EVALUATION OF METHODS TO ASSESS TRANSMISSION POTENTIAL OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS BY MOSQUITOES AND ESTIMATION OF MOSQUITO SALIVA TITERS

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Abstract. Determining the dose of an arbovirus transmitted by a mosquito is important to design transmission and pathogenesis studies simulating natural infection. Several different artificial infection and transmission methods used to assess vector competence and to estimate the dose injected during mosquito feeding have not been fully evaluated to determine whether they accurately reflect natural transmission. Additionally, it is not known whether different mosquito vectors transmit similar amounts of a given virus. Therefore, we compared three traditional artificial transmission methods using Venezuelan equine encephalitis virus (VEEV) and Aedes albopictus and Ochlerotatus taeniopygus mosquitoes. Both the mosquito species and the infection route used affected the amount of virus detected in the saliva after a 10-day extrinsic incubation period. Median titers of virus detected in saliva of Ae. albopictus and Oc. taenio- rhynchus mosquitoes ranged from 0.2 to 1.1 log_{10} (mean 0.7–1.4 log_{10}) and 0.2 to 3.2 log_{10} (mean 1.0–3.6 log_{10}) plaque-forming units, respectively. The results of this study will aid in the design of transmission and pathogenesis studies involving arboviruses.

INTRODUCTION

Estimation of the amount of infectious arbovirus transmitted by a mosquito during a blood meal is important to design transmission and pathogenesis studies simulating natural infection, yet is poorly characterized. Three methods are typically used for experimental infection of adult female mosquitoes: 1) intrathoracic inoculation, 2) oral exposure by using an artificial blood meal, or 3) oral exposure by feeding on a viremic vertebrate host. Different methods can also be used to estimate the amount of virus delivered in a mosquito’s saliva during transmission, including both indirect and direct methods. Indirect methods include comparing the time of death of an animal exposed to a mosquito bite to that of animals infected with a known lethal dose, or comparing the time between mosquito feeding and viremia to the time between a known infectious dose delivered by needle and viremia. Direct methods include detecting virus in hanger drops of blood fed upon by mosquitoes, detection of virus in vertebrate tissue immediately after mosquito feeding, detection of virus in blood-agar fed upon by mosquitoes, and detection of virus after mosquito salivation into a fluid such as immersion oil.1 Fetal bovine serum (FBS) may be a more sensitive collection medium due to its stabilizing properties. However, Chamberlain and others5 compared several indirect and direct methods and concluded that a similar method to saliva collection by capillary tubes may not be an efficient method for virus detection.

Arboviruses encounter several potential infection and dissemination barriers within the mosquito, including the midgut and salivary gland infection barriers, and midgut and salivary gland escape barriers.7 Intra thoracic inoculations, which circumvent the infection and escape barriers but not the salivary gland infection and escape barriers, ensure more uniform infection of some mosquitoes than oral exposure. Oral infection via artificial blood meals or viremic animals more closely resembles natural exposure. Several studies have demonstrated that mosquitoes are less susceptible to infection by artificial than by natural blood meals due to the concentration of virus adjacent to the midgut epithelium that follows natural but not artificial blood meals.7 In contrast, one study of Ae. aegypti mosquitoes infected with Semliki Forest virus found no difference in virus titers in infected mosquitoes after artificial versus natural blood meals except during the first 24 hours, and transmission did not differ significantly.10 However, infection rates, which usually are lowered by artificial blood meals compared with viremic animals, were not reported.

The use of viremic animals for assessing vector competence and transmission potential also has some disadvantages. Animal use in biomedical research is expensive, highly regulated, and requires special facilities. In addition, exposing mosquitoes with a predetermined dose requires knowledge about the animal’s viremia level after a given intrinsic incubation period. Variation in viremia responses among individuals can make the accurate prediction of viremia levels difficult. Also, good laboratory models may not exist because laboratory animals sometimes do not develop sufficient viremia levels to infect even proven vectors.

