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Male Ae. Aegypti in Virus Amplification

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Male Mosquitoes: Involvement in Arbovirus Cycling

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ABSTRACT

Male mosquitoes lack the ability to transfer arboviruses to vertebrates by bite, but they may be able to amplify such virus by alternative mechanisms. One possible scheme for male-involved amplification is male infection with virus by feeding on infected Florida nectars followed by inoculation of female mosquitoes via venereal transmission. In order to determine the ability of orally-infected male mosquitoes to transmit Sindbis virus, virus viability was measured in various nectar solutions. Virus survived under insectary conditions in nectar solutions that varied from pH 5.2 - 7.4 and osmolarity that ranged from 285 - 647 mmol/kg. Virus survived in these nectars for at least 7 days and titer was sustained for at least 24 hours. We have demonstrated that Sindbis virus can survive in solutions other than blood and buffered cell culture media. Preliminary research on the tolerance of Sindbis virus to tangerine, strawberry and white mangrove nectars has been investigated and venereal transfer studies are planned.

KEYWORDS: male mosquitoes, Sindbis virus, venereal transmission

INTRODUCTION

Male and female mosquitoes differ in their nutritional requirements. Females require a blood meal for egg development and will feed on sugar when blood is not easily available (Foster & Takken, 2004). Males must feed on nectar which serves as a carbohydrate source and provides the raw material for males to metabolize sugars for swarming, mating, and flying (Clements, 1999). Males cannot transmit virus to vertebrates, but can venereally transmit virus to female mosquitoes such that virus can next be transmitted to vertebrates following blood feeding (Mavale et al., 2005). Traditional routes of arbovirus transmission are: horizontal, vertical, venereal, and nonviremic transmission (Higgs et at., 2005). Alphaviruses are transmitted primarily by horizontal cycles and the potential of venereal transmission is being investigated using Sindbis virus in *Aedes aegypti* (Brown & Condreay, 1986). Beaty and colleagues (2000) showed a diagram for LaCrosse virus transmission, which includes venereal transmission as a viable mode of transmission. LaCrosse is a Bunyavirus, and we speculate that Alphviruses may also demonstrate meaningful venereal transmission.

Flower nectar as a possible source for transmission by male mosquitoes is the main objective of this research. Flower nectar evolved in plants as a means to attract pollinators, including mosquitoes (De la Barrera & Nobel, 2004). The main components in nectar are glucose, fructose, sucrose, and water in varying proportions (De la Barrera & Nobel, 2004). Research groups have used different percentages of sugars in feeding experiments. Gary and Foster (2004) used a 50% sucrose solution, while Johnson and colleagues (2002) used a 5% sugar solution. We chose a 5% sugar solution in order to have a reasonable osmolarity for the virus. There has been no study to suggest if the mosquitoes feed on one percentage preferentially.

Two studies investigated if availability of sugar reduced virus transmission and if female mosquitoes prefer human scent or nectar scent. Gary and Foster (2004) concluded that nectar-producing plants placed in the vicinity of humans affects the transmission of malaria and the mosquitoes' energy budget because females will reduce

blood feeding and instead feed on nectar. Foster and Takken (2004) determined that up to five days post-emergence of *Anopheles gambiae*, female and male mosquitoes prefer nectar scent and after the first five days, female mosquitoes prefer human scent. This research investigates male mosquitoes and their ability to amplify a variant of Sindbis virus in nature after feeding on infected nectar.

MATERIALS AND METHODS

Nectar Solutions. PBS-based nectars and water-based nectars were prepared by dissolving varying amounts of glucose, fructose, and sucrose in 200ml PBS or water. The formulas for strawberry, tangerine, and white mangrove, which are flowering plants found in Florida, were used because each was greater in one of the three carbohydrates (Van Handel et al., 1972). The nectars were diluted to five percent sugar and ninety-five percent water or PBS in order to ensure that the mosquitoes would feed on the solutions and the osmolarity would not be too great for the Sindbis virus (Johnson et al., 2002). Nectars were filter sterilized by a Zap Cap and stored at 4°C. The pH and osmolarities of the three nectars in PBS and water were recorded.

Virus Viability. The heat resistant variant of Sindbis virus (SVHR) was used. Sterile 1.5mL microcentrifuge tubes were labeled and ninety microliters of each of the six nectars were placed into microcentrifuge tubes. Ten microliters of SVHR ($1.6X10^8$ pfu/ml stock) was then placed into each tube. A positive control was used for both the water and the PBS trials, which contained ninety microliters of PBS or water and ten microliters of SVHR. The negative control contained no virus and one hundred microliters of PBS or water. Upon the addition of SVHR, one of each of the six nectars, a positive control, and a negative control were placed into a -20^{o}C freezer as the zero time point. The remaining time points of 1, 2, 3, 4, 5, 6, 12, 24 hours, and day 5 and 7 were placed into insectary conditions, and following incubation, were placed into the freezer at their respective time points (see above).

