VECTOR COMPETENCE OF *AEDES ALBOPICTUS* FOR A NEWLY RECOGNIZED *BUNYAVIRUS* FROM MOSQUITOES COLLECTED IN POTOSI, MISSOURI[1]

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ABSTRACT. The vector competence of a Kentucky strain of *Aedes albopictus* was assessed for a newly recognized *Bunyavirus* isolated from *Ae. albopictus* collected in Potosi, Missouri. Females are susceptible to peroral infection and 44.7% became infected after ingesting about 15 Vero cell plaque-forming units (PFU) of virus. Virus replicated and reached average titers of $10^{5.4}$–$10^{6.0}$ PFU/mosquito by day 7 postfeeding. Fourteen (40%) of 35 females tested in an *in vitro* virus transmission experiment were infected, and 3 (21.4%) of the infected females transmitted virus. There was no evidence of vertical transmission among 1,196 progeny of a group of mothers exposed to infection perorally or among 6,635 progeny of mothers infected by parenteral inoculation. The absence or infrequency of vertical transmission suggests that the virus was not introduced into Missouri via infected *Ae. albopictus* eggs. Nonetheless, *Ae. albopictus* is a competent vector of this virus and we provide the first experimental evidence for incriminating *Ae. albopictus* as a vector in a natural arbovirus transmission cycle in the United States.

INTRODUCTION

Sixteen isolates of a newly recognized Bunyamwera serogroup (family Bunyaviridae, genus *Bunyavirus*) arbovirus were made from pools of *Aedes albopictus* (Skuse) collected in or near Potosi, Washington County, Missouri, during August and September 1989 (Francy et al., in ms.; C. J. Mitchell, G. C. Smith, T. F. Tsai, and C. Frazier, unpublished data). Ten of the virus isolates came from mosquitoes collected by Mark Niebylski, University of Notre Dame, by sweeping vegetation with a large hand-held vacuum device and by aspirating landing/biting mosquitoes from field workers (Francy et al., in ms.). Six additional virus strains were obtained from 1,039 *Ae. albopictus* females (28 pools) collected in Potosi by aspirator on August 28, 1989, by William B. Kottkamp, St. Louis Health Department. The virus is distinct from known bunyaviruses and is related to Tensaw virus; details concerning the field isolation and identification of the virus will be published separately. We report here on the results of virus infection and transmission experiments conducted with *Ae. albopictus* in the laboratory.

MATERIALS AND METHODS

The virus strain used, 89-3380, was isolated from a pool of 67 *Ae. albopictus* collected on September 21, 1989. The virus, passed twice in Vero cell culture when given to us by Nick Karabatsos of this Division, titered $10^{6.7}$ Vero cell plaque-forming units (PFU)/ml.

Our *Ae. albopictus* colony originated from ovitrap collections made in Lexington, Kentucky, during the summer of 1987. The colony has been maintained in our insectary and cycled 5 or 6 times each year; therefore, our specimens were from approximately the 12th to 15th laboratory generations. All mosquitoes were reared at 26.7 (±0.5°C), 80% RH, and a photoperiod of L:D 16:8. Three- to 5-day old females were used in the feeding trials.

To obtain information about viremias in vertebrate hosts potentially useful for infecting mosquitoes experimentally, we inoculated 4 golden Syrian hamsters, 6–7 weeks of age, subcutaneously with 0.1 ml each of a virus suspension subsequently shown to contain about 17,500 PFU per dose. These hamsters were bled daily for 4 days by cardiac puncture following anesthetization with 0.05–0.07 ml of Ketamine-Rompum (200 mg Ketamine HCl/ml and 0.3 mg Rompum). Whole blood (0.1 ml) was diluted 1:10 in BA-1 diluent (0.2 M Tris, pH 8.0, 0.15 M NaCl, 1% BSA, 10 mg/liter phenol red, 50 mg/liter Gentamicin, and 1 μg/ml Fungizone) and frozen at −70°C until tested for virus. After obtaining information on viremia profiles in hamsters, 6 additional hamsters, 4 weeks of age, were inoculated subcutaneously as follows: 3 were given 0.1 ml estimated to contain 1,750 PFU/dose and 3 were given 0.1 ml estimated to contain 17,500 PFU/dose.

On day 4 postinoculation, 6 groups of approximately 85 *Ae. albopictus* each were allowed to feed for 30 min on each of the potentially viremic, anesthetized hamsters. Postfeeding bloods were drawn from each hamster as described above. Three freshly fed mosquitoes from each group were frozen immediately for subsequent virus assay and the remaining engorged specimens were sorted into 6 cages, given

[1] Use of trade names or commercial sources is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

5% sugar water and incubated at 26.7°C, 80% RH and 16:8 L:D. A few mosquitoes from some groups were frozen on days 2, 4, 7, 9, 12 and 14 postfeeding for virus titration to monitor virus replication.