Many studies have estimated the arbovirus dose delivered by an infected mosquito during feeding.1,2,4,11–18 Chamberlain and others14 estimated the amount of eastern equine encephalitis virus (EEEV) inoculated by orally (viremic chicken) infected Ae. aegypti mosquitoes to be highly variable, ranging from undetectable to 5 log_{10} mouse intracerebral 50% lethal doses (ICLD_{50}). A later study reported up to 3 log_{10} plaque-forming units (pfu) of EEEV in the saliva of Culex melanaura deposited artificially into capillary tubes filled with immersion oil.18 A study of Semliki forest virus transmission by Anopheles albimanus using artificial blood feeding for both infection of mosquitoes and collection of saliva reported that 3.2 to 4 log_{10} mouse LD_{50} were transmitted.13 Using indirect methods, Mellink estimated that 2.7 log_{10} infectious doses of Venezuelan equine encephalitis virus (VVEV) per mg of saliva are transmitted by Ae. aegypti mosquitoes infected intrathoracically. Using quantitative real-time reverse-tran-
scription polymerase chain reaction (RT-PCR) and capillary collection, VanLandingham and others determined that Culex pipiens pipiens mosquitoes infected by an artificial blood meal contain an average of 4.3 $\log_{10}$ pfu of West Nile virus in their saliva with a range of 0.5 to 5.3 $\log_{10}$.

Despite numerous studies cited above, important gaps remain in our knowledge regarding horizontal transmission of arboviruses. Different mosquito species have never been compared directly to determine if they transmit different amounts of a given virus. Different methods for virus exposure to mosquitoes have never been systematically evaluated for their effect on transmission and infectious doses delivered after an appropriate extrinsic incubation period. Also, the effect on transmission of the virus dose used to infect mosquitoes has not been evaluated. Additionally, the media used for saliva collection, oil and FBS, have not been compared. To address these issues, we used VEEV, an emerging arboviral pathogen of humans and equines, and two mosquito species: Aedes albopictus, a species that is susceptible to infection with VEEV and is often used as a model species for arbovirus transmission studies because it feeds readily in the laboratory, and Ochlerotatus taeniorhynchus, a proven VEEV vector. Three different routes for mosquito infection were compared along with two methods for collecting saliva. We determined the effect of mosquito species, route of infection, and the method of saliva collection on the amount of VEEV injected by mosquitoes.

MATERIALS AND METHODS

**Virus**. Venezuelan equine encephalitis virus was rescued from an infectious cDNA clone derived from the 1995 Venezuelan epizootic human strain 3908 (subtype IC, the etiologic agents of all recent VEE epidemics). This strain was passaged once in C6/36 mosquito cells before undergoing RNA extraction and infectious cDNA clone production. Virus recovered from BHK cells electroporated with transcribed RNA was used for all experiments without further passage. The use of virus derived from an infectious clone avoided attenuating mutations that occur when VEEV is passaged in cell culture.

**Viremia determinations.** Six- to eight-week-old female NIH Swiss mice were infected SC with 1,000 pfu of VEEV. Three mice per time point were bled at 10, 14, 18, and 24 hours after inoculation into capillary tubes containing either immersion oil or FBS/glycerol. The largest cohorts for the three feeding times were analyzed by the Mann Whitney test or the Kruskal-Wallis test using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

**Statistics.** Data that passed a normality test were analyzed by an unpaired $t$ test. Data that were not normally distributed were analyzed by the Mann Whitney test or the Kruskal-Wallis test using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

RESULTS

**Comparison of saliva collection methods.** Fetal bovine serum may result in recovery of greater quantities of virus in mosquito saliva than oil due to its stabilizing properties. To test this hypothesis, saliva from infected Aedes albopictus mosquitoes was collected in capillary tubes containing either immersion oil or FBS/glycerol. The largest cohorts for the three different infection routes were used to compare the sensitivity of the two collection media (Figure 1; Table 1). No significant difference ($P > 0.05$) occurred in virus detection rates or in...
saliva titers using immersion oil versus FBS; therefore, these groups were combined for further analysis.

