QUANTITATIVE ANALYSIS OF LA CROSSE VIRUS TRANSCRIPTION AND
REPLICATION IN CELL CULTURES AND MOSQUITOES

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Abstract. La Crosse (LAC) virus (family Bunyaviridae, genus *Orthobunyavirus*) small (S) segment negative-sense RNA genome (vRNA), positive-sense full-length RNA complement (vcRNA), and subgenomic mRNA were assayed in infected cell cultures and female *Aedes* (*Ochlerotatus*) *triseriatus* mosquito tissues using quantitative PCR (Q-PCR). During persistent infection of C6/36 (*Aedes albopictus*) and MAT (*Aedes triseriatus*) cultured cells and cytopathic infection of BHK-21 cultured cells, LAC vRNA was the most abundant RNA species, followed by mRNA and vcRNA. RNA copy numbers per cell were quantified and vRNA correlated to virus titer in cell culture medium. The Q-PCR assay proved more sensitive than reverse transcription (RT)-PCR and immunofluorescence assays (IFA) for detecting LAC virus infection of mosquitoes. After infection of female mosquitoes orally, quantities of LAC RNA increased in ovaries for 6 days, and as ovarian biosynthetic activity quiesced, LAC RNA quantities decreased then remained detectable at a low level. After a second, noninfectious blood meal, quantities of LAC RNA in ovaries increased significantly, quantitatively confirming correlation of LAC virus RNA synthesis with vector metabolic activity. Coregulation of viral replication and mosquito ovary metabolic activity may condition efficient transovarial transmission.

INTRODUCTION

La Crosse (LAC) virus (family Bunyaviridae, genus *Orthobunyavirus*) is an important cause of pediatric arboviral encephalitis in North America. In nature, LAC virus cycles between vertebrate and invertebrate hosts. Infections can cause serious disease in human hosts, but there are few untoward effects in the mosquito vector. Most remarkably, LAC virus can be maintained in nature by transovarial transmission in mosquitoes and can replicate in potentially vulnerable tissues of mosquito ovaries and embryos without deleterious effects.

The LAC virus consists of three single-stranded negative-sense RNA segments in helical nucleocapsids surrounded by a host cell–derived lipid envelope. The genome segments are designated large (L), medium or middle (M), and small (S) and code for four structural proteins: L segment, the RNA-dependent RNA polymerase; M segment, the envelope glycoproteins, G1 and G2; S segment, the nucleocapsid protein, N. Additionally, two nonstructural proteins, NS* and NS*M, are coded from the S and M RNA genome segments, respectively.

Reverse transcription polymerase chain reaction (RT-PCR) was previously used to investigate the possible coregulation of LAC virus transcription and replication with host metabolic activity in tissues of infected female *Aedes triseriatus* mosquitoes. In ovaries of orally infected mosquitoes that became quiescent after a blood meal and oviposition, LAC mRNA and genome-complementary RNA (vcRNA) declined to undetectable amounts; however, genome RNA (vRNA) remained detectable by RT-PCR. After a second, noninfectious blood meal and activation of another gonadotrophic cycle, synthesis of LAC mRNA and vcRNA was induced and both increased to amounts similar to those prior to quiescence. This suggested that the synthesis of LAC virus RNA species was coregulated with the metabolic activity of the mosquito ovary; however, quantitative analysis was not performed.

Real-time quantitative-polymerase chain reaction (Q-PCR) allows quantitation of a specific nucleic acid in a sample. Quantitative assays have been used for *in vitro* and *in vivo* studies of several members of the Bunyaviridae family including Crimean Congo hemorrhagic fever and Rift Valley fever viruses, Tomato spotted wilt virus, and Black Creek Canal, Puumala, and Sin Nombre hantaviruses. The sensitivity of these assays is remarkable, detecting an equivalent of 10–100 tissue culture infectious dose (TCID)₅₀/mL of the respective virus genomes and as few as 1,000 copies of individual RNA species in cell cultures and vertebrate, insect, and plant tissues. These studies have applied Q-PCR in diagnosis, studies of pathogenesis, and determination of effectiveness of antiviral treatment in bunyavirus infections. Q-PCR technology has yet to be used to study the effect of changes in host physiologic and metabolic activity on regulation of bunyavirus replication and transcription in arthropod vectors.

