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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 *Flavivirus*) 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.[5,9,10]

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

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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.[18] 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, dilation 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 others[24] 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 Sindbis[21] and dengue viruses[25,26] and in *Anopheles gambiae* Giles against O'nyong-nyong virus.[27] 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 Duracool 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 pledgets 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[29] and held at −80°C until processing to determine blood

meal titer. 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*g* 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°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 In Genius 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 proprietary protocols.[30] Before reverse transcription-polymerase chain reaction (RT-PCR), the RNA from each sample was treated with DNase to eliminate contamination from DNA. Using 48 13-mer arbitrary primers and oligo dT, RT-PCR reactions were performed and analyzed on a denaturing polyacrylamide gel. Fluorescence was analyzed using a digital Hitachi FMBIO II Fluorescence Imaging System (Hitachi Software Engineering America, Ltd., San Francisco, CA). We chose 11 of the 26 reproducible PCR amplified products showing an increase in expression after blood ingestion, and these were excised from the gel. The fragments were cloned by TA cloning into the pCR2.1 cloning vector (TA Cloning Kit; Invitrogen). Recombinant plasmids were transformed into TOP10 chemically competent *Escherichia coli* cells following the included protocol (TA Cloning Kit; Invitrogen) and grown overnight on Luria broth (LB) plates containing X-gal and ampicillin in a 37°C incubator. White colonies were used to inoculate LB broth containing ampicillin and grown under constant aeration overnight at 37°C. Recombinant plasmid DNA was isolated from the *E. coli* cells using the QIAprep Spin miniprep kit (Qiagen, Valencia, CA). Positive recombinant plasmids were identified by digestion with restriction enzymes, analyzed by agarose gel electrophoresis, and visualized on an InGenius gel documentation system (Syngene, Frederick, MD).

Sequence analysis

One of the PCR amplified products of interest, the cloned differentially expressed product, 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.

Semi-quantitative RT-PCR

In a separate experiment, temporal gene expression of the cloned differentially expressed product, CQ G12A2, was analyzed using semi-quantitative RT-PCR for midguts of mosquitoes fed uninfected or WNV-infected blood meals and allowed to incubate for 10 days. Clone-specific primers were designed (Integrated DNA Technologies, Coralville, IA) and included the following: CQ G12A2 forward primer, 5′-CTTGCAGGAGTCTATATTTGAGTC-3′, CQ G12A2 reverse primer, 5′-ATGAGTTTATCCTGTTGTTTGT GA-3′, to generate a PCR product of 362 bp. RT-PCR was performed on RNA isolated from midguts dissected at different time points (0, 3, 6, and 9 hours and 1–10 days; $N = 20$ midguts/time point) after feeding on uninfected blood or WNV-infected blood, using clone specific primers described previously and following the included protocol of the Enhanced Avian HS RT-PCR kit (Sigma Aldrich, St. Louis, MO). Total RNA from each sample was treated with RQ1 RNase-free DNase (Promega, Madison, WI) before RT-PCR to eliminate contamination from DNA. Semi-quantitative RT-PCR reactions were amplified on an MJ Mini Gradient Thermo Cycler (Bio-Rad Laboratories). All RT-PCR products were analyzed on 2% agarose gels, stained with ethidium bromide, and visualized on an InGenius gel documentation system. All semi-quantitative RT-PCR analyses were repeated three times.

Quantitative RT-PCR

The amount of WNV RNA in blood meals and midgut samples was determined using a LightCycler 480 system (Roche, Mannheim, Germany) and Superscript III One-Step Quantitative RT-PCR kit (Invitrogen) for quantitative real-time TaqMan RT-PCR (qRT-PCR) using methods described elsewhere.[12] Standard curves were based on 10-fold serial dilutions of known WNV titers determined by plaque assay as described elsewhere.[12]