**Effect of infection route on saliva titers.** Saliva virus content was compared among mosquito cohorts infected using three methods: 1) intrathoracic inoculation, 2) artificial blood meal, and 3) viremic animals. Saliva was collected from all orally infected mosquitoes after a 10-day incubation. Significantly lower ($P < 0.001$) saliva titers occurred in *Ae. albopictus* mosquitoes exposed intrathoracically compared with mosquitoes exposed by artificial blood meals and viremic animals. Because intrathoracic exposure accelerates mosquito infection, saliva titers may peak earlier. Therefore, one group of intrathoracically exposed mosquitoes was evaluated after only 5 days of incubation (Figure 2; Table 2), and the difference in saliva titers was no longer significant when compared with mosquitoes exposed by artificial blood meals or viremic animals. However, *Oc. taeniorhynchus* mosquitoes infected intrathoracically and incubated 5 days had higher saliva titers ($P < 0.01$) compared with those exposed to artificial blood meals or viremic animals (Figure 2; Table 2).

### Table 1
Comparison of immersion oil and fetal bovine serum as a saliva collection media using *Aedes albopictus* mosquitoes

<table>
<thead>
<tr>
<th>Infection method</th>
<th>Titer of infection dose (log_{10} pfu/mL)</th>
<th>Saliva collection medium</th>
<th>Percent with disseminated infection (fraction)</th>
<th>Percent of samples negative by CPE assay</th>
<th>Percent of CPE-negative samples that were positive by mouse inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrathoracic</td>
<td>4.0*</td>
<td>Oil</td>
<td>100% (31/31)</td>
<td>52%</td>
<td>81%</td>
</tr>
<tr>
<td>Intrathoracic</td>
<td>4.0*</td>
<td>FBS†</td>
<td>100% (29/29)</td>
<td>45%</td>
<td>92%</td>
</tr>
<tr>
<td>Viremic animal</td>
<td>7.2</td>
<td>Oil</td>
<td>100% (27/27)</td>
<td>22.2%</td>
<td>67%</td>
</tr>
<tr>
<td>Viremic animal</td>
<td>7.2</td>
<td>FBS†</td>
<td>90% (26/29)</td>
<td>42.3%</td>
<td>73%</td>
</tr>
<tr>
<td>Artificial blood meal</td>
<td>8.1</td>
<td>Oil</td>
<td>92% (23/25)</td>
<td>4.3%</td>
<td>0%</td>
</tr>
<tr>
<td>Artificial blood meal</td>
<td>8.1</td>
<td>FBS†</td>
<td>94% (17/18)</td>
<td>6%</td>
<td>100%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>96%</td>
<td>31%</td>
<td>79%</td>
</tr>
</tbody>
</table>

CPE, cytopathic effects; FBS, fetal bovine serum.
* Total dose delivered intrathoracically.
† 50% fetal bovine serum/50% glycerol.
Effect of infection dose on saliva titers. For *Ae. albopictus*, a range of oral doses from artificial blood meals and viremic animals was used: 6.1, 8.1 versus 6.1, 6.8, and 7.2 log_{10} pfu/mL, respectively. The range of oral doses for *Ae. albopictus* exposed to artificial blood meals did affect the saliva titer (*P* < 0.0003). However, this range of oral doses did not significantly affect the amount of virus in the saliva of *Ae. albopictus* exposed to viremic animals (Figure 2; Table 2).

Effect of mosquito species on saliva titers. For *Ae. albopictus*, the median cohort titers of virus in 208 saliva samples tested in cell culture ranged from 0.2 to 1.1 log_{10} pfu (mean range 0.7–1.4 log_{10} pfu), with a total range of 0.2 to 4.2 log_{10} pfu (Figure 2; Table 2). Overall, *Oc. taeniorhynchus* saliva contained larger amounts of VEEV than that of *Ae. albopictus*, with median titers of 0.2–3.2 log_{10} pfu (mean range 1.0–3.6 log_{10} pfu) and total range of 0.2–6.2 log_{10} pfu (Figure 2; Table 2). When individual cohorts were analyzed, the difference in saliva titers between mosquito species was significant for cohorts exposed intrathoracically (*P* < 0.0001) but not between cohorts exposed by artificial blood meals and viremic animals (*P* > 0.05).

