REAL-TIME REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION QUANTIFICATION OF WEST NILE VIRUS TRANSMITTED BY CULEX PIPIENS QUINQUEFASCIATUS

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Abstract. Transmission experiments are a critical component of vector competence studies. In this study, a real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was used to enumerate the amount of West Nile virus (WNV) secreted in mosquito saliva following oral infection. Culex pipiens quinquefasciatus were allowed to feed on WNV-infected blood, and saliva was collected on days 14 and 21 post-infection (pi). The amount of virus at these two time points varied significantly, with mean equivalent plaque-forming units (pfu) of approximately 30,500 on day 14 pi and 5,800 on day 21 pi. Individual mosquitoes secreted up to 2 × 10^5 pfu of virus. Titer of whole mosquitoes and immunofluorescence assay of salivary glands from mosquitoes collected at these two time points were also used for supplemental comparison. This report describes the first use of a real-time RT-PCR to quantify the amount of WNV in mosquito saliva.

INTRODUCTION
Mosquito-borne viruses are emerging and re-emerging in many areas of the world. Several arboviruses, such as o’nyong-nyong virus, have re-emerged after years of apparent inactivity and are increasing in significance to human health.1,2 The re-emergence and spread of arboviral diseases may be the result of many factors,3 including increased travel, encroachment into new areas, and reduced efforts to control arthropod vectors. The introduction and rapid spread of West Nile virus (WNV) throughout the United States since 1999 and the highly publicized discussions regarding the potential use of biologic agents by terrorists has increased public awareness of vector-borne diseases. Due to the complexity and specificity of the relationships between the vector, agent, and vertebrate host, many questions remain unanswered despite at least 100 years of investigators studying arthropod-borne diseases.

Susceptible mosquitoes may become infected after feeding on a viremic host, and following an intrinsic incubation period, salivary glands may become infected. Mosquito must salivate during blood feeding because the saliva contains numerous substances that function to counter vertebrate hemostasis by preventing blood coagulation and enhancing vasodilation.4 The transmission of an arbovirus to the vertebrate host depends upon the secretion of infectious virions in the saliva of the arthropod vector. Several methods have been described to collect saliva and, using in vitro and in vivo assays, to determine the amount of virus transmitted by an arthropod during feeding.5–9 Variations in these systems make comparisons between studies difficult, but it is clear that values range widely and are influenced by vector species, virus, incubation conditions, and the time post-infection (pi).

In this study, Culex pipiens quinquefasciatus were fed on infectious blood meals containing WNV.10 Saliva was collected and analyzed 14 and 21 days pi and the amount of virus secreted was determined using a quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS
Mosquitoes. Laboratory-reared Cx. pipiens quinquefasciatus (Sebring strain) were obtained from the Harris County Mosquito Control District (Houston, TX). The Sebring strain was collected in 1988 from Sebring County, Florida. The colony consists of mosquitoes from F30 or later generations and is maintained at 26°C, with a light/dark cycle of 14 hours: 10 hours with a one hour crepuscular period to simulate dawn and dusk. Larvae were fed a 1:1 mixture of TetraMin fish flakes (Doctors Foster and Smith, Inc., Thinelander WI) and crushed Prolab 2500 rodent diet (PMI Nutrition International LLC, Brentwood, MO). Adults were provided with 10% sucrose ad libitum and fed weekly on anesthetized hamsters as per National Institutes of Health (Bethesda, MD) guidelines for the humane use of laboratory animals.

Virus. A 2002 Houston isolate of WNV (lineage I) prepared as a mixed brain/liver homogenate from an infected Blue jay (Cyanocitta cristata), and designated as strain 114 (GenBank accession number AY187013) was used for all experiments.10 Stock virus was produced following a single passage in Vero (green monkey kidney) cell culture and harvested as a tissue culture supernatant. Sequence analysis of this virus confirmed its homology with the 1999 New York strain (NY99 GenBank accession number AF196835). Prior to feeding, fresh virus was propagated by inoculating a monolayer of Vero cells in T25 tissue culture flasks (Nalge Nunc International, Rochester, NY) at a multiplicity of infection of 2.0 using 1 mL of Leibovitz L-15 medium supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. After rocking for one hour, 4 mL of medium was added and cells were either incubated at 37°C for three days and then harvested for the mosquito blood meal or aliquoted and frozen at −80°C. Based on an optimized plaque assay technique,11 the titer of this frozen stock virus was determined to be 2 × 10^8 plaque-forming units (pfu)/mL.