Statistical analysis

Box plots were used to test viral titers of pooled midgut samples for normality.[31] The lack of normality was verified with Kolmogorov-Smirnov tests. Viral titers at each time point were log-transformed [log ($x + 1$)] before analysis of variance (ANOVA) with the GLM procedure in SAS.[31] If significance was observed, the Duncan multiple range test was used to determine which means were significantly different.[31]

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.[32] A comparison of midgut transcription alterations 4 days after female mosquitoes ingested uninfected blood or blood meals containing ~6.8 plaque-forming units (pfu) WNV/mL showed 26 amplification products with reproducible differences. Of these, 21 cDNAs showed an increase in expression, and 5 showed a decrease in expression as a result of WNV presence. Eleven cDNAs, ranging in size from 190 to 418 bp and exhibiting increased expression after infection with WNV, were cloned. Six of these 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_001650338)[33] (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 d 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; $P \leq 0.001$; 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.[34] 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.[35] 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.[35–37] 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.[37] 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.[38] Toll receptors in *Drosophila* function in development, antifungal, and antibacterial responses.[39] *Drosophila melanogaster* Toll-1 has also been implicated in antiviral responses.[40] Mosquito TLRs that function in antibacterial and antifungal responses have been characterized in *Anopheles gambiae*[41] and *Ae. aegypti*.[42] Additionally, the involvement of the Toll pathway has been shown in regulating resistance to dengue virus infection in *Ae. aegypti* mosquitoes.[26] 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 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.[43] Additionally, the putative expression product of CQ G12A2 may be involved in defense reactions to invading microorganisms ingested with the blood.[18]

The temporal expression of CQ G12A2 in midgut tissue of mosquitoes fed WNV-infected blood seemed to increase at 6, 8, and 10 days after infection. Expression of CQ G12A2 at 6 days after infection suggests that this gene is likely involved in more than blood digestion because all of the blood is already digested by this time.[44] Activation of CQ G12A2 at these times is consistent with the possibility of a role in virus replication and dissemination, because WNV has been shown to replicate and even disseminate in *Cx. p. quinquefasciatus* by 4 days after infection (S. L. Anderson and others, unpublished data).[32] However, because the putative protein product of CQ G12A2 shows similarity to proteins that play a role in immune functions, a role for this product in promoting viral replication is not strongly supported. We studied whether the expression of CQ G12A2 influenced WNV in midguts and found WNV in all time points tested. 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 up-regulated. 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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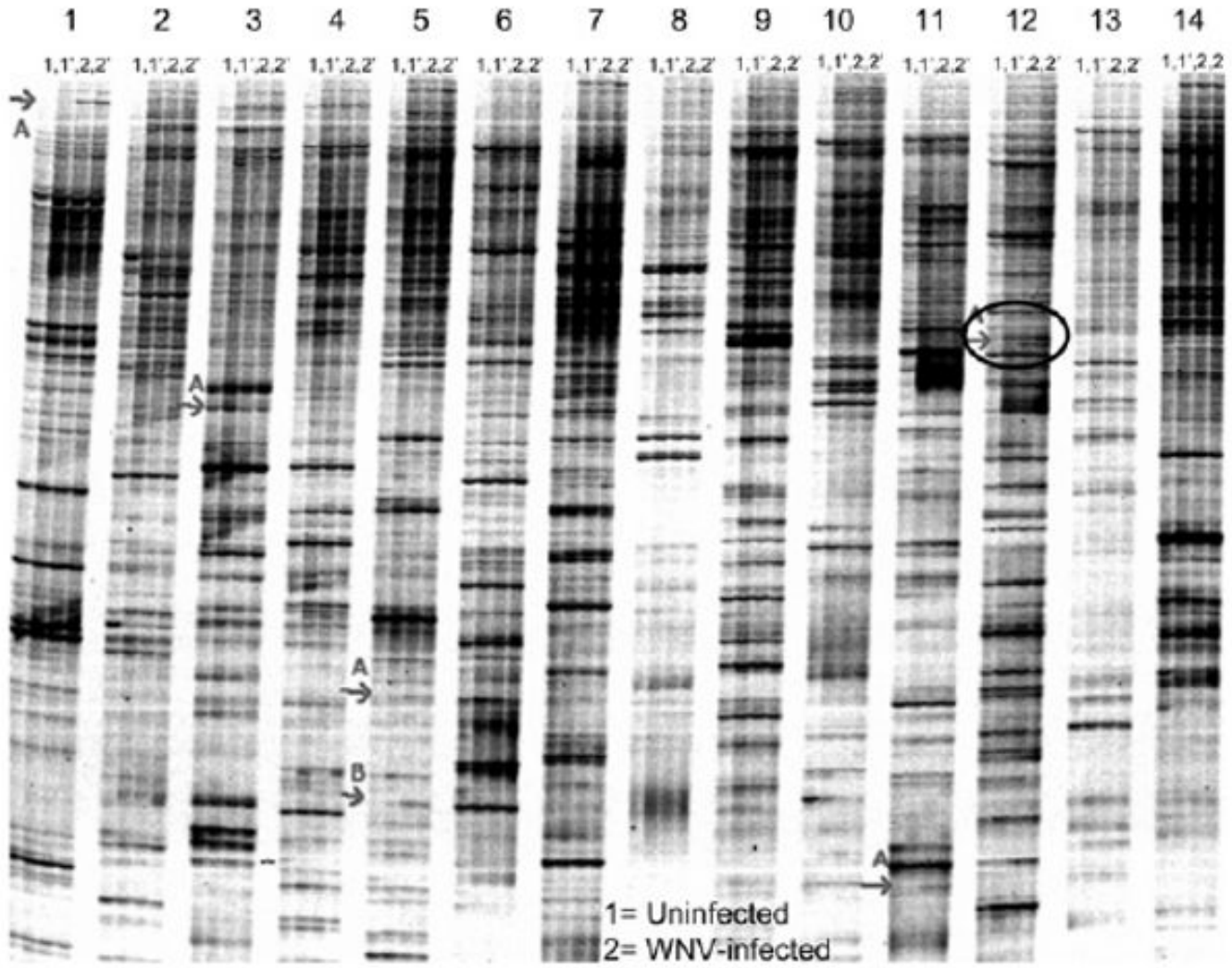