Virus transmission was determined by the *in vitro* feeding technique described by Aitken (1977). Glass capillary tubes drawn to a fine tip at one end were loaded with approximately 5 µl of 2% fetal calf (FCS) serum in PBS, pH 7.2. Each mosquito's wings and legs were removed to immobilize the specimen, then the proboscis of the mosquito was inserted into a loaded capillary tube. Each mosquito was left in place for at least 30 min. The uningested portion of the feeding suspension was expressed onto a microscope slide, loaded into a calibrated capillary needle and injected parenterally into 5 or more *Ae. albopictus*. Parenterally inoculated mosquitoes were given 5% sugar water, incubated at 26.7°C for 6 days, pooled and frozen at −70°C until assayed for virus in Vero cells.

To test for vertical transmission of virus by females infected perorally, eggs were collected on velour paper strips from each of the 6 groups of mosquitoes fed on potentially viremic hamsters and kept until virus infection rates in parental females were determined. First ovarian cycle eggs from the group of mothers with the highest infection rate (44.7%) were hatched, and larvae were reared to adults. Also, F_1 generation progeny from parenterally infected females were tested for virus. One hundred fifty *Ae. albopictus* were inoculated parenterally (Rosen and Gubler 1974) with approximately 300 Vero cell PFU each of virus and incubated under insectary conditions described above. Females were fed on an uninfected hamster 7 days postinoculation and allowed to oviposit, then refed on day 15 postinoculation and allowed to oviposit again. First and second ovarian cycle progeny were reared to adults, with the exception of a few that were pooled as larvae or pupae.

Adult progeny from all vertical transmission experiments were pooled by sex in pools of 50 or less and frozen at −70°C until processed for virus tests. Pools were triturated in 1 ml of BA-1 diluent and centrifuged at 4°C for 30 min at 2,500 rpm. Supernatants were further clarified by centrifugation at 13,000 rpm for 4 min and parenterally inoculated into 3- to 5-day-old *Ae. albopictus* for virus amplification. Suspensions from each pool of potentially infected adults were inoculated into 6 or more mosquitoes. These mosquitoes were incubated for 6 days at 26.7°C under standard insectary conditions, pooled according to the inoculum received and frozen at −70°C until processed and tested for virus.

To confirm that parenteral inoculation of mosquito suspensions of progeny from the vertical transmission experiments would be an efficient method of amplifying small quantities of virus, 4 groups of 3- to 5-day-old *Ae. albopictus* were inoculated with a series of 10-fold, low-titered stock virus (89-3380) suspensions and incubated under standard insectary conditions. Samples of 3 mosquitoes were removed and frozen at −70°C at 8 and 24 h postinoculation and daily thereafter for 6 days. Mosquitoes were sonicated individually in 1 ml of BA-1 diluent and processed for virus testing as described above. Specimens were frozen at −70°C until tested for virus in Vero cells.

Specimens were screened for virus or were titrated as appropriate. Individual adult mosquitoes were disrupted by sonic energy in 1 ml of BA-1 diluent. Suspensions were centrifuged at 2,000 rpm for 20 min, and the supernatants were frozen at −70°C until tested. Each pool of parenterally inoculated mosquitoes was triturated. Following centrifugation, supernatants were frozen at −70°C until tested. Tests for virus were done by plaque assay in Vero cell culture. Briefly, 10-fold dilutions of supernatants were made in BA-1, and samples (0.1 ml) were inoculated into Vero cell cultures in 6-well plates, adsorbed for 1 h at 37°C and overlaid with 1% Noble agar in M-199 supplemented with 2% FCS, 2.0 g/liter of $NaHCO_3$, 150 µg/ml of DEAE-dextran, and 1:4,000 neutral red. Cell cultures were then examined for 10 days for characteristic plaques.

RESULTS

Only 2 of 4 hamsters were viremic in the viremia profile experiment. One hamster was viremic on days 2, 3 and 4 postinoculation ($10^{3.3}$, $10^{4.7}$ and $10^{3.9}$ Vero Cell PFU/ml, respectively), and the other was viremic on days 3 ($10^{2.6}$ PFU/ml) and 4 ($10^{4.0}$ PFU/ml) postinoculation. On the basis of these results, we chose to feed mosquitoes in the next experiment on day 4 postinoculation. Only 3 of 6 hamsters were viremic at this time. Blood samples were tested immediately and mosquitoes that had fed on nonviremic hamsters were discarded when the test results became available.

The 3 viremic hamsters had titers of 4.0×10^2, 1.7×10^3, and 3.0×10^3 PFU/ml. Based on an estimated blood meal volume of 5 µl/mosquito, females that fed on the 3 viremic hamsters would have ingested about 2, 8 and 15 PFU, respectively. Peroral infection rates after 14 days of incubation are summarized in Table 1.