CPE Assay. BHK-21 cells were grown in completed Eagle's minimum essential medium (MEM-E) according to the methods of Renz and Brown (1976). The cells were incubated at 37°C and 5% CO_2. A $75cm^2$ flask of BHK-21 cells underwent a 1:2 split. The cell pellet from the flask was resuspended in five milliliters of MEM-E and placed into a sterile bottle containing 105 milliliters of MEM-E. The bottle was shaken vigorously to ensure the cells were resuspended, and approximately one milliliter of the cell suspension was drawn out and placed into 13 of the 24 wells on a sterile 24-well plate. Ten plates were used to analyze virus viability in water and PBS. The cells were placed into the incubator at 37°C and 5% CO_2 until a preconfluent monolayer formed. Once a preconfluent cell monolayer formed, all media was removed from the wells, and fifty microliters of each thawed sample was placed in its respective well as dictated by the diagram (Figure 1). Upon application to all wells, the plates were placed on a rocker for one hour to promote virus adsorption. Two milliliters of media was then added to each well with cells, the plates were placed in the incubator, and CPE was monitored daily and recorded as absent or present (-/+).

Plaque Assay. The virus-nectar PBS solutions for both trials at the 0 and 24 hour time point from the virus viability study were adsorbed onto a $25cm^2$ flask of preconfluent monolayer of BHK-21 cells. The MEM-E was removed and the cells were inoculated with 200 microliters of the virus-nectar serial dilutions. The flasks were placed on a rocker for 1 hour to enhance virus adsorption. A 1:1 agarose:media overlay was added to the flasks. After a 24 hour incubation period, a second overlay of 1:1 agarose:media and 3% neutral red viable dye was added to the flasks. The flasks were then wrapped in foil and incubated for 24 hours and analyzed for plaques.

RESULTS

Nectar compositions used in this research are recorded in Table 1. All PBS-based nectars had a pH of approximately 7.4 and osmolarities ranging between 585-647 mmol/kg compared to PBS at 461 mmol/kg (Table 2). All water-based nectars had a pH of approximately 5.2 and osmolarities ranging between 390-444 mmol/kg compared to water at 285 mmol/kg. Virus titer for water-based nectar is pending. The experimental design for the CPE assay is shown in Figure 1. The CPE assay was positive for the presence of viable virus in all the nectars and positive controls (Figure 2). The negative controls indicated no CPE. The pHs and osmolarities for some of the normal mediums in which Sindbis virus is found are given in Table 3. The pHs and osmolarities of these normal mediums (human, bovine and avian blood, and MEM-E) are all within the range of or very similar to the pHs and osmolarities of the nectars used in this research. The plaque assay results (Table 4) showed titers in the 10^6 and 10^7range. The zero and 24 hour incubation time points were assayed twice to determine virus titer. There were slight decreases in the titers for the nectars in both trials from the zero to the 24 hour time point. In the first trial, the titers for strawberry nectar were equal, but in the second trial, there was a difference of a log. Even though there was a log decrease, the titer was still high.

DISCUSSION

Sindbis virus is primarily blood-borne in nature. Blood has a pH of 7.4 and an osmolarity of 295 mmol/kg for humans and other vertebrates have blood with similar pHs and osmolarities (Table 3). In the laboratory setting, Sindbis virus is grown in MEM-E, which has a pH of 7.4 and an osmolarity of 540 mmol/kg. We found that Sindbis virus survived an acidic pH of 5.2 for at least seven days. Because the virus is membrane bounded, this was quite unexpected. Acidic pH tends to disrupt the membrane and kill cells, but the fact that SVHR survived seven days in this hostile environment speaks to its physio-chemical properties. This suggests that the virus may be able to survive acidic pHs found in the stomachs of most animals and these animals may be able to get infected via oral route if the virus is able to cross the gut barrier (Hardy et al., 1983). If this is possible, pollinating birds may become infected without the need for a mosquito vector and thus amplify the virus in nature. Male mosquitoes, if they can become orally infected, could lead to amplification of the virus in nature because the virus can survive for long periods of time in nectar. This gives the male mosquito ample time to feed on infected nectar. Also, many male mosquitoes could become infected from the same flower because the virus is shown to survive for at least seven days.

SVHR survives in the osmolarity ranges of 285-647 mmol/kg for seven days. Sindbis virus is purified using a sucrose gradient in the lab. Our experiments implied that the virus infectivity was not affected by the sugar in the nectar solution because sucrose is used to purify the virus. Also, SVHR may be able to survive at even higher osmolarities which would mean that during dry seasons when nectar dilution is not affected by rainfall the virus could survive and be amplified in nature. Sindbis virus is somehow able to tolerate a wide range of osmolarities in order to prevent lysis and death. Further study is needed to understand how the virus can survive in such hostile conditions and to determine what the maximum osmolarity tolerated.