Figure 1.

Results from the fluorescent differential display analysis of the gene expression differences between midgut tissue isolated from *Cx. p. quinquefasciatus* female mosquitoes 4 days after ingestion of uninfected (Sample 1) and WNV-infected (Sample 2) blood. Numbers 1–14 at the top of the figure represent 14 of the 48 primers used in the DD reaction. Each primer was used in a PCR reaction with RNA from uninfected midguts (Sample 1, first two lanes) and infected midguts (Sample 2, next two lanes). Each reaction was duplicated, as shown on the figure as Samples 1′ and 2′. The red arrows indicate those products with differential gene expression. The black circle points out the expression of the product CQ G12A resulting from fluorescent differential display.

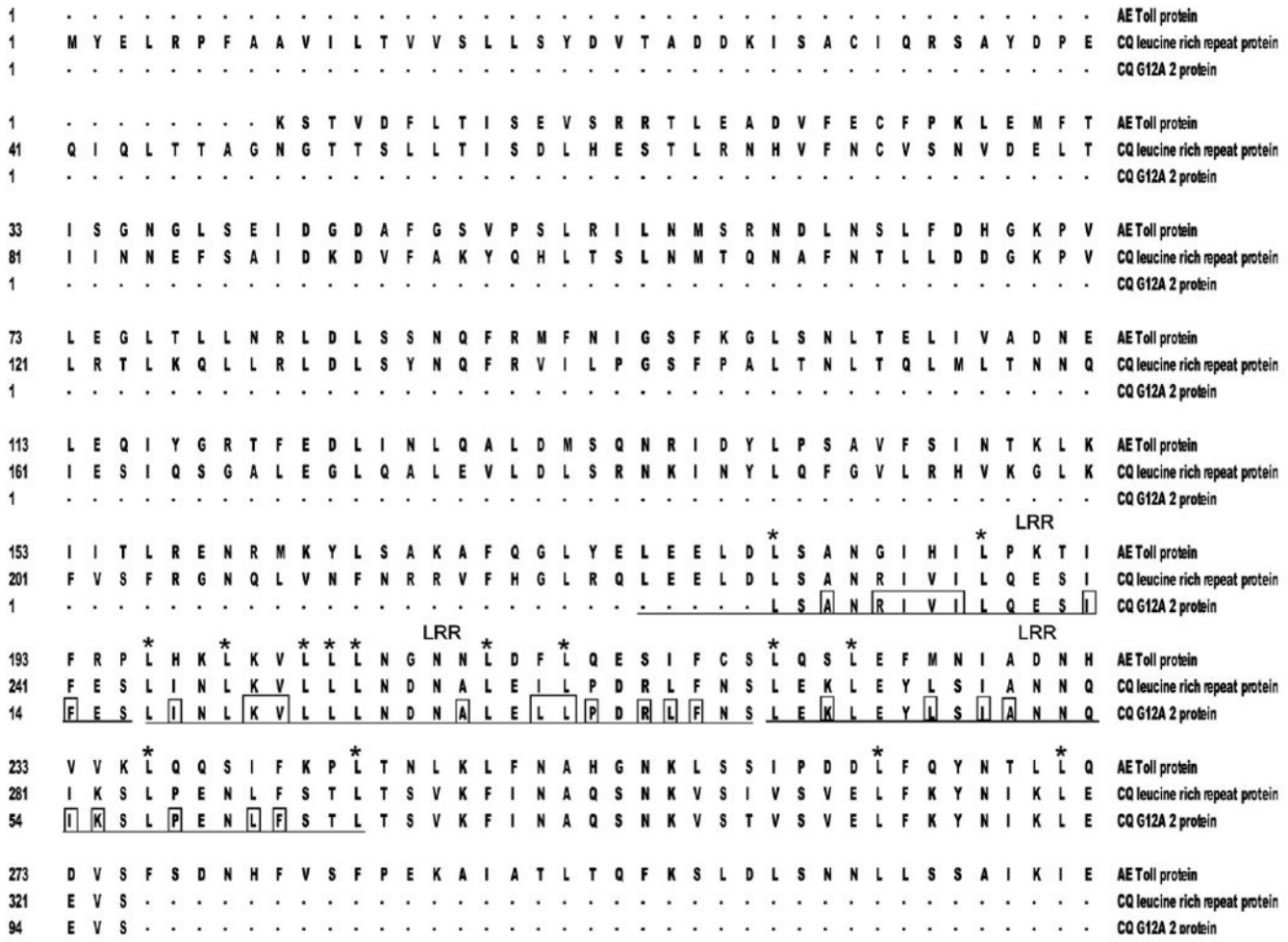

Figure 2.

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.