In these studies, we developed and characterized a Q-PCR assay in cell cultures. We then used Q-PCR to quantitate viral RNAs in mosquito ovaries to test the hypothesis that coregulation of viral transcription and replication with ovarian biosynthetic activity are potential determinants of efficient transovarial transmission of LAC virus by *Ae. triseriatus*.

MATERIALS AND METHODS

Cells. C6/36 (*Aedes albopictus*), MAT (Mather *Aedes triseriatus*), BHK-21 (baby hamster kidney), and Vero (African green monkey kidney) cells were grown in Leibovitz L-15 medium including 10% or 20% (MAT) fetal bovine serum (FBS) and 100 units/mL penicillin plus 100 µg/mL streptomycin in 25 cm² flasks at 28°C (mosquito cells) or 37°C (mammalian cells). Cells were maintained in the same medium with 1% FBS.

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Virus. LAC virus (wt10) was isolated in 1965 from the brain of a patient with a fatal case of LAC encephalitis. The virus had been passed three times in suckling mouse brain and six times in BHK-21 cell culture. Stock virus (LAC wt10) was prepared in BHK-21 cells and titrated by the Karber method in Vero cells in 96-well plates, and had a titer of $10^{8.4}$ TCID$_{50}$/mL.

Mosquitoes. *Ae. triseriatus* mosquitoes (AIDL strain) originated from field material collected near La Crosse, Wisconsin, in 1981, and have been colonized continuously at the Arthropod-borne and Infectious Diseases Laboratory at $70^\circ F$, 70% relative humidity, 16-hour light, 8-hour dark cycles. Adult female *Ae. triseriatus* mosquitoes were infected by ingesting an artificial blood meal consisting of equal parts of defibrinated sheep blood and infected BHK-21 cell culture medium. At predetermined times postinfection (PI) (Days 1, 3, 6, 9, 14, 21, 28, 30), including after a subsequent blood or sugar meal at Day 30 (Day 30 plus 24, 48, 72, and 96 hours), ovaries were dissected for RNA extraction and heads were fixed for fluorescent antibody analysis from 10 infected and 2 uninfected female mosquitoes that had engorged a blood meal.

Direct detection of viral antigen by immunofluorescent analysis (IFA). Mosquito heads were severed from the bodies, squashed on acid washed slides, fixed in cold acetone, and stained with a fluorescein isothiocyanate (FITC)-conjugated anti-LAC mouse polyclonal antibody F92. Slides were examined for fluorescence with an Olympus BH2 epifluorescence microscope.

Experimental infection of cell culture. BHK-21 cells were infected with LAC virus at a MOI of 0.01, C6/36 and MAT cells at a MOI of 10 as described previously. Infection of mammalian cells at a lower MOI precluded aberrant RNA synthesis. At predetermined times PI (1, 3, 6, 9, 14, 21, 28, 30, and 34 days for mosquito cells and 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 hours for mammalian cells), the cells were cultured.
harvested, scraped into the medium, and centrifuged. The supernatant was removed for virus titration, and the cell pellets were stored at −70°C.

**RNA extraction, cDNA synthesis, and PCR amplification.** Total RNA was extracted from cells and tissues with a single-step acid guanidinium thiocyanate-phenol-chloroform method.18 RNA was precipitated in isopropanol, and stored at −70°C. Approximately 3 μg of total cell RNA or one fourth of total RNA from tissues of a single mosquito was mixed with each reverse transcription primer (Table 1 and Figure 1). After reverse transcription, cDNA was purified with the QIAquick kit from Qiagen and PCR amplified for 25 cycles of Taq DNA polymerase according to manufacturers’ instructions.

