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Vector Competence of *Aedes aegypti*, *Culex sitiens*, *Culex annulirostris*, and *Culex quinquefasciatus* (Diptera: Culicidae) for Barmah Forest Virus

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J. Med. Entomol. 37(5): 660–663 (2000)

ABSTRACT *Aedes aegypti* (L.), *Culex sitiens* Weidemann, *Culex annulirostris* Skuse, and *Culex quinquefasciatus* Say mosquitoes colonized at the Queensland Institute of Medical Research, Brisbane Australia, were fed on blood containing Barmah Forest virus (BF). Only *Cx. annulirostris* was susceptible to infection, with a median cell culture infectious dose ($CCID_{50}$) of $10^{3.36}$ per mosquito. *Ae. aegypti* and *Cx. quinquefasciatus* were infected experimentally, but at rates of <9%. *Cx. sitiens* did not become infected. Infection rates for *Cx. annulirostris* fed $10^{3.5}$ $CCID_{50}$ of virus per mosquito, varied from 9 to 50% between 2 and 13 d after infection. Virus transmission to suckling mice by *Cx. annulirostris* occurred from 2 d after infection. Transmission of BF virus by *Cx. annulirostris* was 10% at 2 d after infection and did not exceed 8% thereafter. Although *Cx. annulirostris* may be infected and is able to transmit BF virus to suckling mice, it is nonetheless a relatively inefficient vector of the virus.

KEY WORDS *Aedes aegypti, Aedes notoscriptus, Culex annulirostris, Culex sitiens, Culex quinquefasciatus,* Barmah Forest virus

BARMAH FOREST (BF) Virus (Togaviridae: *Alphavirus*) has been linked with human infection in Australia since 1986 (Vale et al. 1986) and with human disease since 1988 (Phillips et al. 1990). The clinical symptoms of BF virus are similar to those of Ross River (RR) virus and include polyarthritis, myalgia, arthralgia, rash, and fever. Symptoms may persist for up to several months (Boughton et al. 1988, Phillips et al. 1990, Mackenzie and Smith 1996, Flexman et al. 1998). During 1996, 837 cases of BF infection were reported in Australia (Curran et al. 1997).

Of the four alphaviruses in Australia (Getah, Sindbis, Ross River, and Barmah Forest), only RR and BF are frequently the causative agents of human disease (Lindsay et al. 1994). RR and BF viruses are common etiological agents of epidemic polyarthritis and polyarticular disease in Australia, with RR virus the most common agent of arbovirus mediated disease in Australia (Hargreaves and Hall 1992). RR and BF viruses account for 90 and 10%, respectively, of Australian cases of polyarthritis caused by mosquito-borne viruses (Phillips et al. 1990).

The increasing prevalence of BF virus is of concern, particularly because isolates have been recorded from mosquitoes from five of six Australian mainland states (Russell 1994). BF virus appears to have a broad range of vectors based on isolations from 16 species of wild-caught mosquitoes, including *Aedes vigilax* Skuse (Russell 1994, Lindsay et al. 1995, Clancy and Russell 1997, Russell et al. 1997), *Culex annulirostris* Skuse and *Culex quinquefasciatus* Say (Lindsay et al. 1995). The range of potential vectors (including four genera: *Aedes*, *Culex*, *Anopheles*, *Coquillettidia*) is similar to that of RR virus and is likely to be large.

The isolation of an arbovirus from wild-caught mosquitoes indicates that a particular species may be susceptible to infection, and therefore may be classified as a suspected vector (WHO 1985). Experimental infection studies are needed to confirm the vector status of mosquito species with high field virus isolation rates. The experimental feeding of virus mixtures in blood to mosquitoes is an essential prerequisite to determining vector status. This is because experimental infections of mosquitoes, through feeding virus/blood mixes or via viremic animals, provide direct evidence of the susceptibility to infection at particular levels of viremia. Pledget and membrane feeding of arboviruses to mosquitoes may result in higher minimum infection thresholds and lower infection rates for some viruses compared with feeding on viremic animals (Jupp 1976, Turell 1988, Weaver et al. 1993). This is thought to be because additives in artificial bloodmeals (sucrose and heparin) affect the deposition of the bloodmeal in the mosquito. Sucrose, used to increase palatability, can reduce the degree of virus concentration adjacent to the abdominal midgut epithelium—that may result in decreased infection of some viruses (Turell 1988, Weaver et al. 1993). For this reason, the sucrose content of the bloodmeals used in this and similar experiments was limited to 1%.