Virus replication in mosquitoes from the time of feeding through 14 days of incubation is shown in Table 2. Titers shown for mosquitoes on the day of feeding (day 0) are based on the

titers of the infectious blood meals and estimated blood meal volumes of 5 μl/female. Only 8 of 9 mosquitoes that were frozen immediately after feeding and subsequently tested for virus were shown to contain virus. Also, the amounts of virus found in these freshly fed mosquitoes were less than expected in most cases, i.e., expected 2 PFU/female, observed 1, 1, and 2; expected 8 PFU/female, observed 0, 2, and 2; expected 15 PFU/female, observed 3, 3, and 6. The observed results are based on testing the entire samples of virus suspensions and counting PFU. The virus replicated to high titer in each of the 3 groups of mosquitoes (Table 2).

Thirty-eight females, fed on the blood meal with a titer of 3.0×10^3 Vero cell PFU/ml, were used in the *in vitro* transmission experiment on day 14 postfeeding. Three of these females were excluded from the analysis because they ingested most or all of the feeding suspension used to collect salivary secretions and there was not enough remaining to inject into other mosquitoes for virus amplification. Fourteen (40%) of the remaining 35 females were subsequently shown to be infected; virus titers ranged from $10^{5.0}$ to $10^{5.9}$ PFU per female. Three of 14 infected females transmitted virus in the *in vitro* assay for a transmission rate of 21.4%.

First ovarian cycle progeny from the group of females with a 44.7% infection rate (Table 1) were tested to determine whether they were infected with virus. A total of 1,196 specimens (469 females and 727 males) were tested in 25 pools (10 pools of females and 15 pools of males) with negative results. One hundred forty-five pools comprised of 4,123 (2,175 males and 1,948 females) first ovarian cycle and 2,491 (1,348 males and 1,148 females) second ovarian cycle adult progeny from parenterally infected females were tested for virus with negative results. Five additional pools made up of 19 late instar larvae and 2 pupae also were negative for virus. To confirm that the mothers parenterally inoculated with virus and used in the vertical transmission experiment were infected, 10 mothers that survived the second ovarian cycle were tested for virus. Each female was shown to be infected.

Table 3 summarizes data on replication of the newly recognized *Bunyavirus* following parenteral inoculation of small quantities of virus into *Ae. albopictus*. Following a typical eclipse phase, when virus could not be detected at 8 h postinoculation, all specimens inoculated with 11.0 and 1.3 Vero cell PFU each were shown to be infected. Virus replicated to high titers in infected specimens.

Table 1. Peroral infection rates of Lexington, KY, *Aedes albopictus* for a newly recognized *Bunyavirus*.

Titer of infectious meal*	No. tested	% infected day-14 incubation
2 PFU	54	11.1
8 PFU	48	22.9
15 PFU	38	44.7

* Number of Vero cell plaque-forming units (PFU) per 5 μl, i.e., estimated blood meal volume.

DISCUSSION

The significance of our findings relates to the first recognized involvement of *Ae. albopictus* in a virus transmission cycle in the United States. Since the discovery of this species in Harris County, Texas, in 1985 (Sprenger and Wuithiranyagool 1986), studies have shown that subpopulations of *Ae. albopictus* established in the United States and elsewhere in the Western Hemisphere can become infected with and transmit several arboviruses under experimental conditions (Boromisa et al. 1987, Mitchell et al. 1987, Miller and Ballinger 1988, Miller et al.

Table 2. Replication of a newly recognized *Bunyavirus* in perorally infected *Aedes albopictus*.

	Titers[a] of infectious meals					
	2 PFU/female		8 PFU/female		15 PFU/female	
Day postfeeding	No. positive/ No. tested	titer x̄ (SE)	No. positive/ No. tested	Titer x̄ (SE)	No. positive/ No. tested	Titer x̄ (SE)
0	3/3	0.3[a]	2/3	0.9[a]	3/3	1.2[a]
2	0/3		1/3	3.6[b]	1/3	3.5[b]
4	0/3		0/2		0/3	
7	1/10	6.0[b]	2/10	5.5[b] (0.2)	6/10	5.4[b] (0.8)
9	0/3		0/3		1/3	5.5[b]
12	Not done		Not done		1/3	5.7[b]
14	6/54	5.5[b] (0.3)	11/48	5.5[b] (0.1)	17/38	5.5[b] (0.3)

[a] Based on $\log_{10}$ Vero cell PFU titer of infectious blood meals and estimated blood meal volume of 5 μl per mosquito.

[b] Log_{10} Vero cell PFU/female.

Table 3. Replication of a newly recognized *Bunyavirus* following parenteral inoculation into *Aedes albopictus*.