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**Q-PCR analysis.** One-fourth of cDNA prepared from each reverse transcriptase reaction was used for Q-PCR analysis. Samples were amplified in 50 μL with optimized forward and reverse primer and probe concentrations. Serial 10-fold dilutions of known copy number plasmids (1 × 10^12 to 1 × 10^4 copies) were amplified simultaneously to generate standard curves. For accurate comparison of LAC RNA values, the actin mRNA values within each sample were normalized. The mRNA copy number was calculated after subtraction of vcRNA copy number. Q-PCR primers and labeled probes were purchased from Applied Biosystems and Q-PCR reagents were obtained from Stratagene (Brilliant Q-PCR reagents with SureStart Taq DNA polymerase) and used according to manufacturers’ instructions.

| Statistical analysis.** Comparisons of the copy number of each LAC RNA species in ovary samples at sequential time points were performed using SAS statistical analysis software. Differences in RNA quantities between time points, as well as between blood-fed and sugar-fed mosquitoes at the same time point, were analyzed statistically using Scheffe’s t test and Tukey’s t test based on their controlled conservative comparisons.
FIGURE 2. Q-PCR analysis of LAC virus RNA species in infected cultured cells and titration of LAC virus in cell culture medium. Copy number of each LAC S segment-specific RNA species was calculated per cell from Q-PCR analysis of total RNA extracted from pelleted cell cultures at times PI shown. Virus titer in cell culture medium was determined by end-point titrations and expressed as TCID$_{50}$/mL. Each copy
RESULTS

Q-PCR analysis of LAC RNA species in cultured cells. LAC virus S segment vRNA, mRNA, and vcRNA in infected C6/36, MAT, and BHK-21 cells were quantitated at predetermined time points PI and copy numbers per cell were calculated. Each RNA species was detectable throughout the time course of infection.

In C6/36 cells, the copy number of LAC S segment mRNA peaked at $2.7 \times 10^4$ copies per cell on Day 9 PI, then stabilized between $1.0 \times 10^4$ and $1.5 \times 10^4$ copies per cell by Day 14 PI; vRNA peaked at $5.1 \times 10^4$ copies per cell on Day 6 PI, then stabilized between $1.6 \times 10^4$ and $1.8 \times 10^4$ copies per cell by Day 9 PI; vcRNA peaked at $6.6 \times 10^3$ copies per cell on Day 6 PI, then stabilized between $1.2 \times 10^3$ and $1.5 \times 10^3$ copies per cell by Day 9 PI. At each time point throughout the course of infection, the quantity of LAC vRNA exceeded that of LAC mRNA, which exceeded the quantity of vcRNA (Figure 2A).

In MAT cells, which are derived from the natural vector of LAC virus, the full-length LAC S segment-specific RNA copy numbers were greater than in C6/36 cells, although mRNA copy numbers were lower. S segment mRNA peaked at $1.1 \times 10^5$ copies per cell on Day 9 PI, then stabilized between $6.2 \times 10^4$ and $7.2 \times 10^4$ copies per cell by Day 14 PI; S segment vRNA peaked at $2.2 \times 10^5$ copies per cell on Day 6 PI, then stabilized between $1.0 \times 10^5$ and $1.5 \times 10^5$ copies per cell by Day 9 PI; vcRNA peaked at $3.4 \times 10^4$ copies per cell on Day 6 PI, then stabilized between $1.3 \times 10^4$ and $2.9 \times 10^4$ copies per cell until 48 hours PI. The quantity of LAC vRNA exceeded that of LAC mRNA, which exceeded the quantity of vcRNA except at Days 6 and 30, when the amount of vcRNA was higher than mRNA (Figure 2B).

