Vector Competence of Australian Mosquitoes (Diptera: Culicidae) for Japanese Encephalitis Virus

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ABSTRACT Australian mosquitoes were evaluated for their ability to become infected with and transmit a Torres Strait strain of Japanese encephalitis virus. Mosquitoes, which were obtained from either laboratory colonies and collected using Centers for Disease Control and Prevention light traps baited with CO_2 and octenol or reared from larvae, were infected by feeding on a blood/sucrose solution containing $10^{4.5\pm0.1}$ porcine stable-equine kidney (PS-EK) tissue culture infectious $dose_{50}$/mosquito of the TS3306 virus strain. After 14 d, infection and transmission rates of 100% and 81%, respectively, were obtained for a southeast Queensland strain of *Culex annulirostris* Skuse, and 93% and 61%, respectively, for a far north Queensland strain. After 13 or more days, infection and transmission rates of >90% and ≥50%, respectively, were obtained for southeast Queensland strains of *Culex sitiens* Wiedemann and *Culex quinquefasciatus* Say, and a far north Queensland strain of *Culex gelidus* Theobald. Although infection rates were >55%, only 17% of *Ochlerotatus vigilax* (Skuse) and no *Cx. quinquefasciatus*, collected from far north Queensland, transmitted virus. North Queensland strains of *Aedes aegypti* L., *Ochlerotatus kochi* (Dönitz), and *Verrallina funerea* (Theobald) were relatively refractory to infection. Vertical transmission was not detected among 673 F_1 progeny of *Oc. vigilax*. Results of the current vector competence study, coupled with high field isolation rates, host feeding patterns and widespread distribution, confirm the status of *Cx. annulirostris* as the major vector of Japanese encephalitis virus in northern Australia. The relative roles of other species in potential Japanese encephalitis virus transmission cycles in northern Australia are discussed.

KEY WORDS Japanese encephalitis, mosquitoes, vector competence, Australia

JAPANESE ENCEPHALITIS (JE) VIRUS is considered to be the leading cause of viral encephalitis in Southeast Asia, with more than 50,000 cases reported annually, particularly in young children (Burke and Leake 1988, Vaughn and Hoke 1992, Solomon 1997). However, the actual number of cases is estimated to be much higher, because many cases are misdiagnosed or not officially notified (Tsai 2000). Although the virus has a high case-fatality rate (25%) in unvaccinated populations, permanent neurological sequelae occur in a further 50% of cases (Solomon et al. 2000). In addition to the disease burden in humans, JE causes fatal encephalitis in horses and fetal abortion in infected sows (Burke and Leake 1988). Of particular concern has been the continued geographical spread of JE into new areas of Asia, such as Pakistan (Igarashi et al. 1994) and parts of India (Victor et al. 2000). This expansion has been linked to deforestation and construction of dams and irrigation schemes, which have expanded vector habitats and increased exposure to humans (Tsai 1997). Discrete outbreaks of JE have also occurred on the Pacific islands of Guam (Hammon et al. 1958) and Saipan (Paul et al. 1993).

The geographical distribution of JE recently has expanded into the Australasian region, after an outbreak in the Torres Strait, northern Australia, in 1995 (Hanna et al. 1996). To date, five human cases with two deaths have been recorded (Hanna et al. 1996, Hanna et al. 1999). The emergence of JE in Australia was unexpected, because the previous closest foci was in Bali, Indonesia, 3,000 km west of the Torres Strait (Mackenzie 1999). In Southeast Asia, JE is maintained in a natural cycle between rice field mosquitoes and wading birds and/or domestic pigs (Burke and Leake 1988, Vaughn and Hoke 1992). Although JE can be maintained in a natural bird-mosquito cycle, pigs are considered essential for preepidemic viral amplification (Tsai 1997). Indeed, the Torres Strait outbreaks have been attributed to the high domestic pig population on many of the island communities (Hanna et al. 1996, Ritchie et al. 1997b).