Virus infectivity was not affected by the pH or the osmolarities examined because after a seven-day incubation, cytopathic effect was observed in the BHK-21 cells for all nectar solutions. Within 24 hours, massive CPE could be seen across the cell monolayer in the well, which implies that the virus was not hindered by the sugar solution or the pH. The media which was added after the adsorption process also aided in the neutralization of the water-based nectars because the cells would not have been able to

survive at a pH of 5.2. Because CPE was not observed in the mock controls but was observed when virus was present, it can be determined that the virus was causing the cell lysis and not the initial application of the acidic water-based nectar solution. A plaque assay will further reinforce this hypothesis if the virus forms plaques.

The titers obtained from the plaque assay show no significant difference between the nectars and PBS-virus solutions, which implies that the virus was not affected by the sugar solutions. No significant decrease was observed between the 0 hour time point and the 24 hour time point. The strawberry nectar on the second trial did decrease by a log, but the 24 hour time point titer is believed to be high enough to infect the male mosquitoes orally. Female *Aedes aegypti* mosquitoes imbibe approximately 2 microliters of blood when feeding, so if males imbibe one-twentieth of this volume, or 0.1μL, the male will be infected with at least 200 virions (Ogunrinade, 1980). The virus titers are believed to be high enough to infect the male mosquito, but proffering to the male mosquitoes needs to be conducted to determine validity.

The next step in this research is to proffer the infected nectar to the male mosquitoes and determine if they are infected orally by leg assay. If it is shown that the male mosquitoes are infected via the nectar, infected males will be mated with unmated uninfected females to determine the success of venereal transmission. From this data, the amplification of virus due to the male can be determined and the male mosquito will no longer be viewed as only a reproductive necessity.

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TABLES

Nectar Solution	Sucrose g/100ml	Dextrose g/100ml	Fructose g/100ml
Tangerine	3.1	0.95	0.95
Strawberry	0.35	2.65	2.0
White Mangrove	1.1	1.6	2.3

TABLE 1. Nectar formulas for three plants found in Florida were made-up at 5% original concentration. Each was chosen because they were higher in one of the three sugars. Tangerine is high in sucrose; strawberry is high in dextrose; white mangrove is high in fructose.

Solution	**pH in PBS**	**pH in Water**	**Osmolarity (mmol/kg) in PBS**	**Osmolarity (mmol/kg) in Water**
PBS	**7.39**	**N/A**	**460.5**	**N/A**
Water	**N/A**	**5.25**	**N/A**	**285**
Strawberry	**7.38**	**5.22**	**647**	**444**
Tangerine	**7.38**	**5.21**	**585**	**390**
White Mangrove	**7.38**	**5.21**	**625**	**422**

TABLE 2. Mean pH and osmolarity for nectar solutions in PBS and water. pH remains similar to PBS and water in the nectar solutions. Osmolarities change with the addition of sugar, which is to be expected because sugar is a molecule.

Blood	pH	Osmolarity (mmol/kg)
Human	7.39	295
Avian	7.54	340
Bovine	7.38	270-300
Cell Culture Media		
MEM-E	7.43	540

TABLE 3. The pH and osmolarity values of some common fluids in which SVHR is found.

	1st Trial		2nd Trial		
Solution	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**Day 5, Day 7**
Tangerine	**5.0×10^6**	**2.5×10^6**	**1.2×10^7**	**8.0×10^6**	**Pending**
Strawberry	**3.5×10^6**	**3.5×10^6**	**1.5×10^7**	**4.5×10^6**	**Pending**
White Mangrove	**6.5×10^6**	**2.0×10^6**	**1.15×10^7**	**8.5×10^6**	**Pending**
Mock	**0**	**0**	**0**	**0**	**Pending**
PBS/Virus	**3.5×10^5**	**0***	**1.0×10^7**	**1.0×10^7**	**Pending**

TABLE 4. Virus titer results for 0 and 24 hours time points in PBS. No significant changes are seen in the virus titer between 0 and 24 hours. Virus titers did decrease by a log in the second trial with strawberry, but it is still believed to be an infectious titer.
* = technical error therefore no data was obtained