In the acute, cytolytic infection of BHK-21 cells, LAC S segment-specific mRNA and vcRNA quantities were 5- to 10-fold higher than in mosquito cells. At the MOI used, the mRNA quantity peaked at $7.9 \times 10^5$ copies per cell at 16 hours PI, then remained between $1.7 \times 10^6$ and $2.5 \times 10^6$ copies per cell until 48 hours PI; vRNA peaked at $1.8 \times 10^5$ copies per cell at 16 hours PI, then remained between $9.3 \times 10^4$ and $1.1 \times 10^5$ copies per cell until 48 hours PI; vcRNA peaked at $3.4 \times 10^4$ copies per cell at 28 hours PI, then remained between $1.3 \times 10^4$ and $2.9 \times 10^4$ copies per cell until 48 hours PI. The quantity of LAC vRNA exceeded that of LAC mRNA, which exceeded the quantity of vcRNA except at 28 and 36 hours PI (Figure 2C); the relative copy numbers of each RNA were the same as in mosquito cells.

LAC virus titer in C6/36 cell culture medium peaked at $10^{7.3}$ TCID\(_{50}\)/mL by Day 14 and then decreased to a plateau of $10^{6.0}$ TCID\(_{50}\)/mL by Day 28. LAC virus titer in MAT cell culture medium peaked at $10^{7.6}$ TCID\(_{50}\)/mL by Day 6 and then decreased to $10^{6.0}$ TCID\(_{50}\)/mL by Day 34. LAC virus titer in BHK-21 cell culture medium remained at peak titers of $10^{7.4}$ TCID\(_{50}\)/mL to $10^{8.0}$ TCID\(_{50}\)/mL from 16 hours to 48 hours PI. As the RNA copy numbers in mosquito cells stabilized, the virus titer in cell culture medium also stabilized (Figures 2A–2C).

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number data point is from a single amplification. Each titration data point is an average from eight replicates on a single 96-well plate. A, C6/36 cells; B, MAT cells; C, BHK-21 cells.
Correlation coefficients were determined for each set of values between virus titers and copy numbers of intracellular vRNA. For C6/36 cells, MAT cells, and BHK-21 cells, r values were 0.7724, 0.5925, and 0.6699, respectively.

Sensitivity of detection of LAC virus disseminated infection in *Aedes triseriatus* mosquitoes by IFA and RT-PCR. Each mosquito used in standard RT-PCR or Q-PCR analysis was assayed for disseminated infection by IFA of head tissues prior to ovary dissection. Viral antigen was first detected in head tissues at Day 6 PI. By Day 28 PI, 100% (10 of 10) of mosquitoes had detectable viral antigen in heads (data not shown).

To compare the sensitivity of standard RT-PCR to Q-PCR analysis, LAC mRNA, vRNA, vcRNA, and actin mRNA from ovaries of individual infected mosquitoes were RT-PCR amplified and products were visualized after agarose gel electrophoresis. LAC mRNA was first detectable by RT-PCR in mosquito ovaries at 6 days PI, vRNA at 1 day PI, and vcRNA at 3 days PI. The number of mosquito ovaries with LAC RNA detectable by standard RT-PCR varied between 20% and 100%, with vRNA being detectable in 100% of ovary samples from Day 9 onward. The detection limit of RT-PCR for a pair of ovaries, determined by visual examination of products amplified in 25 cycles from known concentrations of plasmid DNA, was found to be between $1 \times 10^8$ and $1 \times 10^9$ copies for each RNA species (results not shown). The detection limit for Q-PCR was approximately $4 \times 10^4$ copies per ovary pair (data not shown). Q-PCR thus was approximately 244 to 2,440 times more sensitive than traditional RT-PCR.

Q-PCR analysis of LAC infected *Aedes triseriatus* ovary tissues. The copy numbers of LAC virus S segment vRNA, mRNA, and vcRNA in ovaries of each infected female *Ae. triseriatus* mosquito were determined by Q-PCR analysis, and the average copy number of each LAC RNA in each sample is shown in Figure 3. In contrast to standard RT-PCR, LAC virus S segment mRNA and vcRNA were detectable in all 10 ovary samples at each time point from Day 1 PI throughout the time course of infection, and vRNA was detectable in every ovary sample at each time point from Day 6 PI onward. In the ovary samples between Day 1 and Day 30, the only significant difference in copy numbers between sequential time points occurred between Day 3 and Day 6 (mRNA $P = 0.0002$, vcRNA $P < 0.0001$).