Based on laboratory experiments, *Ae. vigilax* (Boyd and Kay 1999) has been implicated as an important

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Table 1. Barmah Forest virus percentage infection rates (*n*) in mosquitoes, processed at 8 d after pledget feeding

Log virus dose	*Ae. aegypti*	*Cx. annulirostris*	*Cx. sitiens*	*Cx. quinquefasciatus*
3.5	8.5 (200)	40 (57)	0 (50)	2.1 (92)
2.5	1.4 (74)	50 (48)	0 (40)	0 (72)
1.5	0.9 (108)	0 (75)	0 (21)	0 (56)
0.5	0 (119)	0 (42)	0 (12)	0 (24)
ID_{50}	NA	3.36	NA	NA

Infection = % infection (*n*). Log virus dose = Log_{10} Vero $CCID_{50}$ per mosquito. NA = not applicable. *n* = number of mosquitoes tested.

vector of BF virus. *Aedes notoscriptus* (Skuse) also may be a vector in urban Australia based on laboratory results (Watson and Kay 1999). *Aedes aegypti* (L.), *Culex sitiens* Weidemann, *Cx. annulirostris,* and *Cx. quinquefasciatus* are common pest species in Australia. *Ae. aegypti* is a highly efficient dengue vector and common in peridomestic environments in some urban areas of north Queensland, Australia, whereas *Cx. annulirostris,* a freshwater species found throughout Australia, is a major vector of RR virus. *Ae. aegypti* is also a proven laboratory vector of RR virus (Kay et al. 1979, Gubler 1981, Ballard and Marshall 1986, Mitchell and Gubler 1987) and, given its close association with humans, is a potential vector for BF virus. Although not implicated as important RR vectors, the coastal mosquito *Cx. sitiens* and the urban freshwater *Cx. quinquefasciatus* are common in their respective environments, and RR virus has been isolated from wild-caught specimens of both species (Lindsay et al. 1992, Ritchie et al. 1997). The current study seeks to define the vector competence of these four species through oral infection, and to demonstrate their ability to transmit virus orally as a further step in determining their vector status.

Materials and Methods

An isolate of BF virus (BF2078/120) from *Ae. vigilax* collected in 1996 at Mount Coolum, ≈100 km north of Brisbane, Australia, was used. Stock virus (C6–36 passage 2) had a titer of 10^7 Vero $CCID_{50}$ (median cell culture infectious dose) per 100 μl, and was stored in aliquots at –70°C until required.

Adult female mosquitoes from laboratory colonies at the Queensland Institute of Medical Research (QIMR) had the following origins: *Ae. aegypti* (1989), from eggs collected in Townsville, north Queensland, Australia; *Cx. annulirostris* (1998), from adults collected at Brisbane, Queensland, Australia; *Cx. sitiens* (1996), from adults collected at Coomera Island, Queensland, Australia; and *Cx. quinquefasciatus* (1999), from larvae collected at Brisbane, Queensland, Australia.

Newly emerged mosquitoes were maintained at 28°C, 70% RH, and a photoperiod of 12:12 (L:D) h, with a 10% sucrose solution. Before infection, 2- to 3-d-old females were starved for 24 h. To determine susceptibility to oral infection, groups of mosquitoes were offered four 10-fold dilutions of BF virus (10^{-2} to 10^{-5}) in heparinized rabbit blood (25 U/ml) and 1% sucrose for 4 h. To determine the virus dose ingested per mosquito, blood-virus mixtures were titrated on Vero (African green monkey) cells. The $CCID_{50}$ was calculated based on an average *Ae. vigilax* bloodmeal volume of 3 μl, and ranged from $10^{0.5}$ to $10^{3.5}$ $CCID_{50}$ per mosquito.