FIGURES

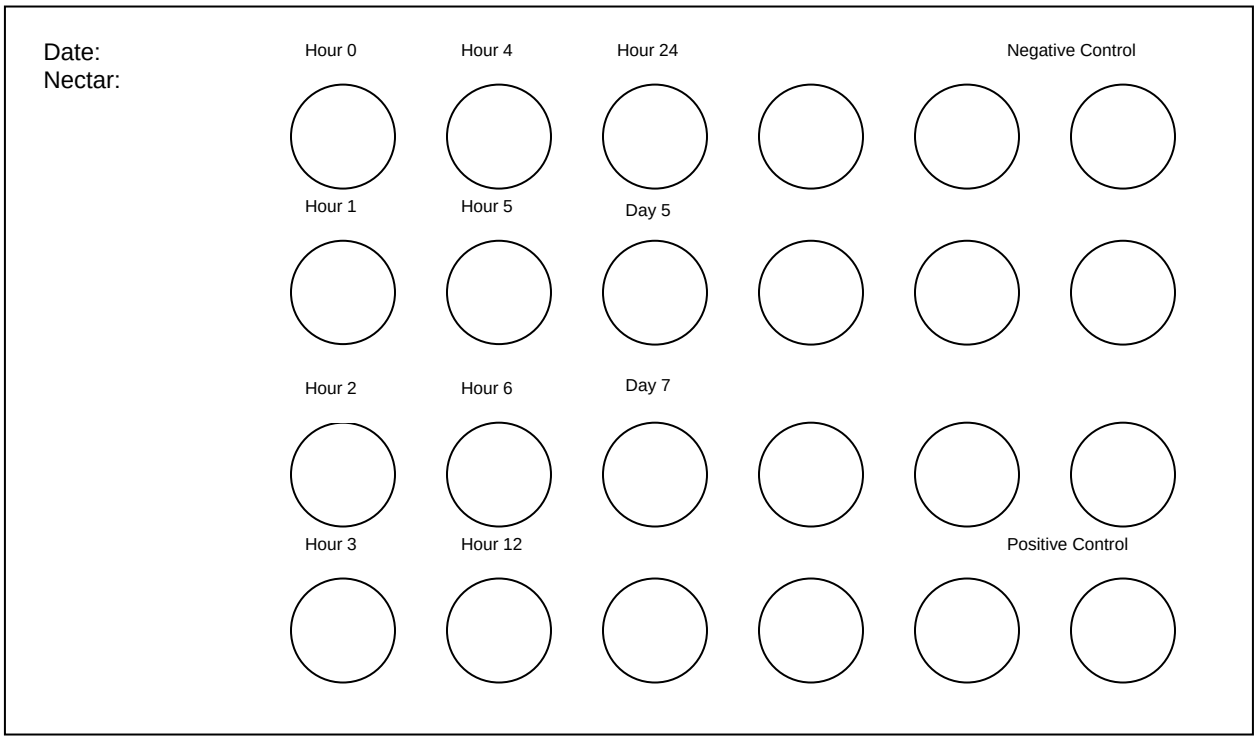

FIGURE 1. Diagram of 24-well plate showing the positions of the time points for virus-nectar solutions. Experimental controls were present on every plate.

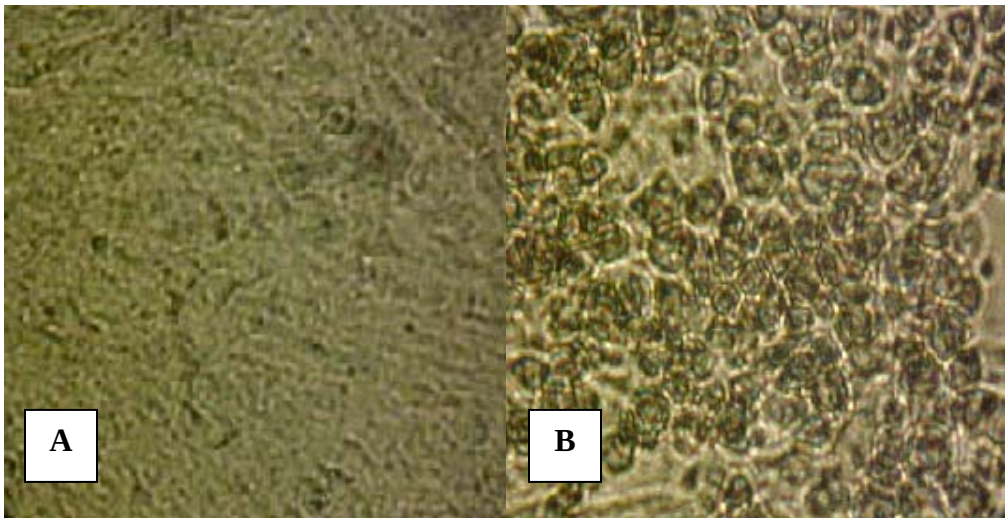

FIGURE 2. A. CPE was not observed in confluent monolayer of BHK-21 cells following adsorption of negative control. B. Evident CPE was observed in confluent monolayer of BHK-21 cells following adsorption of nectar solutions and positive control.