Blood meal preparation. At three days pi, tissue culture supernatant was harvested from the flasks and mixed with an equal volume of defibrinated sheep blood (Colorado Serum Company, Denver, CO). Adenosine triphosphate was added as a phagostimulant to produce a final concentration of 2 mM.

Mosquito infection and maintenance. The infectious blood was heated to 37°C and transferred to the chamber of a Hemotek feeding apparatus (Discovery Workshops, Accrington,
Lancashire, United Kingdom) housed in an isolation glove box within a Biosafety Level 3 (BSL-3) insectary. The chamber was placed on top of the mesh of one-pint cartons containing 50 3–5-day-old female mosquitoes that had been deprived of sucrose for 12 hours prior to presentation of the blood meal. Following a one-hour feeding period, cartons of mosquitoes were chilled, and fully engorged females were transferred to new cartons. Cartons containing the experimental mosquitoes were placed in a sealed, humidified, plastic box and maintained with 10% sucrose in a Precision model 818 environmental chamber (Precision, Winchester, VA) at 26°C, with a 12 hour:12 hour light:dark cycle. A sample of the blood meal was collected and stored at −80°C for later titration. Pre-feeding and post-feeding samples were not collected since previous analysis had determined that there was no significant change in the titer during the one-hour feeding period.

**Saliva collection.** Uninfected control and 14 and 21 days pi female mosquitoes were chilled, and wings and legs were removed and discarded. The proboscis was inserted into 10-µL capacity capillary tubes (Drummond Micropocs, Broomall, PA) filled with Nujol mineral oil (Perkin Elmer, Boston, MA). After 30 minutes, female mosquitoes were placed in labeled storage tubes, and salivary glands were dissected for an immunofluorescence assay (IFA). Oil containing the saliva was expelled under pressure into 1.7-mL tubes for analysis. Saliva was collected from 53 mosquitoes on day 14 pi, 49 mosquitoes on day 21 pi, and 3 control mosquitoes from the same time points. Samples were frozen immediately after collection at −80°C pending analysis.

**Mosquito analysis by titration and IFA.** For titration, mosquitoes collected at 0, 6, 14 and 21 days pi were individually triturated in 1 mL of L-15 medium, filtered through a 0.22-µm syringe filter (Millipore, Carrigwohill, Cork, Ireland), and titrated as serial 10-fold dilution on Vero cells as previously described. Salivary glands were dissected in phosphate-buffered saline and allowed to dry on glass microscope slides. Following fixation in cold acetone for 10 min, WNV antigen was detected with monoclonal antibody H86.13B4 using a standard indirect IFA as previously described. Glands were examined using an Olympus IX-71 inverted microscope with FITC filters and a DP-11 digital camera (Olympus America, Inc., Melville, NY).

**Real time RT-PCR assays.** Viral RNA from saliva samples and seed virus was extracted using the QiAamp Viral RNA Kit (Qiagen, Valencia, CA) following manufacturer’s protocol. The TaqMan One-Step RT-PCR (Applied Biosystems, Foster City, CA) was used for amplification. The reverse transcriptase was performed at 50°C for 20 minutes, 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds and 60°C for one minute. A standard curve (y = −0.287x + 12.053, r² = 0.993) was generated using three replicates of a standard curve created with 10-fold serial dilutions of RNA extracted from virus stock with a known titer of 2 × 10⁸ pfu/mL. Since the standard curve was created from virus stock, actual values are expressed as equivalent pfu, which represents a close estimation of viable virus. Quantification of WNV in saliva samples was calculated by comparison of the threshold cycle (Ct) values of the samples to the standards using the Cepheid software. Real-time viral values are expressed as the average of three wells.