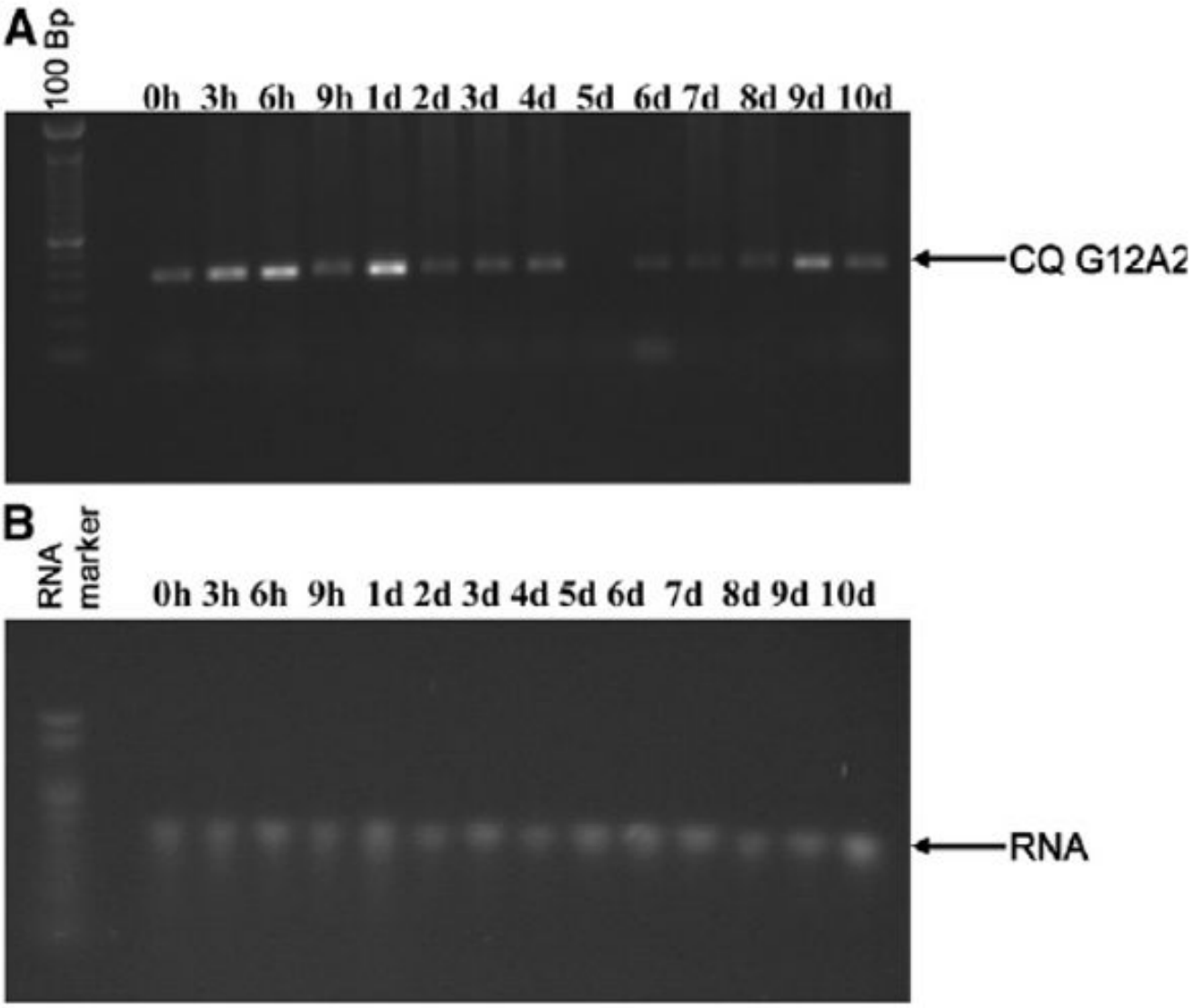

Figure 3.
Semi-quantitative RT-PCR analyses of DD clone CQ G12A2 in *Cx. p. quinquefasciatus* mosquito midgut RNA isolated from females fed an uninfected blood meal (**A**). **B**, Integrity of the RNA. Bp, base pairs; h, hours; d, days.