All animal experiments were approved by the University of Queensland Animal Ethics Committee (Approval number MICRO/412/01/NHMRC/QH/APR) and Queensland Health Scientific Services Animal Ethics Committee (Approval number UQ 1/99/27).

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The major vectors of JE are *Culex* mosquitoes, of which *Culex tritaeniorhynchus* Giles is considered to be the primary vector throughout much of Southeast Asia (Burke and Leake 1988, Vaughn and Hoke 1992). Other species, such as *Culex vishnui* Theobald, *Culex fuscocephala* Theobald, and *Culex gelidus* Theobald are considered to be important secondary vectors in certain areas (Rosen 1986, Vaughn and Hoke 1992). Virus isolations in the Torres Strait and the Western Province of Papua New Guinea have indicated that *Culex annulirostris* Skuse is the most likely vector of JE in northern Australia (Ritchie et al. 1997a, Johansen et al. 2000, Johansen et al. 2001). Single isolates from *Cx. gelidus* and *Ochlerotatus vigilax* (Skuse) collected from Badu Island indicate that they could play a secondary role in JE transmission (Johansen et al. 2001, van den Hurk et al. 2001).

To confirm its status as the major vector of JE in Australia, we undertook vector competence experiments to assess the ability of *Cx. annulirostris* to become infected with and transmit a Torres Strait strain of JE. Numerous other mosquito species, obtained from southeast and northern Queensland also were evaluated as potential laboratory vectors.

Materials and Methods

Mosquitoes. Mosquitoes used for the infection experiments originated from either established laboratory colonies or were collected as adults or larvae from various locations in far north Queensland.

Colony Mosquitoes. Mosquitoes were obtained from laboratory colonies housed at the Queensland Institute of Medical Research and the Australian Army Malaria Research Unit. The origins of the colonies were: *Cx. annulirostris* from adults collected from Brisbane, Queensland; *Culex sitiens* Wiedemann from larvae collected from Coomera Island, Queensland; *Culex quinquefasciatus* Say from larvae collected from the Gold Coast, Queensland; *Ochlerotatus notoscriptus* (Skuse) from Closeburn, Queensland and supplemented with Brisbane field material; *Oc. vigilax* from larvae collected from Redlands Shire, Queensland; and *Aedes aegypti* L. from eggs collected from Townsville, north Queensland. Newly emerged mosquitoes were maintained on 10% sucrose at 28°C and 70% humidity, with a 12:12 (L:D) photoperiod.

Field-Collected Mosquitoes. With the exception of *Cx quinquefasciatus*, wild-caught mosquitoes used in the infection experiments were collected as adults using Centers for Disease Control and Prevention light traps (John W. Hock Company, Gainesville, FL) baited with CO_2 (1 kg dry ice) and 1-octen-3-ol (release rate: 4.5 mg/h) and operated for 12 h (1800–0600 hours) (Ritchie and Kline 1995). Adult mosquitoes were collected from Bamaga, Cairns and Normanton in far north Queensland in 2001. It was expected that these mosquitoes would be free from natural infection, because JE activity has not been detected on mainland Australia since 1998. Adult mosquitoes were sealed in a polystyrene cooler and transported to Brisbane for the vector competence experiments. *Culex quinquefasciatus* fourth instar larvae were collected from settling ponds at Mareeba, far north Queensland and shipped to Brisbane where they were reared to adults.

Virus. An isolate of JE (TS3306) obtained from *Oc. vigilax* collected from Badu Island in 1998, was used for all mosquito infections. This isolate shared between 99.1% and 100% sequence homology with 1995 Badu Island isolates and PNG isolates from 1997 and 1998 (Johansen et al. 2001). The virus was passaged once in C6–36 (*Aedes albopictus* (Skuse)) cells and twice in porcine stable-equine kidney (PS-EK) cells (Gorman et al. 1975) and had a final titer of 10^8 PS-EK $CCID_{50}$ (cell culture infectious $dose_{50}$)/ml.