Time points were visualized using SigmaPlot version 8.0 (SPSS Science, Chicago, IL), and significance was determined by Student’s unpaired t-test.

**RESULTS**

Three hundred *C. pipiens quinquefasciatus* were fed a blood meal with a WNV titer of 7.9 log¹₀ 50% tissue culture infectious doses (TCID₅₀)/mL, and after natural mortality during the maintenance period, 172 were analyzed. Titration of 4 whole mosquitoes following oral infection demonstrated all of these to be infected with WNV, with an average titer of 5.5 log¹₀ TCID₅₀/mL on day 0 pi. Whole mosquitoes were titrated on days 6, 14, and 21 pi; all titrated mosquitoes were found to have imbibed infectious WNV, with average titers of 4.4, 6.4, and 6.3 log₁₀ TCID₅₀/mL, respectively (Table 1). Titers ranged from 3.3 to 5.5 log₁₀ TCID₅₀/mL for whole mosquitoes on day 6 pi, from 4.5 to 7.5 log₁₀ TCID₅₀/mL on day 14 pi, and from 5.5 to 7.5 log₁₀ TCID₅₀/mL on day 21 pi (Table 1). Uninfected control mosquitoes (n = 3) from each time point were confirmed to be negative by titration on Vero cell culture.

A real-time RT-PCR was used to measure WNV in saliva from mosquitoes that were fed a blood meal with or without WNV. Saliva from 53 and 49 WNV-exposed female mosquitoes was collected on days 14 pi and 21 pi, respectively. Additionally, saliva from three uninfected control mosquitoes was collected at these time points.

The titers of WNV in saliva were calculated by correlation with the generated standard curve. The quantity per sample was determined by the Cepheid software and ranged from 3 to 198,866 pfu/sample (11–198,866 for day 14 and 3–50,850 for day 21). Ninety-three percent (46 of 53) of the mosquito saliva collected on day 14 pi and 81% (40 of 49) of the mosquito saliva collected on day 21 pi had detectable amounts of WNV. The mean titer for days 14 and 21 was 30,532 and 5,846 pfu/sample, respectively (Figure 1). The difference between these time points (five-fold) was significant (*P = 0.0025*).

All mosquito salivary glands were collected individually and matched with the saliva samples. The correlation be-

## Table 1

<table>
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<tr>
<th>Days pi</th>
<th>No. positive/total</th>
<th>Percent</th>
<th>No. positive/total</th>
<th>Percent</th>
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<tr>
<td>21</td>
<td>1</td>
<td>100/100</td>
<td>49/49</td>
<td>49/49</td>
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* pf = postinfection; ND = not determined.

† Log₁₀ 50% tissue culture infectious dose/mL.
between the RT-PCR and the salivary gland IFA was 0.93. Seven salivary glands that were positive by IFA had no detectable virus in the saliva, whereas one mosquito had no detectable virus by IFA in the salivary glands with measurable virus in the saliva.

**DISCUSSION**

This report describes the first analysis of WNV in mosquito saliva using a quantitative RT-PCR. This technique appears to be valuable for laboratory studies on vector competence and pathogen transmission. For example, the technique negates the requirement for a susceptible vertebrate to demonstrate transmission, and is not influenced by problems of contamination that are often encountered during analysis of mosquito saliva by *in vitro* cell culture methods. However, the assay may not be practical for analysis in the field or for routine epidemiologic evaluation of mosquito involvement in ongoing transmission cycles.

During feeding, mosquitoes such as *Aedes aegypti* salivate at a rate of approximately 0.03 nL/minute with an average total of 4.7 nL of saliva secreted during the blood feeding process. Novak and others collected only 2 nL of saliva from *Ae. aegypti* over a 10-minute period. Although inoculation of serotonin to induce salivation did not significantly increase the yield, this chemical did increase the relative amount of the female gland-specific apyrase. Saliva was collected in oil during a 30-minute period, without chemical induction. Since mosquitoes were not observed to imbibe the oil, this method appears to allow the maximum harvest of WNV that the mosquito would secrete during natural feeding upon a host. Analysis of saliva to determine the amount of transmitted virus has been described in several reports and reviewed by Hurlbut. The abundance of contaminating bacteria on mosquito mouthparts often precludes direct titration of saliva on cell cultures and the small volumes of collected saliva collected makes filtration impractical. Due to low viral titers in saliva, amplification of the dilutions is necessary to obtain detectable levels. Therefore, inoculations of either naive mosquitoes or susceptible vertebrates are used, with incubation required to produce either detectable viral/antibody titers or symptomatic disease. However, these effective methods are labor-intensive, relatively slow due to the incubation periods for either mosquitoes or cell cultures, and subject to problems of contamination from bacterial and fungal flora associated with insect mouthparts. The technique evaluated during this study eliminates many of the problems associated with the previously used techniques and with appropriate primer design can be readily manipulated to provide the desired level of virus specificity.