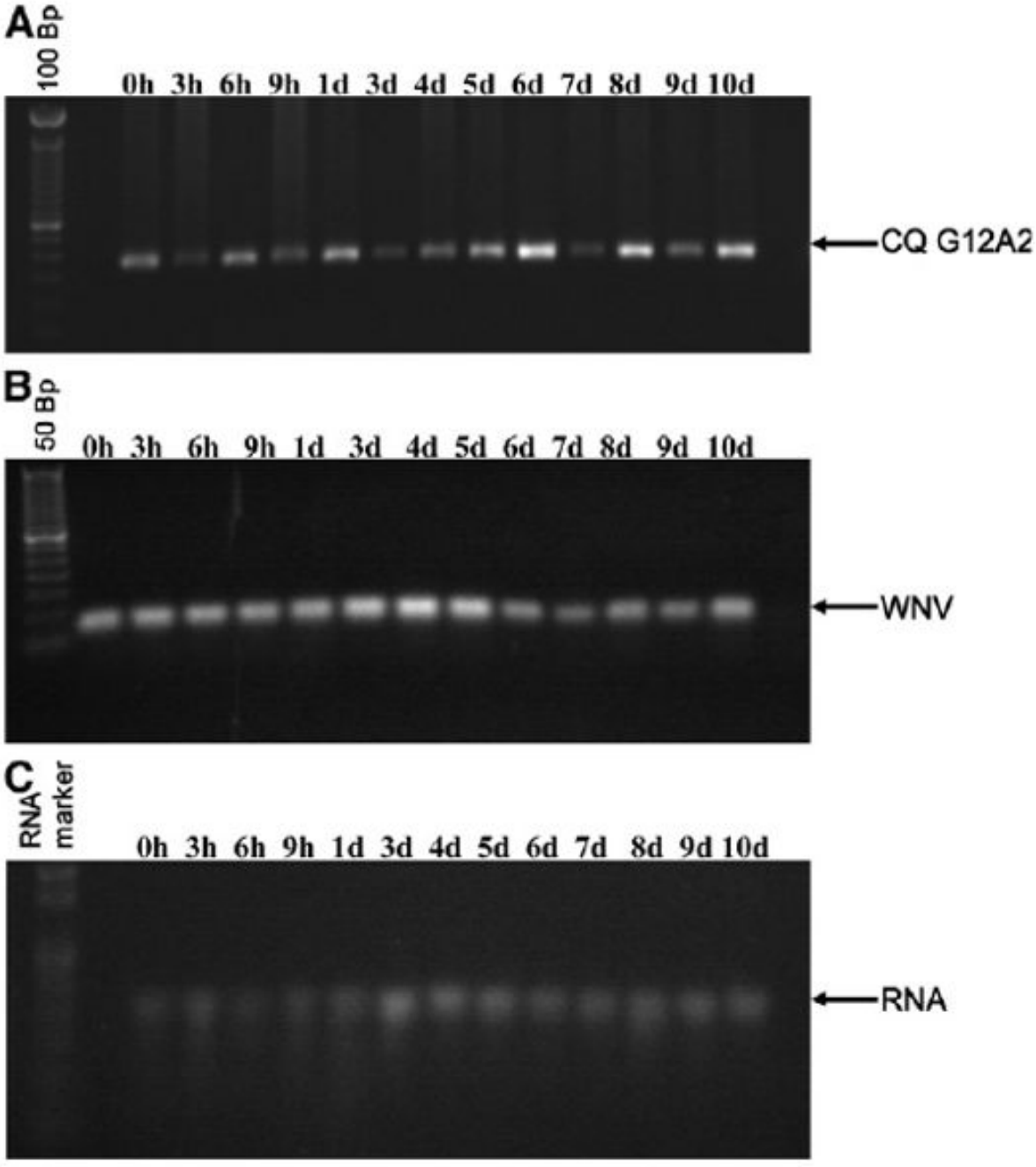

Figure 4.

Semi-quantitative RT-PCR analyses of DD clone CQ G12A2 in *Cx. p. quinquefasciatus* mosquito midgut RNA isolated from females fed a WNV-infected blood meal (**A**). **B**, Agarose gel of the qRT-PCR analysis of WNV titer in the same RNA used in **A. C**, Integrity of the RNA. Bp, base pairs; h, hours; d, days.