After feeding, engorged mosquitoes were sorted on a chill table, held for 8 d extrinsic incubation period at 28°C, and then killed and stored at –70°C before processing. Individual mosquitoes were homogenized separately in sterile 1.5-ml microfuge tubes in 500-μl diluent (RPMI 1640, 10% fetal calf serum, glutamine, penicillin, streptomycin and fungizone) and then centrifuged at 8,000 × *g* for 5 min at 4°C. Supernatant was transferred into sterile microfuge tubes and respun. One hundred microliters of the resultant supernatant were transferred to duplicate wells of a 96-well microtiter plate and titrated as 10-fold dilutions. Each well was seeded with 1.5 × 10^5 Vero cells/ml. Unfed mosquitoes were processed with each batch of plates as negative controls. Plates were incubated at 37°C and were examined daily for cytopathic effect, which was confirmed by staining with 0.5% crystal violet in 10% formalin after 7 d. The median infectious dose (ID_{50}) and $CCID_{50}$ end points were calculated by Probit analysis (Gillespie 1993). Infection rates were expressed as the number of infected mosquitoes per total tested.

Transmission rates for *Cx. annulirostris* were determined by inducing individual mosquitoes that had fed on the 10^{-2} ($10^{3.5}$ $CCID_{50}$ per mosquito) dilution to feed on 2-d-old Quackenbush mice at regular intervals after infection (Queensland Institute of Medical Research animal ethics protocol A97011). After feeding, each mosquito was assayed for virus, as above, to confirm virus infection and to monitor the rate of viral growth, except that legs were removed and processed separately to determine virus dissemination from the mesenteron. Mice were inspected daily for any clinical signs, such as hind limb flaccid paralysis consistent with alphavirus infection (Mims et al. 1973, Seay and

Table 2. Infection and transmission of Barmah Forest virus by *Cx. annulirostris* fed $log^{3.5}$ $CCID_{50}$ (Vero) per mosquito, processed at different incubation periods at 28°C

Time (days)	*n* tested	% infected (*n*)	% disseminated infection (*n*)	% trans. (*n*)	% transmission by infected mosquitoes (*n*)
2	10	10 (1)	10 (1)	10 (1)	100 (1)
3	14	14 (2)	14 (2)	7 (1)	50 (1)
5	11	18 (2)	0 (0)	0 (0)	0 (0)
6	14	50 (7)	14 (2)	7 (1)	14 (1)
8	11	9 (1)	9 (1)	0 (0)	0 (0)
10	12	33 (4)	17 (2)	0 (0)	0 (0)
13	13	46 (6)	23 (3)	8 (1)	16 (1)
Total	85	27 (23)	13 (11)	5 (4)	17 (4)

% Infection = % mosquitoes infected. % Disseminated infection = % with virus present in legs. % Transmission rate = % of mosquitoes transmitting virus to mice. *n* = number of mosquitoes tested.

Wolinsky 1982). After 21 d, all mice were euthanized and their sera tested for neutralizing antibody to BF virus by the varying virus/constant sera microneutralization method (Lennette 1969). Each test-plate had cell (media plus cells) and sera (sera, media, plus cells) control rows, and a virus control column (media, virus titration plus cells). Plates were incubated at 37°C and examined daily for cytopathic effect, which was confirmed by staining with 0.5% crystal violet in 10% formalin after 7 d. Neutralization tests were considered positive if cell and sera control cells were normal and the log neutralization index was >2.0 (Reed and Muench 1938). Transmission rates were calculated as the number of 21-d-old mice with neutralizing antibody, plus any euthanized after clinical signs, divided by the number of mice tested.

Results

Infection Thresholds. After an extrinsic incubation period of 8 d, *Cx. annulirostris* showed greatest susceptibility, with infection rates of up to 50% and an ID_{50} of 3.36 $CCID_{50}$ (Vero) per mosquito (Table 1). In contrast, *Ae. aegypti* and *Cx. quinquefasciatus* had poor susceptibility, with maximum infection rates of 8.5% and 2.1%, respectively. *Cx. sitiens* did not become infected.