Vector Competence Experiments. Because JE is considered an exotic virus in Australia, all infection and transmission experiments were undertaken at the Pathogen Containment Level Three Insectary at Queensland Health Scientific Services, Brisbane. Upon emergence, all colony mosquitoes and the Mareeba strain of *Cx. quinquefasciatus* were held for 2–3 d before being exposed to the virus. No attempts were made to age-grade the field-collected adult mosquitoes. Mosquitoes were starved for 24 h before being allowed to feed for 2–4 h on a virus suspension using a glass membrane feeder and pig intestine as the membrane. The virus suspension contained JE stock virus diluted in heparinized rabbit blood and 1% sucrose, and was maintained at 37 ± 1.0°C during feeding. To determine the virus titer of the blood at the time of feeding and if there was a reduction in virus titer, a sample of the blood/virus suspension was taken before and after feeding, diluted 1:20 in tissue culture media (RPMI 1640, with 10% fetal bovine serum, antibiotics, and fungizone) and stored at –70°C for later titration.

After 18 h, blood-engorged mosquitoes were sorted by species and placed into 1-liter containers within an environmental growth cabinet (Sanyo Electric, Gunma, Japan). Mosquitoes were maintained at 28°C, 70%–75% RH, and 12:12 (L:D) with 45-min crepuscular periods, and offered 10% honey water.

The majority of mosquitoes collected from Bamaga and Normanton died within 2–3 d of being exposed to the virus, and few survived the extrinsic incubation period of 14–15 d, when transmission was attempted. Similarly, survival of *Mansonia septempunctata* Theobald collected from Cairns declined rapidly, so mosquitoes were killed at 9 d postvirus exposure, and stored at –70°C to await virus assay.

Transmission. Transmission of JE was determined by allowing individual mosquitoes to feed on Quackenbush suckling mice. For the colony strains of *Cx. annulirostris* and *Cx. sitiens*, transmission was attempted at 7, 10, and 14 d after virus exposure. For all other mosquitoes, transmission was attempted 13 or more days after virus exposure. Individual mosquitoes were placed in 150-ml plastic containers with 0.5-mm gauze covering one end. A single suckling mouse was held against the gauze until the mosquito had probed or fed on the mouse. Mice then were marked and returned to their mother. After the transmission at-

tempt, mosquitoes were killed and frozen at –70°C to await virus assay.

Suckling mice then were examined twice daily for neurological symptoms, including hunching, twitching of limbs, flaccid paralysis, and ataxia. Symptoms usually appeared at 3–4 d postinfection. Symptomatic mice were euthanized and their brains removed and stored at –70°C to await virus assay. Mice that did not develop symptoms were monitored for 21 d, after which they were euthanized and their sera tested for JE-specific antibodies using an indirect fluorescent antibody test (Herrmann 1988). Briefly, 15 μl of serum, diluted 1:5 in phosphate-buffered saline (PBS) pH 7.4, was placed on fixed JE infected cells on an 18-well slide. The slides were then incubated for 30 min at 37°C before being washed for 10 min in PBS. Slides were air dried, then a drop of fluorescein-labeled anti-mouse conjugate was added to each well. After 30 min incubation, slides were again rinsed in PBS for 10 min, air dried, and a coverslip was added using two drops of fluorescent mounting media (Dako Corporation, Carpintaria, CA). The stained antigen/antibody slides were examined using a fluorescent microscope, and positive samples were identified as a green or yellow-green fluorescence within infected cells.

Virus Assay. The blood/virus mixture was titrated as 10-fold dilutions in a 96-well microtiter plate containing confluent PS-EK cell monolayers. Plates were incubated at 37°C with 5% CO_2 and checked daily for any cytopathic effect. After 7 d, plates were fixed in PBS/acetone and stored at –20°C. The amount of virus ingested by individual mosquitoes was based on an average bloodmeal volume of ≈3 μl (Turell and Kay 1998).

