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Reduced infection in mosquitoes exposed to blood meals containing previously frozen flaviviruses

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Abstract

The increased difficulty and expense of using live animals for delivering infectious blood meals in arthropod-borne virus vector competence experiments has resulted in an increase in the use of artificial feeding systems. Compared to live hosts, artificial systems require higher viral titers to attain mosquito infection, thereby limiting the utility of such systems with low or moderate titer virus stocks. Based on the report that freshly propagated virus is more infectious than previously frozen virus, we determined whether such a preparation would enhance the ability to use artificial feeding systems. *Culex quinquefasciatus* and *Aedes aegypti* mosquitoes were offered blood in artificial membrane feeders containing freshly collected or previously frozen St. Louis encephalitis and dengue serotype-2 viruses (family *Flaviviridae*), respectively. Infection rates and estimates of vector competence were significantly lower ($P < 0.05$) for mosquitoes feeding on blood meals containing frozen–thawed compared to freshly collected virus. We indicate that the use of freshly propagated virus in artificial feeding systems can be an effective blood delivery method for low-titer viruses and viruses that are otherwise inefficient at infecting vectors in such systems. Fresh viruses used in artificial feeding systems may be a viable alternative to the heavily regulated and expensive use of live animals.

Keywords

Flavivirus; Artificial feeding; Mosquito; Arthropod-borne virus

In recent years, the use of live animals has become more difficult and expensive, resulting in an increase in utility of artificial feeding systems for vector competence experiments. Artificial membrane feeding systems are an alternative to live animals for delivering viremic blood meals in vector competence experiments. Infection rates tend to be reduced in mosquitoes exposed to artificial feeding systems, compared to live hosts, as was reported for *Culex quinquefasciatus* exposed to St. Louis encephalitis virus (SLEV) (family *Flaviviridae*) via pledget versus viremic chick (Meyer et al., 1983). Compared to live hosts, artificial systems require higher viral titers to attain mosquito infection, thereby limiting the use of virus stocks with low titers (Jupp, 1976; Meyer et al., 1983). In a study of *Culex pipiens* and *Aedes taeniorhynchus* exposed to Rift Valley fever virus (RVFV) (family *Bunyaviridae*) via blood-soaked pledgets as compared to viremic hamsters, infection rates were lower in the artificial system when viral titer ingested by mosquitoes was <4.7 logs plaque-forming units (pfu) RVFV/mosquito, but were equivalent in both systems when mosquitoes ingested >4.7 logs pfu RVFV/mosquito (Turell, 1988).

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Previous studies have attributed diminished rates of mosquito infection using artificial versus live feeding systems to the use of frozen–thawed virus stocks mixed with blood prior to mosquito feeding. Investigations comparing vector competence of mosquitoes exposed to artificial systems with fresh (i.e. virus-infected tissue culture supernatant collected immediately before use and never frozen) versus frozen–thawed virus mixed with blood are restricted to a limited number of viruses. One study implied difficulty infecting *Aedes aegypti* with dengue virus serotype-2 (DENV-2) (family *Flaviviridae*) from frozen–thawed virus stocks compared with fresh stocks, but provided no evidence (Miller et al., 1982). A subsequent investigation showed that *Ae. aegypti* fed through a membrane system containing blood mixed with relatively low-titered fresh yellow fever virus (YFV) (family *Flaviviridae*) exhibited approximately a five-fold increase in infection rates versus mosquitoes fed blood containing frozen–thawed YFV (Miller, 1987). The same study reported no significant difference in infection rates of *Ae. aegypti* exposed to an artificial membrane system containing blood mixed with comparatively high-titered fresh versus frozen–thawed YFV. Reduced infection rates in mosquitoes fed blood meals containing frozen–thawed versus fresh virus were also reported for *Cx. pipiens* ingesting artificially administered blood meals containing moderate quantities of RVFV (Turell, 1988). It may be that the use of freshly propagated virus in artificial blood feeding experiments could significantly enhance vector infection rates for low and moderate titer virus stocks and those that are otherwise inefficient at infecting in such studies.

Consequently, we evaluated the ability of *Cx. quinquefasciatus* and *Ae. aegypti* to become infected following exposure to artificial blood meals containing frozen–thawed or freshly propagated SLEV or DENV-2, respectively. Previous studies have established vector competence of *Ae. aegypti* for DENV-2 and *Cx. quinquefasciatus* for SLEV (Armstrong and Rico-Hesse, 2003; Chamberlain et al., 1959; Gubler et al., 1979; Sudia, 1959). We also determined whether this difference in virus preparation had an effect on dissemination of infection within the mosquito. Finally, we evaluated the effects of a virus stabilizer (gelatin, phosphate-buffered saline, bovine serum albumin and sucrose) mixed with SLEV prior to freezing on infection of *Cx. quinquefasciatus*.

1. Mosquitoes

Five- to six-day old female *Cx. quinquefasciatus* and *Ae. aegypti* from well established lab colonies were utilized for this experiment and maintained under a 14 h light:10 h dark cycle simulating a long day photoperiod. Adult mosquitoes were housed in 1-L cardboard cages with mesh screening and provided 20% sucrose and water *ad libitum*.