Hours post-inoculation	Infective dose[1] per 0.17 μl per mosquito							
	0.01 PFU		0.1 PFU		1.3 PFU		11.0 PFU	
	Number positive[2]	Titer[3] x̄ (SE)	Number positive	Titer x̄ (SE)	Number positive	Titer x̄ (SE)	Number positive	Titer x̄ (SE)
8	0		0		0		0	
24	0[4]		1	2.4	3	1.8 (0.3)	3	3.5 (0.9)
48	0		0		3	4.3 (0.2)	3	4.6 (0.4)
72	0		2	4.8 (0.4)	3	4.8 (0.4)	3	4.7 (0.2)
96	0		1	5.1	3	4.6 (0.6)	3	4.7 (0.2)
120	0		1	4.0	3	4.9 (0.2)	3	4.6 (0.6)
144	1	4.9	2	5.0 (0)	3	4.9 (0.1)	3	4.9 (0.3)
168	0		1	4.8	3	5.0 (0.3)	3	5.0 (0.3)

[1] Based on backtitration of inocula in Vero cells.
[2] Three specimens tested per time interval and virus dose unless otherwise indicated.
[3] Log_{10} Vero cell PFU/ml.
[4] Only 2 specimens tested.

1989, Turell et al. 1988, Grimstad et al. 1989, and Mitchell and Miller 1990). However, isolation of a newly recognized *Bunyavirus* from *Ae. albopictus* from Washington County, Missouri (Francy et al., in ms), represents the first recognized involvement of this species in a natural arbovirus transmission cycle in the Western Hemisphere. Our experimental results corroborate evidence from the field (Francy et al., in ms.) indicating *Ae. albopictus* may be a natural vector of this virus.

Chamberlain et al. (1954) defined the "threshold of infection" as the lowest concentration of virus capable of causing an infection in 1–5% of mosquitoes ingesting it. Our results indicate the threshold of infection of virus strain 89-3380 in *Ae. albopictus* is less than 400 Vero cell PFU/ml in hamster blood. In fact, 11.1% of mosquitoes feeding on this concentration became infected despite ingesting only an estimated 2 PFU/female and 44.7% did so after ingesting an estimated 15 PFU/female (Table 1). The susceptibility of *Ae. albopictus* to peroral infection with the virus, as measured by the threshold of infection, compares favorably with the most susceptible mosquito species tested by Chamberlain et al. (1954) in their studies with eastern equine encephalitis and western equine encephalitis viruses. A more exact comparison is difficult because their virus titers were expressed as mouse intracerebral LD_{50}.

The virus replicated to high titer in perorally infected *Ae. albopictus* irrespective of the titer of the infectious meal. Virus titers appeared to reach a plateau in infected mosquitoes on or before day 7 postfeeding (Table 2). Consequently, it would be worthwhile to determine the length of the incubation period required before mosquitoes can transmit virus by bite, i.e., the extrinsic incubation period. This period may be somewhat shorter than the 14-day period that we arbitrarily selected for our virus transmission trial.

Among the mosquitoes tested for virus transmission in the *in vitro* assay, 40% were subsequently shown to be infected, and 21.4% of the infected females transmitted virus. Therefore, this would result in a population transmission rate of 8.6%, i.e., 21.4% of 40%. It is possible that in nature infected vertebrate hosts circulate virus at higher titers than did our hamsters. If so, virus infection rates in exposed mosquitoes would be expected to be higher. In any event, it is clear that *Ae. albopictus* is susceptible to peroral infection and that almost one-quarter of mosquitoes infected by feeding on low titered blood meals can transmit virus by bite after a 2-week incubation period.

Vertical transmission of virus to progeny of infected *Ae. albopictus* females was not demonstrated despite the proven sensitivity of our assay system for amplifying small quantities of virus (Table 3). The failure to demonstrate vertical transmission of virus among 1,196 first ovarian cycle progeny of mothers exposed to infection by the oral route is not surprising. Most of the eggs would have been laid within a few days after the mothers took the infectious blood meal and before widespread dissemination of virus would have been expected to occur. Also, since only 44.7% of the mothers in this group were infected, more than one-half of the progeny probably came from uninfected mothers. However, the apparent lack of vertical transmission of virus to 6,635 first and second ovarian cycle progeny of mothers uniformly infected by parenteral inoculation suggests that vertical transmission of virus is unlikely to occur among Lexington, Kentucky, *Ae. albopictus*. If this also is the case for the Potosi, Missouri, mosquito

strain, initial introduction of the virus into Potosi by importation of infected *Ae. albopictus* eggs in used tires seems improbable. Conversely, the existence of an ancillary, silent cycle involving another vector species seems more likely. Further studies in the field and the laboratory will be required to answer these questions.

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