The body and legs of the colony mosquitoes were assayed separately, because the recovery of virus from the legs demonstrates that the mosquito possesses an infection where the virus has escaped through the midgut and disseminated throughout the hemocoel (Turell et al. 1984). Similarly, the body, legs, and head plus salivary glands of the species for which transmission was not attempted were removed and assayed separately. Field-collected mosquitoes that were used in the transmission attempts were not tested for disseminated infection.

To determine whether individual mosquitoes were infected or possessed a disseminated infection, either whole mosquitoes, or the body, legs, and head plus salivary glands were homogenized separately in 600-μl tissue culture media using a SPEX 8000 mixer/mill (Spex Industries, Edison, NJ). Homogenates were transferred to a 1.5-ml Microfuge tube and centrifuged at 2,290 g for 10 min, after which 50 μl of supernatant were transferred to duplicate wells of a 96-well microtiter plate containing confluent PS-EK cell monolayers. Plates were incubated at 37°C with 5% CO_2 and checked daily for signs of cytopathic effect. After 7 d, plates were fixed in PBS/acetone and stored at –20°C.

Mouse brain aspirates were homogenized in 1 ml of tissue culture media using the SPEX 8000/mixer mill and centrifuged at 2,290 g for 10 min. Fifty microliters of the supernatant was inoculated onto PS-EK cells and incubated and fixed as described above. Infection in the mosquitoes and mouse brains and the results of the blood/virus titration were confirmed using the JE-specific monoclonal antibody 995 in a cell culture enzyme immunoassay (CCEIA) (Gould 1991, Broom et al. 1998). Any contaminated samples were filtered through a 0.2-μm filter before being re-assayed.

Infection rates were expressed as the percentage of infected mosquitoes of the total tested. Transmission rates were expressed as the percentage of mice from which virus was recovered from brain aspirates, in addition to animals positive for JE antibodies, of the total number of mice that were probed or fed upon by mosquitoes. For the colony mosquitoes and the mosquito species that did not feed on recipient mice, dissemination rates were calculated separately for the legs and head plus salivary glands, and expressed as the number of mosquitoes with virus recovered from the legs or head plus salivary glands per total number of mosquitoes tested.

Growth of JE in Mosquitoes. A separate experiment was undertaken to examine the growth of JE in the colony strains of *Cx. annulirostris* and *Cx. sitiens* at selected times postvirus exposure. Five individual *Cx. sitiens* were sampled daily from day 0–15 d after being exposed to the virus and frozen at –70°C. Because limited feeding on the blood/virus mixture was achieved with *Cx. annulirostris*, only eight mosquitoes were tested for viral titer at 10 d after being exposed to the virus.

To determine viral titers, whole individual mosquitoes were homogenized in 1 ml of diluent, centrifuged at 2,290 × *g* for 10 min, before being titrated as 10-fold dilutions on confluent PS-EK cell monolayers. Inoculated cells then were processed using the CCEIA as described above. Only infected mosquitoes were included in the JE growth analysis.

Vertical Transmission by *Oc. vigilax*. To assess whether wild-caught *Oc. vigilax* could transmit JE vertically to its progeny, mosquitoes were offered a second blood meal at 11 d after virus exposure. Both engorged and non-engorged mosquitoes then were placed individually into 30-ml tubes, containing 2 ml of 50% seawater into which a small plug of cotton wool was inserted to soak up the water. The mouth of the tube was sealed with gauze. The mosquitoes were held at 28°C and 70% RH for 2 d, after which ≈90% had oviposited. Eggs were stored in a humidified chamber at 28°C for 21 d, before being hatched in oxygenated 50% seawater. Larvae were reared at 28°C in 700-ml containers and maintained on Sealion floating fish pellets (Sealion Feed Stuff, Taipei, Taiwan). For emergence, pupae were placed in 150-ml containers sealed with gauze at one end. Adults were maintained on 10% honey water for 3 d, before being killed with CO_2 and stored at –70°C to await virus assay. F_1 adults from each individual parent were grouped in pools of 10 mosquitoes or less, homogenized in 2 ml of tissue culture media, before being assayed in the CCEIA as described above.