2. Viruses

The Florida TBH28 strain of SLEV used was passaged twice in African green monkey kidney (Vero) cells. A Southeast Asian DENV-2 (16803 strain) was passaged once in the mosquito *Toxorhynchites amboinensis*, nine times in Vero cells and twice in C6/36 cells.

3. Virus stock preparation

Individual T-75 cm^2 flasks containing monolayers of Vero cells in Leibovitz-15 medium with 10% fetal bovine serum and 50 μg/mL gentamicin were inoculated with either SLEV at a multiplicity of infection (moi) of 2.0 or DENV-2 at a moi of 1.0 and held at 35 °C. Low feeding success expected for *Ae. aegypti* made it necessary to inoculate a second set of flasks with DENV-2 24 h after the first flask was inoculated so that another feeding could be attempted the day after the initial feeding. For flasks inoculated with DENV-2, supernatant was removed and replaced with fresh media 72 h post-inoculation (hpi) and 144 hpi, followed by harvesting at 240 hpi. For flasks inoculated with SLEV, supernatant was removed and replaced with fresh

media 48 and 144 hpi, followed by harvesting at 192 hpi. We created fresh virus blood meals by mixing one-half SLEV or DENV-2 supernatant with one part or four parts citrated bovine blood, respectively. To create frozen–thawed virus blood meals, we froze the other half of the fresh supernatant at −80 °C for 30 min, and then thawed at room temperature prior to mixing with citrated bovine blood.

4. Mosquito infection

Mosquitoes were sugar starved 24 h prior to blood feeding. *Cx. quinquefasciatus* and *Ae. aegypti* were allowed to feed for 30 min on membrane feeders (Alto et al., 2003) containing citrated bovine blood (35 °C) with frozen–thawed or fresh SLEV or DENV-2, respectively. After feeding, mosquitoes were immobilized with cold, fully engorged specimens transferred to new cages, held at 28 °C and provided sucrose. Due to low feeding success of *Ae. aegypti*, a second feeding was attempted 24 h later.

5. Mosquito processing

After a 13-day incubation period, all surviving mosquitoes were removed from each cage, legs and bodies triturated separately in 0.9 mL BA-1 (Lanciotti et al., 2000) and stored at −80 °C for later processing.

6. Virus assays

Individual mosquitoes were ground in diluent with 4.5 mm zinc-plated beads, homogenized at 25 Hz for 3 min (TissueLyser; Qiagen, Inc., Valencia, CA), and centrifuged at 4 °C and 3000 × *g* for 4 min. Viral RNA was extracted from 250 μL of samples using the MagNA Pure Instrument with the MagNA Pure LC Total Nucleic Acid Kit (Roche, Mannheim, Germany) and eluted in 50 μL of buffer. The Superscript III One-step quantitative RT-PCR (qRT-PCR) system (Invitrogen, Carlsbad, CA) was utilized to amplify viral RNA as described previously (Callahan et al., 2001; Lanciotti and Kerst, 2001). Samples were amplified using the LightCycler® 480 Instrument (Roche, Mannheim, Germany) programmed as follows—DENV-2 samples: 48 °C for 30 min, 95 °C for 2 min, 45 cycles at 95 °C for 15 s and 60 °C for 30 s and finally 40 °C for 30 s; SLEV samples: 48 °C for 30 min, 95 °C for 2 min, 45 cycles at 95 °C for 10 s and 60 °C for 15 s and finally 50 °C for 30 s. Quantification of SLEV and DENV-2 in samples was determined by comparing crossing point values to standard curves (Bustin, 2000) based on data acquired from 10-fold serial dilutions of virus stocks with known concentrations.

A separate experiment was conducted to compare virus titers of frozen–thawed and fresh stocks of SLEV and DENV-2. Virus stocks were prepared as described above, except that cells were inoculated with SLEV and DENV-2 at a moi of 2.3 and 2.0, respectively. Samples were tested by qRT-PCR as described previously, and by plaque assay (Gargan et al., 1983) on Vero cell monolayers, except that a second overlay, containing neutral red, was added 6 days after the initial overlay.

7. Statistical analyses

Infection rates were the percentage of mosquitoes tested with infected bodies and dissemination rates were the percentage of mosquitoes tested that had infected legs. Differences in rates between virus preparations were examined using Chi-square analyses (SAS, 1999).

8. Effects of virus preparation on titer

Viral titers of blood meals containing fresh or frozen–thawed virus and fed to mosquitoes were equivalent for SLEV and approximately equivalent for DENV-2 as measured by qRT-PCR (Table 1). A separate experiment using both qRT-PCR and plaque assay to compare viral titers of fresh and frozen–thawed virus stocks revealed similar titers for SLEV (Table 2). For DENV-2, similar titers were observed for stocks measured by qRT-PCR, while a higher titer was observed in the fresh compared to frozen–thawed DENV-2 measured by plaque assay (Table 2).