Virus detection by inoculation of mice. Intracerebral inoculation into suckling mice is more sensitive for detecting VEEV compared with vertebrate cell culture methods, as shown in Table 1. The limit of detection for our CPE assay using the volumes available was 0.5 log_{10} pfu; therefore, suckling mice were used for some saliva samples if the mosquitoes were positive for viral dissemination to the legs and/or wings. Numbers to the right of the symbols with values of 0.2 indicate the number of samples that are in between zero and the limit of detection of the assay, 0.5 log_{10} pfu. 

**Figure 2.** Comparison of route and titer of mosquito infection. Titers were determined by cell culture assays (plaque and CPE assay). Cohorts are labeled as follows: infection titer (log_{10})-method of infection. Mosquitoes tested were *Ae. albopictus* unless noted as *Oc. taeniorhynchus* (*Oc. taen.*). The bar indicates the median saliva titer. The dashed bar indicates the geometric mean saliva titer. Numbers in parentheses to the right of the symbols with values of 0.5 indicate the number of samples with titers of 0.5 log_{10} pfu. The dotted line at 0.5 log_{10} pfu indicates the limit of detection for the cell culture assays. The symbols below this line indicate samples negative by the CPE assay, indicating that these samples were below the limit of detection for the assay, but the mosquitoes were positive for viral dissemination to the legs and/or wings. Numbers to the right of the symbols with values of 0.2 indicate the number of samples that are in between zero and the limit of detection of the assay, 0.5 log_{10} pfu. IT, intrathoracic; ABM, artificial blood meal; VA, viremic animal.
negative by the CPE assay, 38 (79%) were positive by mouse inoculation, resulting in a 25% increase in sensitivity. The data gathered initially using mouse inoculation were not quantitative. Therefore, we determined the titers of saliva samples from *Ae. albopictus* mosquitoes by calculating sucking mice ICLD_{50} values for some saliva samples. Simultaneous titrations in Vero cells and mice indicated that the ICLD_{50}/pfu ratio was approximately 200:1 (data not shown). Of 16 CPE-negative saliva samples from mosquitoes with disseminated infections, 3 tested positive in sucking mice and the ICLD_{50} values converted to pfu were 0.065, 0.054, and 0.065 log_{10} pfu. Five samples contained less than one mouse ICLD_{50} (less than 0.04 pfu). The addition of the mouse ICLD_{50} assay decreased the geometric mean titer of saliva by one tenth of a log_{10} pfu, and increased sensitivity by 2–31%.

**DISCUSSION**

Of the methods typically used for experimentally infecting mosquitoes with arboviruses, intrathoracic inoculations, artificial blood meals and viremic animals, all have advantages and disadvantages as discussed above. However, it is unknown if the infection method affects the dose of an arbovirus delivered in the saliva of a mosquito vector. We therefore systematically evaluated the effect of these infection methods, as well as the virus doses used for infection, on saliva titers using VEEV. Two methods for saliva collection were also compared, and saliva titers in two different mosquito species were compared to determine if they transmit different amounts of VEEV.

**Effect of saliva collection medium.** Our results indicate no difference in immersion oil versus FBS for the detection and quantification of VEEV in mosquito saliva. Silinized (to reduce possible virus adhesion) capillary tubes were also compared with non-silinized tubes spiked with VEEV, and no difference was noted (data not shown). The problems of contamination of the medium by mosquito mouthparts during *in vitro* collection of saliva, as discussed by Vanlandingham and others, were not experienced in our studies.