After the noninfectious blood meal at Day 30, the average mRNA copy number increased by 24 hours, then began to decline at 96 hours. After a sugar meal, the average copy number continued to decline for 24 hours, then increased sharply until 72 hours. The vcRNA and vRNA showed similar patterns, with sharp increases within 24 hours after the blood meal. The increase in vcRNA was delayed to 48 hours after a sugar meal and a modest increase in vRNA occurred at 72 hours after the sugar meal. In sugar fed mosquitoes, between Day 30 and Day 31 samples, the copy numbers of mRNA ($P = 0.0317$) and vcRNA ($P = 0.0057$) differed significantly, and between Day 32 and Day 33 vcRNA differed significantly ($P = 0.0183$). None of the sequential vRNA copy numbers from either blood- or sugar-fed mosquitoes differed significantly. Additionally, at Day 31, the copy numbers of vcRNA significantly.
from blood-fed mosquitoes differed statistically from those of sugar fed mosquitoes ($P = 0.0169$); at Day 32, vRNA and vcRNA differed statistically ($P = 0.0360$ and 0.0322, respectively); and at Day 34, vcRNA differed statistically ($P = 0.0022$). In contrast, the copy numbers of mRNA of blood and sugar fed mosquitoes did not differ significantly.

**DISCUSSION**

The ability of LAC virus to be transovarially transmitted in *Ae. triseriatus* mosquitoes without causing extensive pathogenicity, even in diapausing embryos, is truly remarkable. One mechanism that could condition this is co-regulation of viral transcription and replication with host metabolic activity.

Previous studies used either IFA$^{22-24}$ or RT-PCR$^4$ to examine virus infection and replication in mosquito tissues after an infectious blood meal. In this study, a Q-PCR assay was used to accurately and specifically assay quantities of the three LAC virus S segment RNAs (mRNA, vRNA, and vcRNA) in infected mosquito and mammalian cell cultures and in *Ae. triseriatus* ovary tissues. In both persistently infected mosquito cell cultures and cytolically infected mammalian cell cultures, the three RNA species could be distinguished and copy number per cell was calculated. After an initial increase in copy number within 3 days PI (mosquito cells) or 8 hours PI (mammalian cells), the quantities of LAC RNA declined in mosquito cells and remained at maximum levels in mammalian cells throughout the remainder of infection. In all cell types, vRNA was more abundant than mRNA, which was more abundant than vcRNA.

The sensitivity of the Q-PCR analysis developed for this study was compared with IFA and traditional RT-PCR. Disseminated infection could be detected in mosquito head tissue by IFA by Day 9, whereas infection of ovaries could be detected by RT-PCR and Q-PCR by Day 1. RT-PCR and Q-PCR were more sensitive than IFA, with both detecting infection in mosquitoes not positive by IFA. Q-PCR was more sensitive than RT-PCR, detecting a greater number of infected mosquitoes per time point and a smaller quantity of RNA per ovary.

LAC RNA from *Ae. triseriatus* ovaries was quantitated by Q-PCR at predetermined time points post-infectious blood meal. As observed in LAC virus infected cell cultures, the abundance of viral RNA species in ovary tissues was vRNA > mRNA > vcRNA. Between Days 3 and 6 PI, the quantities of each LAC RNA species in ovaries decreased significantly (Figure 3). This decrease could be due to oviposition of eggs containing LAC RNA. After oviposition and prior to a second blood meal, the quantities of each RNA species in ovaries immediately increased and then remained stable through Day 30. After a second, noninfectious blood meal, quantities of each LAC RNA species in ovaries increased within 48 hours and then decreased by 96 hours (Figure 3), again most likely due to oviposition. Surprisingly, after a sugar meal at 30 days PI, mRNA and vcRNA increased by 72 hours, then decreased to quantities similar to those observed at Day 30 after the initial blood meal (Figure 3). The upregulation of viral RNA synthesis could have resulted from many factors, including hormonal activation of ovarian macromolecular synthesis, distension of the abdomen due to quantity of sugar solution ingested, or from uptake of the sugar meal into the midgut (as observed by inclusion of green food coloring in the meal).