9. Effects of virus preparation on vector competence

For mosquitoes fed blood meals containing either SLEV or DENV-2, rates of infection and dissemination were significantly different ($P < 0.05$) in mosquitoes exposed to freshly collected versus frozen–thawed virus (Table 1). Virus stabilizer did not have a significant effect on vector competence of *Cx. quinquefasciatus* for SLEV that had been previously frozen (data not shown).

Dissemination rates for *Cx. quinquefasciatus* and *Ae. aegypti* fed blood meals containing SLEV or DENV-2 were significantly ($P < 0.05$) lower in mosquitoes fed blood meals containing frozen–thawed versus fresh virus. These results are similar to those reported for *Ae. aegypti* fed YFV (Miller, 1987) and for *Cx. pipiens* and *Ae. taeniorhynchus* fed RVFV (Turell, 1988). After freezing, we found that titers of SLEV stocks approximated fresh stocks. Similar results have been reported with RVFV propagated *in vivo* and *in vitro* (Turell, 1988). For DENV-2 tested via plaque assay, we found a higher titer in fresh versus frozen–thawed virus, while frozen–thawed versus fresh virus titers were similar when tested via qRT-PCR.

The reason for diminished infectivity in fresh compared to frozen–thawed virus is unknown; although it has been hypothesized that structural changes in virions due to freezing may inhibit subsequent binding to mosquito cells (Miller, 1987). Data pertaining to changes in flavivirus morphology due to freezing are lacking. Nevertheless, a study using electron microscopy found that another enveloped virus, herpes simplex virus 2 (HSV-2) (family *Herpesviridae*), that had been frozen and slowly thawed (4 °C) exhibited more irregularly shaped particles with damaged binding proteins, compared to quickly thawed (37 °C) HSV-2 (Hansen et al., 2005). The same study indicated no significant change in HSV-2 titers due to freezing; however, more viable virus particles were recovered when liquid nitrogen versus slow freezing was utilized. The virus stabilizer we utilized had no effect on mosquito infection rates. Additional cryoprotectants should be evaluated for their ability to shield viral particles from detrimental freezing effects. Although viral titers in frozen–thawed versus fresh virus stocks were virtually identical for SLEV and somewhat lower for DENV-2 *in vitro*, in the mosquito there seems to be a barrier to midgut infection by frozen–thawed SLEV and DENV-2. Understanding interactions between virions and mosquito midgut receptors is essential for understanding these vector–virus relationships.

These findings have important practical and biological implications for experimental design of vector competence studies. In this and other studies, similar consequences for five different mosquito species and four viruses suggest that this is a generalizable phenomenon impacting studies of mosquito vector competence that use frozen–thawed virus stocks. The dramatic difference observed in rates of infection and dissemination between virus treatments is notable. We acknowledge that neither fresh nor frozen–thawed virus stocks are circulating in the environment; however, the use of fresh virus stocks may be more applicable to real-world situations than frozen–thawed virus stocks when live animals are not accessible due to animal use regulations, lack of animal care facilities or prohibitive costs. Despite reports that artificial viremic blood meal delivery systems generally result in decreased mosquito infection rates

when compared to live hosts, there are some cases that necessitate the use of artificial systems. An advantage of artificial systems is the ability to accurately calculate the infectious dose being utilized. This benefit is somewhat diminished when using freshly propagated virus stocks, as titers are less predictable. We suggest that feeding and virus preparation methods be taken into consideration when designing and generalizing vector competence experiments.

Even though this phenomenon is known by some researchers and has been reported previously, those published reports are restricted by small sample sizes and represent a limited number of viruses (neither SLEV nor DENV-2 had been examined). The current study indicates that the use of freshly propagated virus in artificial feeding membrane systems can be an effective delivery method for low or moderate titer viruses and viruses that are otherwise inefficient at infecting vectors in such systems. Fresh viruses used in artificial feeding systems may be a viable alternative to the heavily regulated and expensive use of live animals.

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Table 1

Infection and dissemination rates for *Culex quinquefasciatus* and *Aedes aegypti* fed respective freshly collected or frozen–thawed artificial blood meals containing SLEV or DENV-2 after an extrinsic incubation period of 13 days at 28 °C and tested by qRT-PCR

Virus	Blood meal virus titer (logs pfu/mL)	No. tested	No. infected (%)	No. disseminated (%)
Culex quinquefasciatus				
SLEV (frozen)	3.4	113	0	0
SLEV (fresh)	3.4	116	112 (97)	87 (78)
Aedes aegypti				
DENV-2 (frozen)	5.4 ± 0.5	100	1 (1)	0
DENV-2 (fresh)	5.5 ± 0.02	100	56 (56)	17 (30)

Table 2

Comparison of viral titers of fresh and frozen–thawed SLEV and DENV-2 tested by plaque assay and qRT-PCR

Virus	Fresh virus titer[a,b] (logs pfu/mL)	Frozen–thawed virus titer (logs pfu/mL)
SLEV	6.0[a] (6.6)[b]	5.9 (6.6)
DENV-2	7.3 (6.6)	6.2 (6.8)

[a] Plaque assay.

[b] qRT-PCR.