Table 1. Infection and transmission of Japanese encephalitis virus by five colony mosquito species fed $10^{4.5 \pm 0.1}$ $CCID_{50}$ of virus per mosquito, after 5–19 d extrinsic incubation

Species	Days post infection	% Infection[a]	% Dissemination[b]	% Transmission[c]
Culex annulirostris	5	78 (14/18)	6 (1/18)	NT[d]
	7	89 (16/18)	33 (6/18)	24 (4/17)
	10	94 (17/18)	78 (14/18)	57 (8/14)
	14	100 (36/36)	64 (23/36)	81 (13/16)
Cx. sitiens	5	83 (15/18)	6 (1/18)	NT
	7	83 (15/18)	28 (5/18)	13 (2/15)
	10	89 (16/18)	33 (6/18)	7 (1/15)
	14	92 (33/36)	11 (4/36)	67 (10/15)
Cx. quinquefasciatus	17–19	98 (50/51)	28 (14/51)	50 (4/8)
Ochlerotatus vigilax	9	19 (12/62)	18 (11/62)	NT
	13	39 (5/13)	39 (5/13)	0 (0/4)
Oc. notoscriptus	13–14	27 (13/48)	8 (4/48)	27 (3/11)

[a] Percentage of mosquitoes containing virus in their bodies (number positive/number tested).
[b] Percentage of mosquitoes containing virus in their legs (number positive/number tested).
[c] Percentage of mosquitoes transmitting virus (number of mice with symptomatic or asymptomatic infection/number tested).
[d] Not tested.

Results

Virus Dose. The mean virus titer at the time of feeding was $10^{7.1 \pm 0.1}$ $CCID_{50}$/ml, with a mean reduction in titer during feeding of $10^{0.1 \pm 0.1}$ $CCID_{50}$/ml. Thus, an average mosquito blood meal of 3 μl would contain $\approx 10^{4.5 \pm 0.1}$ $CCID_{50}$ of virus.

Colony Mosquitoes. After a minimum extrinsic incubation period of 13 d, all six colony species were infected, although infection rates differed among species (Tables 1 and 2). Infection rates >90% were obtained for the three species of *Culex*, but infection rates for *Oc. vigilax*, *Oc. notoscriptus*, and *Ae. aegypti* were <40%. All species developed disseminated infections (i.e., virus detected in the legs), although in some cases, mosquitoes without detectable disseminated virus transmitted the virus to susceptible mice.

After 14 d, evidence of JE transmission was observed with *Cx. annulirostris*, *Cx. sitiens*, *Cx. quinquefasciatus*, and *Oc. notoscriptus* (Table 1). Both *Cx. annulirostris* and *Cx. sitiens* transmitted virus after 7 d. JE-specific antibodies were not detected in any of the surviving mice. None of the *Oc. vigilax* used in the transmission attempts were infected, so transmission was not demonstrated in this species. However, 39% of *Oc. vigilax* had evidence of a disseminated infection, with virus detected in the legs and head plus salivary glands. The dissemination rates for *Ae. aegypti* were 17% and 25% for the legs and head plus salivary glands, respectively (Table 2).