**Effect of infection route on saliva titers.** Significantly less VEEV was detected in *Ae. albopictus* saliva after intrathoracic infection and a 10-day incubation period versus by an artificial blood meal and the same incubation. In contrast, when *Ae. albopictus* was infected intrathoracically and incubated 5 days, no significant difference in titers occurred compared with the artificial blood meal infection (Figure 2; Table 2). This difference in results with different incubation times should be assessed with other viruses in their mosquito vectors to determine if it represents a general phenomenon. The difference in *Ae. albopictus* is most likely due to different kinetics of viral replication in mosquitoes infected intrathoracically versus orally; VEEV titers peak earlier in intrathoracically infected mosquitoes because the midgut infection and escape barriers are circumvented. Our data suggest that when using intrathoracically infected mosquitoes, a shorter extrinsic incubation period should be used to generate peak saliva titers comparable to oral infection. Surprisingly, *Oc. taeniorhynchus* infected intrathoracically had a significantly higher mean saliva titer than those exposed via an artificial blood meal or viremic animal. Although mosquitoes can be less susceptible to infection using artificial blood meals compared with natural infection, our study found no difference in the dissemination rates observed after oral infection using the 2 methods, although there was a a 10-fold difference in the infectious dose.

**Effect of infection dose on saliva titers.** Varying oral doses had a significant effect on *Ae. albopictus* saliva titers for mosquitoes exposed by artificial blood meal, but no difference was noted for mosquitoes exposed to varying oral doses from a viremic animal (Figure 2; Table 2).

**Effect of mosquito species on saliva titers.** The possibility that different mosquito species may transmit different amounts of virus has never been addressed. Our results demonstrate that saliva from *Oc. taeniorhynchus* mosquitoes contains on average 1.2 log_{10} pfu more VEEV than that of *Ae. albopictus* infected using the same methods and doses. Previous studies suggest that some epizootic VEEV strains undergo adaptation for *Oc. taeniorhynchus* transmission, and this adaptation may be a significant factor in epidemic emergence. The higher saliva titers in this proven vector, compared with *Ae. albopictus* that are susceptible to infection but have not been implicated in natural transmission, further support this hypothesis for VEEV emergence, although further studies examining the relationship of saliva titers to transmission efficiency are needed.

A wide range (0.2–4.2 log_{10} pfu) of VEEV titers was detected in the saliva of individual *Ae. albopictus* and *Oc. taeniorhynchus* (0.2–6.2 log_{10} pfu) mosquitoes, consistent with previous studies using other arboviruses. The amount of VEEV in the saliva did not correlate with the amount in the

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**Table 2**

<table>
<thead>
<tr>
<th>Infection method</th>
<th>Mosquito species</th>
<th>Infectious dose (log_{10} pfu/mL)</th>
<th>Percent with disseminated infection*</th>
<th>Percent negative by CPE assay</th>
<th>Median saliva titer (log_{10} pfu)</th>
<th>Mean saliva titer (log_{10} pfu)</th>
<th>Range of saliva titers (log_{10} pfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrathoracic</td>
<td><em>Ae. albopictus</em></td>
<td>4.0†</td>
<td>100% (172/172)</td>
<td>57%</td>
<td>0.2</td>
<td>1.2</td>
<td>0.2–2.9</td>
</tr>
<tr>
<td>Intrathoracic</td>
<td><em>Ae. albopictus</em></td>
<td>4.0†</td>
<td>100% (22/22)</td>
<td>18%</td>
<td>0.9</td>
<td>1.6</td>
<td>0.2–3.6</td>
</tr>
<tr>
<td>Intrathoracic</td>
<td><em>Oc. taeniorhynchus</em></td>
<td>4.0†</td>
<td>100% (32/32)</td>
<td>3%</td>
<td>3.2</td>
<td>3.7</td>
<td>0.8–6.2</td>
</tr>
<tr>
<td>Artificial blood meal</td>
<td><em>Ae. albopictus</em></td>
<td>6.1</td>
<td>57% (36/63)</td>
<td>63%</td>
<td>0.2</td>
<td>1.6</td>
<td>0.2–3.9</td>
</tr>
<tr>
<td>Artificial blood meal</td>
<td><em>Ae. albopictus</em></td>
<td>8.1</td>
<td>92% (61/66)</td>
<td>17%</td>
<td>0.9</td>
<td>1.5</td>
<td>0.2–3.8</td>
</tr>
<tr>
<td>Artificial blood meal</td>
<td><em>Oc. taeniorhynchus</em></td>
<td>8.1</td>
<td>61% (17/28)</td>
<td>30%</td>
<td>1.9</td>
<td>2.9</td>
<td>1.5–5.9</td>
</tr>
<tr>
<td>Viremic animal</td>
<td><em>Ae. albopictus</em></td>
<td>6.1</td>
<td>80% (20/25)</td>
<td>60%</td>
<td>0.2</td>
<td>1.9</td>
<td>0.2–3.9</td>
</tr>
<tr>
<td>Viremic animal</td>
<td><em>Ae. albopictus</em></td>
<td>6.8</td>
<td>100% (10/10)</td>
<td>30%</td>
<td>1.1</td>
<td>1.5</td>
<td>0.9–2.2</td>
</tr>
<tr>
<td>Viremic animal</td>
<td><em>Ae. albopictus</em></td>
<td>7.2</td>
<td>94.6% (53/56)</td>
<td>32%</td>
<td>0.6</td>
<td>1.3</td>
<td>0.2–3.2</td>
</tr>
<tr>
<td>Viremic animal</td>
<td><em>Oc. taeniorhynchus</em></td>
<td>7.2</td>
<td>53% (17/32)</td>
<td>59%</td>
<td>0.2</td>
<td>2.0</td>
<td>0.2–3.1</td>
</tr>
</tbody>
</table>

