, peritrophic matrix formation, and immune responses.16 Ingestion of a blood meal containing viruses also elicits a change in midgut morphology and gene expression.17,21

Studies investigating the morphologic alterations that take place after a mosquito becomes infected with virus have shown that *Aedes aegypti* L. cells infected with replicating yellow fever virus exhibited rough endoplasmic reticulum swelling, vesicle formation, dilatation of perinuclear space, and vacuolization.22 Similar cellular changes were seen in *Aedes albopictus* Skuse C6/36 cells infected with the flavivirus, Kunjin virus.23 Girard and others24 studied the effects of virus replication on membrane induction, cellular organization, and cell viability in midgut and salivary gland tissues of *Cx. p. quinquefasciatus* and found that WNV induced significant membrane proliferation in the midgut epithelium, muscle, and salivary glands. Transmission electron microscopy showed a laboratory strain of *Cx. p. pipiens* refractory to WNV transmission caused by apoptosis of midgut cells, suggesting apoptosis as a potential antiviral mechanism.17

Antiviral responses, including the expression of genes encoding proteins of the innate immune pathway, have been detected in *Ae. aegypti* against Sindbis18 and dengue viruses25,26 and in *Anopheles gambiae* Giles against O’nyong-nyong virus.25 However, the effect of arbovirus infection on gene expression in midgut tissue of *Culex* spp. mosquitoes has not been studied.

This study investigated gene expression alterations in midgut tissue of *Cx. p. quinquefasciatus* after exposure to a WNV-infected blood meal. Results from this project will contribute to our understanding of the physiological process and molecular interactions affected in the midgut of *Cx. p. quinquefasciatus* after infection with WNV.

**MATERIALS AND METHODS**

**Mosquitoes and virus.** *Culex p. quinquefasciatus* established in 1995 from a collection from Alachua County in north-central Florida (F > 50) were reared at 28°C and 70–75% humidity under a 14:10-hour light/dark cycle in a Harford Duracoal Biochamber (Bio-Temp Scientific Inc., Sarasota, FL) with procedures described elsewhere.28

The WN-FL03p2-3 strain of WNV (passaged four times in Vero cells and one time in BHK cells) was isolated from a pool of *Cx. nigripalpus* mosquitoes from Indian River County, FL, in 2003 (A. Doumbouya, unpublished data).

**Blood-meal preparation and mosquito feeding.** Freshly propagated WNV stock was mixed with citrated bovine blood before mosquito feeding to create infected blood meals. Five-to 6-day-old mosquitoes were allowed to feed on cotton pledges containing either infectious or non-infectious citrated bovine blood (Hemostat, Dixon, CA) warmed (35°C) for 10 minutes. After heating, two aliquots of 0.1 mL of infected
blood were added to 1 mL BA-1 diluent\(^9\) and held at \(-80^\circ\)C until processing to determine blood meal titers. Subsequent to feeding, mosquitoes were immobilized with cold, and fully engorged specimens were transferred to 1-L cardboard cages with mesh screening and maintained in incubators for experiment-specific extrinsic incubation periods of either 4 or 10 days after infection at 28°C and provided 20% sucrose ad libitum.

**Mosquito RNA isolation.** Total RNA was extracted from *Cx. p. quinquefasciatus* adult female mosquito midguts using the TRIzol Reagent (Invitrogen, Carlsbad, CA) following the included protocol with the following modifications: dissected midgut tissues were ground in 0.8 mL of TRIzol reagent. After homogenization, samples were allowed to incubate at 23°C for 10 minutes, followed by a debris clearing spin at 12,000 rpm for 10 minutes at 4°C. Then, RNA samples were allowed to dry on ice for 1 hour and resuspended in 0.05 mL of diethylpyrocarbonate (DEPC)-treated water. To aid in resuspension, samples were incubated for 10 minutes at 60°C. All RNA samples were stored at \(-80^\circ\)C until needed. All RNA samples were quantified using a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA). RNA integrity was verified by separating the RNA on an agarose/formaldehyde gel, and the ribosomal bands were visualized using an InGenius gel documentation system (Syngene, Frederick, MD).

**Fluorescent differential display gene expression.** RNA from 200 pooled midguts dissected 4 days after female mosquitoes ingested either uninfected blood or WNV-infected blood was isolated using TRIzol Reagent (Invitrogen). Fifty micrograms of this female midgut RNA was sent to GenHunter (Nashville, TN), where differential display (DD) was performed using the TRIZol Reagent (Invitrogen, Carlsbad, CA) following the included protocol with the following modifications: dissected midgut tissues were ground in 0.8 mL of TRIZol reagent. After homogenization, samples were allowed to incubate at 23°C for 10 minutes, followed by a debris clearing spin at 12,000 rpm for 10 minutes at 4°C. Then, RNA samples were allowed to dry on ice for 1 hour and resuspended in 0.05 mL of diethylpyrocarbonate (DEPC)-treated water. To aid in resuspension, samples were incubated for 10 minutes at 60°C. All RNA samples were stored at \(-80^\circ\)C until needed. All RNA samples were quantified using a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA). RNA integrity was verified by separating the RNA on an agarose/formaldehyde gel, and the ribosomal bands were visualized using an InGenius gel documentation system (Syngene, Frederick, MD).