Field-collected mosquitoes. A total of seven species of field-collected mosquitoes were tested for their ability to transmit JE to susceptible suckling mice 14–15 d postvirus exposure (Table 3). Six of seven species used for the transmission experiments were infected, with highest infection rates observed for *Cx. annulirostris* (93%) and *Cx. gelidus* (100%). Similarly, highest transmission rates were obtained for *Cx. annulirostris* (61%) and *Cx. gelidus* (100%), although only one individual of the latter species fed on a recipient mouse. The transmission rates of *Ochlerotatus kochi* (Dönitz), *Oc. vigilax* and *Verrallina funerea* (Theobald) were all <20%. Virus was not transmitted by the Mareeba strain of *Cx. quinquefasciatus.*

Seven other field-collected species that did not feed on recipient mice were tested for the presence of JE in body, legs, and head plus salivary glands (Table 2). Although only low numbers were tested, all of the bodies and head plus salivary glands of *Cx. sitiens*

Table 2. Infection and dissemination of Japanese encephalitis virus by both colony- and field-collected mosquitoes fed $10^{4.5 \pm 0.1}$ $CCID_{50}$/mosquito that did not probe recipient suckling mice[a]

Species	Source	% Infection	% Dissemination (legs)	% dissemination (head + salivary glands)
Aedes aegypti[b]	Townsville	27 (16/60)[c]	17 (10/60)[d]	25 (15/60)[e]
Ochlerotatus notoscriptus	Cairns	20 (1/5)	20 (1/5)	20 (1/5)
Oc. normanensis	Normanton	0 (0/1)	0 (0/1)	0 (0/1)
Oc. purpureus	Bamaga	100 (2/2)	0 (0/2)	100 (2/2)
Culex sitiens	Cairns	100 (1/1)	100 (1/1)	100 (1/1)
Mansonia septempunctata	Cairns	67 (16/24)	54 (13/24)	54 (13/24)
Ma. uniformis	Cairns	100 (1/1)	100 (1/1)	100 (1/1)
Verrallina carmenti	Bamaga	0 (0/2)	0 (0/2)	0 (0/2)

[a] Mosquitoes were tested at 14–15 d post virus exposure, except for *Ma. septempunctata*, which was tested at 9 d post virus exposure.
[b] With the exception of *Ae. aegypti*, all mosquitoes were field-collected.
[c] Number of mosquitoes with virus detected in the body per number tested.
[d] Number of mosquitoes with virus detected in the legs per number tested.
[e] Number of mosquitoes with virus detected in the head plus salivary glands per number tested.

Table 3. Infection and transmission of Japanese encephalitis virus to suckling mice by field-collected mosquitoes fed $10^{4.5 \pm 0.1}$ $CCID_{50}$/mosquito after 14–15 d extrinsic incubation

Species	Source	% Infection	% Transmission
Culex annulirostris	Bamaga	100 (2/2)[a]	0 (0/2)[b]
	Cairns	93 (51/55)	61 (14/23)
Cx. quinquefasciatus	Mareeba	56 (15/27)	0 (0/16)
Cx. gelidus	Cairns	100 (4/4)	100 (1/1)
Ochlerotatus kochi	Bamaga	21 (6/28)	0 (0/8)
	Cairns	11 (1/9)	13 (1/8)
Oc. vigilax	Cairns	57 (43/75)	17 (3/18)
Verrallina funerea	Cairns	11 (4/36)	7 (1/15)
Coquillettidia xanthogaster	Cairns	0 (0/1)	0 (0/1)

[a] Number of mosquitoes infected per number tested.
[b] Number of mice with symptomatic or asymptomatic infection per number tested.

Wiedemann, *Mansonia uniformis* (Theobald) and *Ochlerotatus purpureus* (Theobald) were infected. The infection rate of *Oc. notoscriptus* was 20%, whereas *Ocheleroatus normanensis* (Taylor) and *Verrallina carmenti* Edwards were not infected. After 9 d, the infection rate in *Ma. septempunctata* was 67%, and 81% of these mosquitoes contained disseminated virus.

Vertical Transmission. A total of 673 F_1 progeny of 42 female *Oc. vigilax* were tested for the presence of JE virus, comprising 302 adult females, 295 adult males, and 76 larvae. However, only 14 (33%) of the parental females were infected. JE was not detected in any of the pools of progeny of infected and uninfected females tested.