* Numbers tested exceed totals in Table 1 because only a fraction of samples were tested by mouse inoculation, whereas all were tested using cell culture assays.
† Total dose delivered intrathoracically.
body or legs (data not shown). A previous study also showed no correlation between the amounts of dengue-2 virus transmitted by *Ae. albopictus* mosquitoes and the amounts of salivary gland tissue infected.13 The wide range of virus transmitted by mosquitoes is probably determined by factors other than the degree of salivary gland tissue infected after dissemination into the hemocoel.

Detection of low virus titers in saliva using mouse inoculation. As seen in Table 1, a large number of mosquitoes was positive for disseminated infection (CPE-positive legs), but contained saliva that was negative by the CPE assay (31.4%). Inoculation of suckling mice demonstrated that small amounts of virus were present in many of these saliva samples, leaving only 6.5% samples negative for virus using both assay methods. The limit of detection for the CPE assay was 0.5 log10 pfu, but sucking mice inoculated IC proved to be much more sensitive than cell culture assays (Table 1).

An important remaining question is whether the amount of virus a mosquito salivates into a capillary tube accurately reflects the amount transmitted while feeding on a vertebrate host. To collect mosquito saliva in a capillary tube, the mosquito must first be cold anesthetized and have its legs and wings removed, traumatic events that may affect salivation. Additionally, mosquitoes are usually allowed to salivate into the capillary tubes for a much longer time than is required for engorgement on a host. To address these limitations of the current study, further experiments to assess amounts of VEEV transmitted *in vivo* are underway in our laboratory.

In conclusion, the VEEV titer used for the infection of mosquitoes appears to have little or no effect on the amount of virus found in saliva after extrinsic incubation. The method of oral infection (artificial blood meal versus viremic animal) also does not appear to affect the titer of VEEV in saliva. However, intrathoracic inoculation generates lower saliva titers when using the same incubation period (10 days). *Ae. albopictus* saliva (median titer of 0.6 log10 pfu, mean titer of 1.0 log10 pfu, range = 0.2 to 4.2 log10 pfu) contains significantly less VEEV than that of *Oc. taeniorhynchus* (median titer of 1.8 log10 pfu, mean titer of 2.2 log10 pfu, range = 0.2 to 6.2 log10 pfu), demonstrating that the mosquito species used in transmission studies can affect the quantity of virus transmitted and possibly the resultant pathogenesis. The results of this study should be considered when designing transmission and pathogenesis studies to mimic natural infection by arboviruses.

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