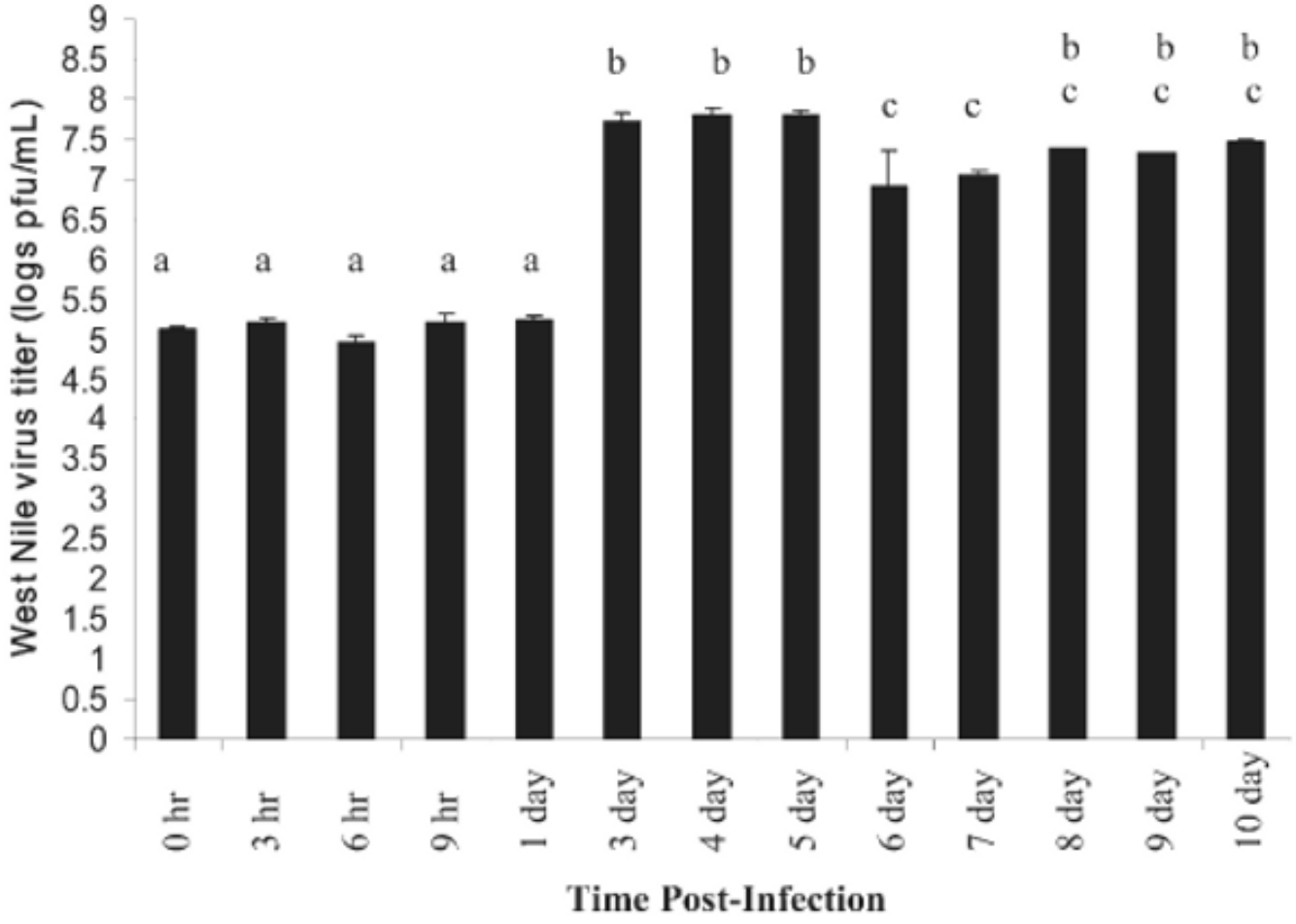

Figure 5.

Culex p. quinquefasciatus midgut WNV titer at different time points after infection. When analyzed using analysis of variance, there is a significant difference in WNV titer in the mosquito midgut at different times after infection ($F = 70.54$, df = 12.38, $P \leq 0.001$). Means with the same letter are not significantly different using the Duncan multiple range test.[31]