Growth of JE in Mosquitoes. After 2 d, the virus titer in *Cx. sitiens* decreased to $10^{1.8 \pm 0.0}$ $CCID_{50}$/mosquito, before increasing to peak at $10^{5.0 \pm 0.1}$ $CCID_{50}$/mosquito at day 11, after which no further increase was observed (Fig. 1). The titer of virus in *Cx. annulirostris* was $10^{5.5 \pm 0.3}$$CCID_{50}$/mosquito at day 10 postinfection.

Discussion

In Southeast Asia, rice field mosquitoes, such as *Cx. tritaeniorhynchus* and *Cx. vishnui*, are the main vectors of JE (Burke and Leake 1988, Vaughn and Hoke 1992). Because these species do not occur in northern Queensland, entomological investigations have been undertaken to elucidate the vectors of JE in Australia. In the current study, highest rates of infection and transmission were observed in the species of *Culex*. We used a slightly higher titer of JE ($10^{7.1 \pm 0.1}$ $CCID_{50}$/ml) than has been recorded in pigs (10^6 suckling mouse intracranial lethal $dose_{50}$/ml; Burke and Leake 1988), because a greater amount of virus is required to

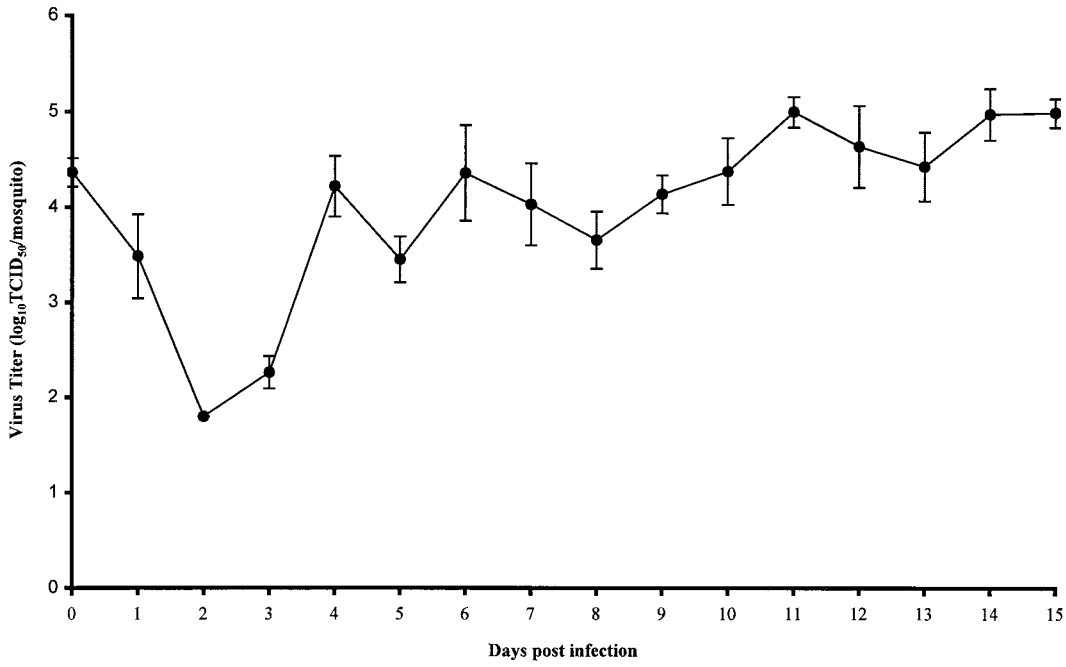

Fig. 1. Growth of JE virus in *Cx. sitiens* (mean $\log_{10}$ titer ± SE per mosquito) fed $\approx 10^{4.5+0.1}$$CCID_{50}$/mosquito.

infect mosquitoes using artificial infection techniques (Jupp 1976, Turell 1988).