Our studies indicate that the *Cx. pipiens quinquefasciatus* mosquitoes infected with WNV are capable of transmitting an average of 4.3 logs of virus with a range of 0.5–5.3 logs. Previous studies have used a variety of technical approaches, and methods to quantify the amounts of virus secreted in saliva. Therefore data are difficult to compare. One criticism of PCR-based viral analysis is that data include both infectious and non-infectious RNA molecules, which leads to an overestimate of true viral content in a sample. Here we used a real-time PCR with reference to a standard sample of known infectivity. Our analysis provided an estimate of the quantity of infectious WNV secreted in mosquito saliva. These data are consistent with estimates of previous studies. Davis allowed yellow fever virus–infected mosquitoes to feed on baby mice and then immediately processed the mice and inoculated extracts into rhesus monkeys. The complex study demonstrated that mosquitoes probably inject approximately 1% of their total virus content, and at least 100 infective doses of virus could be transmitted. Ross calculated that *Ae. aegypti* transmitted between 40 and 130 50% lethal doses (LD50) of chikungunya virus. LaMotte estimated that between 10,000 and 100,000 mouse LD50 of Japanese encephalitis virus was contained in the salivary glands of *Culex pipiens*, while Collins determined that the salivary glands of *Culex tarsalis* contain 1,000–2,500 LD50 of Western equine encephalitis virus. Hurlbut described a new technique to collect saliva into oil in a capillary tube and then measured the amount of virus present by titration in mice. *Culex pipiens pipiens* were infected by feeding upon St. Louis encephalitis virus–infected chickens, and saliva was collected by placing the exposed stylets into a capillary tube containing mineral oil for 3 minutes, after incubation of the mosquitoes at 30°C for up to 28 days. Afterwards, oil was expelled into the aqueous diluent, and serial dilutions of this were inoculated into suckling mice. Saliva titers were calculated to range from 7.9 to 10.6 log10LD50/mL. Gubler and Rosen allowed *Ae. albopictus* to feed on blood droplets and then quantified the amount of dengue 2 virus by intrathoracically inoculating dilutions of the blood into naive mosquitoes. The amount of virus transmitted ranged from 300 to 200,000 mosquito ID50; however, some mosquitoes with infected salivary glands failed to transmit. Chamberlain and others estimated that while most *Ae. aegypti* inoculated less than 100 mouse intracerebral LD50 of eastern equine encephalitis virus, a few inoculated 1,000, and in rarer instances 10,000 LD50 or more. Our detection of WNV in one saliva sample from a mosquito that had no detectable antigen in the salivary glands is probably a reflection of the greater sensitivity of the RT-PCR, and not contaminating bacteria.
tion since uninfected controls were always negative by both methods.

The understanding of viral replication dynamics and dissemination in vector mosquitoes is critical for vector competence studies. The time required for salivary gland infection, and subsequent virus transmission (the extrinsic incubation period) is often species-specific, and is an important factor in determining the relative importance of different species in the natural transmission cycle. Since different vertebrates vary in their susceptibility to WNV infection, it is important to know the relative efficiency of different vectors with different host preferences, to transmit the virus, and to know how much virus they are capable of transmitting. In this study, we demonstrated rapid quantification of virus present in saliva, and observed that the amount of virus in saliva may decrease with time. This age-dependent effect on potential WNV transmission warrants further study since control efforts need to target mosquitoes that pose the greatest risk.

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