Viral replication, indicated by mRNA and vcRNA, was detected in ovaries prior to Day 6 PI, confirming previous studies$^{25}$ that demonstrated viral antigen in the calyx but not in follicles prior to dissemination from the midgut. This may be an artifact resulting from ingestion of a high-titered artificial blood meal (10$^{6.2}$ TCID$_{50}$/mL, compared with viremia titers of 10$^{1.5}$ to 10$^{4.5}$ SMICLD$_{50}$/0.02 mL in chipmunks and squirrels in nature$^{25}$). This could also be due to escape of virus from the midgut in tracheoles. Baculoviruses (family Baculoviridae)$^{26}$ and Sindbis virus (family Togaviridae, genus *Alphavirus*)$^{27}$ can infect and replicate in tracheole cells. Dissection of the ovaries did not remove the tracheoles. A “leaky” midgut$^{28,29}$ could also allow for rapid systemic infection prior to normal routes of dissemination$^{30,31}$ Shortly after ingesting a blood meal the mosquito’s ovaries become highly permeable, with the intercellular spaces of the follicular epithelium and the ovarian sheath becoming larger in preparation for the transport of vitellogenin and other nutrients from fat body into the developing oocyte.$^{32-35}$ Finally, contamination of the tissue during dissection was possible, although unlikely. Ovaries were dissected separate from midguts containing the infectious blood meal and were rinsed twice in sterile PBS. If virus contamination occurred during dissection, only vRNA would be expected and not replicative forms.

When the ovaries became quiescent after oviposition, the copy numbers of the three LAC RNA species stabilized at reduced levels, suggesting co-regulation of LAC virus transcription and replication with vector metabolic activity. Similar results were observed with stabilization of LAC RNA quantities during persistent infections in *C6/36* and MAT mosquito cells. When the mosquito ovary became metabolically active after a second blood meal, LAC RNA transcription and replication were up-regulated (Figure 3), resulting in increased production of LAC virus. The co-regulation of host metabolic activity and LAC transcription and replication could condition noncytopathic persistence and efficient transovarial transmission. Peaks in LAC RNA quantities after a second blood meal (Figure 3) correlate with peak levels of RNA and protein synthesis observed at 36-48 hours and 72 hours in *Aedes aegypti* after a blood meal.$^{36}$ Increased amounts of host mRNA in ovaries could provide targets for scavenging of 5′ nonviral sequences to prime LAC mRNA transcription. Our previous study$^4$ using RT-PCR analysis suggested that virus RNA species involved in replication could be cleared from tissues. However, the more sensitive Q-PCR assay (Q-PCR detection limit between 10$^3$ to 10$^6$ copies as compared with RT-PCR detection limit of 10$^7$ to 10$^8$ copies) demonstrated that LAC virus RNA species persisted in all tissues examined after infection, although in low quantities.

Interestingly, the sugar meal also resulted in an increase of LAC virus mRNA and vcRNA synthesis in the mosquito ovaries. The sugar meal contained green food coloring and was presented through a parafilm membrane that had been “scented” by rubbing on human skin. Approximately 50% of mosquitoes presented with the sugar meal had all of the green-colored fluid in their midguts. Diversion of the sugar meal to the midgut could have resulted in hormonal changes that also up-regulated LAC virus activity. The “host” scent could play a role in ovarian metabolic activity. Increases in
LAC RNA levels in ovaries after a sugar meal were similar to those in mosquito controls exposed to, but not refed on, a vertebrate host in the previous study. Q-PCR analysis is a powerful tool for quantifying copy numbers of LAC virus RNA in specific tissues and organs. The Q-PCR analysis confirms correlation of LAC virus RNA synthesis with host metabolic activity. This demonstration of coregulation of LAC virus RNA synthesis with host metabolic activity provides insight into the molecular basis of a remarkable arbovirus-vector relationship, transovarial transmission, and provides a base of knowledge to further explore these interactions.

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