Transmission Studies. *Cx. annulirostris*, the only species susceptible to infection, was tested for transmission. In response to a per os infectious dose of $10^{3.5}$ BF virus, the overall infection rate of *Cx. annulirostris* ($n = 85$) was 27%; however, some of these females were processed as early as 2 d after infection. Overall, 13% of females tested had infected legs; i.e., virus has disseminated from the infected mesenteron into the hemocoel. Transmission was recorded from 2 d after infection (Table 2) and varied between 0 and 10% through 13 d, with no transmission detected from mosquitoes on days 5, 8, and 10. None of the suckling mice developed clinical signs, but four of 85 (5%) had detectable BF antibody, with log neutralization indexes ranging from 2.0 to 4.0 at 21 d.

Discussion

Aedes aegypti, *Cx. quinquefasciatus*, and *Cx. sitiens* should not be considered vectors of BF strain used in our experiments. Given these results, *Ae. vigilax* remains the major potential coastal vector of BF virus (Boyd and Kay 1999). In peridomestic situations, *Ae. notoscriptus* is the only species identified thus far as a potential vector of BF virus (Watson and Kay 1999).

Culex annulirostris was moderately susceptible to laboratory infection with BF, but transmitted the virus to suckling mice at rates $\leq 10\%$. *Cx. annulirostris* had apparent midgut infection barriers to BF, with $\geq 50\%$ of the mosquitoes that fed on bloodmeals containing virus not becoming infected. However, the overall dissemination rate of 13% and transmission rate to suckling mice of 5% indicated that the mesenteronal escape and salivary gland infection and escape barriers were not completely expressed in our test population.

The very short extrinsic incubation period of 2 d before transmission of BF virus by *Cx. annulirostris* is of interest. Although transmission was low at 2 d (10%), this short extrinsic incubation period equates to a reduced adult mosquito survival time required to transmit BF virus. An infected *Cx. annulirostris* could potentially infect a susceptible host within 2 d of feeding on a viremic host; that is, within a single gonotrophic cycle. Despite this, our data indicated that *Cx. annulirostris* is a relatively inefficient vector of BF virus. No more than 10% of *Cx. annulirostris* transmitted virus to mice, and the maximum infection rate of 50% of *Cx. annulirostris* occurred when fed a relatively high virus dose. At doses of BF virus $10^{1.5}$ $CCID_{50}$ per mosquito (and lower), none became infected, compared with 35% of *Ae. vigilax* infected after ingestion of the same BF virus dose and strain (Boyd and Kay 1999). This indicates that relatively high titered viremias would be required in vertebrate hosts in order for *Cx. annulirostris* to act as a significant vector in a natural system.

The QIMR colony *Cx. annulirostris* had an overall transmission rate of only 5%, and maximum transmission of 10%, compared with *Ae. vigilax* (55%, maximum 100%) (Boyd and Kay 1999) and *Ae. notoscriptus* (34%, maximum 50%) (Watson and Kay 1999) fed the same strain and titer of virus. Infection data from wild-caught *Cx. annulirostris* support these results. Wells et al. (1992) found that the BF virus laboratory infection rate of NSW wild-caught *Cx. annulirostris* was 27%, compared with 63% of *Ae. vigilax* fed the same dose. Ryan and Kay (1999) found that wild-caught *Cx. annulirostris* fed $10^{3.5}$ BF 1611 (another mosquito isolate from Mt. Coolum) had a maximum infection rate of 8% after 11 d, and did not transmit BF virus to mice.

Although *Cx. annulirostris* is known to be an important vector of RR virus, this study provided surprising evidence that, although being capable of transmitting BF virus to vertebrates, it is unlikely to be a major vector of this BF strain.

Acknowledgments

We thank Kay Marshall for technical assistance and Allan Saul for manuscript review. This study was funded by the National Health and Medical Research Council, Australia.

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Received for publication 17 August 1999; accepted 6 April 2000.