**Sequence analysis.** One of the PCR amplified products of *CQ G12A2*, was sequenced using the CEQ DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA) following the included protocol. The remaining 10 cloned amplified PCR products are presently being sequenced and will not be discussed in this manuscript. Sequencing reactions were analyzed on a Beckman Coulter CEQ 8000 Genetic Analysis System. BLAST was used to find regions of local similarity between the cloned sequence and sequences in the GenBank and VectorBase databases.

**RESULTS**

**Differential display of gene expression 4 days after ingesting uninfected or WNV-infected blood.** The effect of WNV infection on gene expression in midgut tissue of *Cx. p. quinquefasciatus* mosquitoes was studied using a fluorescent DD approach to show broad changes in transcription.\(^30\) Mosquito midguts used in the DD analysis were dissected at 4 days after WNV exposure, a time shown to coincide with the presence of virus in midgut cells.\(^3\) A comparison of midgut transcription alterations 4 days after female mosquitoes ingested uninfected blood or blood meals containing \(\sim 6.8\) plaque-forming units (pfu) WNV/mL showed 26 amplification products showing an increase in expression after infection with WNV, were cloned. Six of these sequences in the GenBank and VectorBase databases.
11 amplification products are shown on Figure 1. One of the 11 clones, CQ G12A2 (GenBank accession no. GO254244), contained a 418-bp insert and, through sequence analysis, was found to encode a putative translation product of 131 amino acids, and was incomplete at the 5' and 3' ends (Figure 2). The Cx. p. quinquefasciatus CQ G12A2 putative protein has about three repetitive stretches of amino acids containing leucine residues (Figure 2). VectorBase, Pfam, and BLAST tblastx database searches with the putative translation product of CQ G12A2 showed that it shares 94% identity with a leucine-rich repeat-containing protein from Cx. p. quinquefasciatus (accession no. XP_001846467; unpublished) and shares 32% identity with Toll-like receptors (TLRs) from Ae. aegypti (accession no. XP_0001650338) (Figure 2; GenBank tblastx searches performed on April 23, 2009). Phylogenetic analysis of blastp results indicated that CQ G12A2 clusters with a TLR from Ae. aegypti (data not shown).

**Temporal gene expression in midgut tissue of mosquitoes fed uninfected or WNV-infected blood.** To characterize the temporal expression of CQ G12A2 in midguts of blood-fed mosquitoes, we performed semi-quantitative RT-PCR on RNA extracted from midguts of female Cx. p. quinquefasciatus at different times after exposure to uninfected blood (i.e., 0, 3, 6, and 9 hours and 1–10 days after feeding). Expression of CQ G12A2 was detected at low levels in midgut tissues at each time; however, an increase in transcription was also detected (Figure 3). The up-regulation of transcription coincided with the early time periods after blood feeding, specifically at 3–6 hours, with an additional increase at 1 and 9 days after feeding (Figure 3A).

To determine whether WNV infection affects temporal expression of CQ G12A2 in midgut tissue, we performed semi-quantitative RT-PCR on RNA extracted from midguts dissected from female Cx. p. quinquefasciatus at different times after exposure to blood containing 7.4 ± 0.1 logs pfu WNV/mL (i.e., 0, 3, 6, and 9 hours and 1–10 days after feeding; Figure 4). Expression of our gene of interest was seen in midguts at each of the times sampled. However, there were visible increases in mRNA in midguts dissected 6, 8, and 10 days after infection (Figure 4A).

To determine CQ G12A2’s influence on WNV in midgut tissue, if any, we quantified the amount of WNV RNA in the midgut samples from female Cx. p. quinquefasciatus at different times after exposure to WNV-infected blood (Figure 5). Our qRT-PCR results showed that WNV was detected at all times tested, and significant differences in WNV titer were shown at different times after infection (F = 70.54; df = 12,38; P ≤ 0.001; Figure 5). We analyzed the qRT-PCR samples by agarose gel electrophoresis and found that the intensity of the PCR products coincided with the WNV titers observed using qRT-PCR analysis (Figure 4B). WNV titer increased when transcription of CQ G12A2 was low, as seen in samples dissected at 3–5 days after infection. However, the WNV titer in midguts dissected 6 days after infection showed the lowest titer, but this corresponded to the highest visible expression of CQ G12A2 (Figures 4A and B and 5). These findings are consistent with the possibility that CQ G12A2 plays a role in antiviral responses and consistent with its similarity with the TLR family.

**DISCUSSION**

Proteins with leucine-rich repeats (LRRs) are involved in protein–protein interactions and are of diverse structure, localization, and function. There are seven classes of leucine-rich repeats, and they share the characteristic structure of repetitive stretches of amino acids of variable length containing precisely positioned hydrophobic residues, usually leucines. The Cx. p. quinquefasciatus CQ G12A2 putative protein has about three repetitive stretches of amino acids containing leucine residues. Leucine-rich repeats are found in proteins, both intracellular and extracellular, that function in innate immunity and nervous system development in bacteria, fungi, plants, and animals. The similarity of CQ G12A2 to proteins containing LRR is consistent with the possibility that it plays a role in mosquito innate immunity or development. This will be assessed in future studies.

Extracellular LRR proteins in mammals involved in innate immunity include TLR and are characterized by an LRR region, a trans-membrane domain, and a cytoplasmic Toll/IL-1 receptor domain. Although it has been suggested that TLR proteins in insects such as Drosophila and mosquitoes evolved independently of mammals, dipteran TLRs retain some of the structure and function seen in vertebrate TLRs. Toll receptors in Drosophila function in development, antifungal, and antibacterial responses. Drosophila melanogaster Toll-1 has also been implicated in antiviral responses. Mosquito TLRs that function in antibacterial and antifungal responses have been characterized in Anopheles gambiae and Ae. aegypti. Additionally, the involvement of the Toll pathway has been shown in regulating resistance to dengue virus infection in Ae. aegypti mosquitoes. The similarity of the translation product of CQ G12A2 to a TLR of Ae. aegypti provides additional support for a probable role in innate immune responses in Cx. p. quinquefasciatus mosquitoes that needs further study.

We looked at the temporal gene expression in midgut tissue of mosquitoes fed uninfected blood and found visible support for a probable role in innate immune responses in Cx. p. quinquefasciatus mosquitoes that needs further study.
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increases in expression after the blood meal. The apparent change in expression at the earlier time points after blood ingestion suggests a role of CQ G12A2 early in digestion or in synthesis of proteins used for peritrophic matrix formation. Additionally, the putative expression product of CQ G12A2 may be involved in defense reactions to invading microorganisms ingested with the blood. At different times after infection, we cannot entirely attribute these differences to the expression of CQ G12A2. There may be other unknown factors interacting with WNV and contributing to this phenomenon. Further studies are underway to address these possibilities. Interestingly, we did observe that WNV titer was lowest when the expression of CQ G12A2 seemed to be upregulated. We believe that these findings are consistent with the possibility that CQ G12A2 plays a role in antiviral responses, which is comparable to other gene functions in the TLR family. These studies showed that WNV infection alters the expression of genes that may be involved in antiviral responses in the midgut tissue of female Cx. p. quinquefasciatus. The similarity of the CQ G12A2 putative protein to LRR-containing proteins and its unique expression pattern in WNV-infected Cx. p. quinquefasciatus female mosquitoes suggests a potential role in antiviral responses, perhaps as a protein of the innate immune pathway. Further studies are warranted to characterize this gene and define its involvement in WNV infection of CX. p. quinquefasciatus, which could enhance our understanding of Culex spp.–WNV interactions and contribute to our understanding of vector competence.

Figure 2. Multiple protein sequence alignment of a portion of the Cx. p. quinquefasciatus CQ G12A2 putative translation product (94 amino acids; CQ G12A2 protein; accession no. GO254244), with partial sequences representing the Toll protein (AE Toll protein; accession no. XP_001650338.1) and leucine-rich repeat protein (CQ leucine-rich repeat protein; accession no. XP_001846467.1) from Ae. aegypti and Cx. p. quinquefasciatus, respectively. The numbering represents the amino acid number. The amino acids that are underlined represent the putative conserved region containing leucine repeats in the CQ G12A2 translation product. The boxed residues represent the hydrophobic amino acids contained in the underlined region. The stars indicate leucine residues conserved in all three aligned amino acid sequences. LRR, leucine-rich repeat region.

Although we did detect significant differences in WNV titer at different times after infection, we cannot entirely attribute these differences to the expression of CQ G12A2. There may be other unknown factors interacting with WNV and contributing to this phenomenon. Further studies are underway to address these possibilities. Interestingly, we did observe that WNV titer was lowest when the expression of CQ G12A2 seemed to be upregulated. We believe that these findings are consistent with the possibility that CQ G12A2 plays a role in antiviral responses, which is comparable to other gene functions in the TLR family. These studies showed that WNV infection alters the expression of genes that may be involved in antiviral responses in the midgut tissue of female Cx. p. quinquefasciatus. The similarity of the CQ G12A2 putative protein to LRR-containing proteins and its unique expression pattern in WNV-infected Cx. p. quinquefasciatus female mosquitoes suggests a potential role in antiviral responses, perhaps as a protein of the innate immune pathway. Further studies are warranted to characterize this gene and define its involvement in WNV infection of Cx. p. quinquefasciatus, which could enhance our understanding of Culex spp.–WNV interactions and contribute to our understanding of vector competence.
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