The high infection and transmission rates presented in this study confirm the status of *Cx. annulirostris* as the major vector of JE in Australia. However, a number of other factors contribute to the vectorial capacity of a species, including host feeding patterns, longevity, abundance and ability to become infected in natural transmission cycles. *Culex annulirostris* has yielded the majority of isolates obtained from the Torres Strait and PNG (Ritchie et al. 1997a, Johansen et al. 2000, Johansen et al. 2001). This species is the primary vector of the closely related Murray Valley encephalitis and Kunjin viruses, two Australian members of the JE serological complex of flaviviruses (Mackenzie et al. 1994). Furthermore, *Cx. annulirostris* and *Cx. tritaeniorhynchus* are classified within the *Cx. sitiens* group (Lee et al. 1989) and both species have evolved to become the major vectors of JE serological complex viruses throughout their respective geographical distributions. Mosquitoes classified as *Cx. annulirostris marinae* previously have been implicated as vectors on Guam and have been shown to experimentally transmit JE (Hurlburt and Thomas 1949, Reeves and Rudnick 1951). Finally, *Cx. annulirostris* is a widespread and abundant species in northern Queensland (van den Hurk and Ritchie 1997), and readily feeds on pigs and humans during recorded JE incursions (van den Hurk et al. 2001).

The results of this study indicate that *Cx. sitiens* should be considered a potential vector of JE in Australia. Indeed, in the presence of low numbers of *Cx. annulirostris*, this species, along with *Cx. quinquefasciatus*, was implicated as a vector on the Eastern Islands of the Torres Strait (van den Hurk and Ritchie 1997). To date, no isolates have been obtained from *Cx. sitiens* in northern Australia, possibly because only low numbers have been processed for virus isolation (Ritchie et al. 1997a, Johansen et al. 2001). However, isolates have been obtained from *Cx. sitiens* collected from Malaysia and Taiwan (Vythilingam et al. 1994, Weng et al. 1999). *Culex sitiens* is a common coastal species in Australia (Lee et al. 1989), and has been recorded feeding on pigs in Southeast Asia (Vythilingam et al. 1994).

The colony strain of *Cx. quinquefasciatus* from southeast Queensland was highly susceptible to infection and readily transmitted the virus. However, the north Queensland strain was a relatively inefficient laboratory vector, because only 56% became infected and transmission did not occur. The differences in infection and transmission rates between these two populations could be a result of inherited genetic mechanisms, as has been demonstrated with *Cx. tritaeniorhynchus* collected from different geographical areas of Asia (Takahashi 1980, 1982). Alternatively, the fact that one strain was obtained from a colony and the other from the field may have contributed to the observed differences in vector competence. Isolates of JE have been obtained from *Cx. quinquefasciatus* in Asia, although infection rates generally were low when compared with *Cx. tritaeniorhynchus* (Vythilingam et al. 1997, Weng et al. 1999). However, no isolates have been obtained from this species in Australia, as only low numbers of *Cx. quinquefasciatus* have been processed for virus isolation. Host feeding studies have demonstrated that, although *Cx. quinquefasciatus* has been recorded feeding on pigs, humans and dogs are the main blood meal hosts in northern Australia (Kay et al. 1979).

An established vector of JE in Southeast Asia, *Cx. gelidus* only recently has been recognized in Australia (Muller et al. 2001, Ritchie et al. 2001). Isolates of JE have been obtained from this species from a wide geographical area (Gould et al. 1974, Peiris et al. 1992, Vythilingam et al. 1995), and a Malaysian strain was found to be an efficient laboratory vector (Gould et al. 1962). The ability for *Cx. gelidus* to transmit the Torres Strait strain of JE, coupled with evidence of field infection and a propensity to feed on pigs (van den Hurk et al. 2001), indicate that this species should be considered an important vector in northern Australia.

Our study confirms the ability of *Oc. vigilax* to become infected with JE, indicating that the isolate from Badu Island in 1998 did not result from an infected blood meal or contamination from the hemolymph of another species (Johansen et al. 2001). However, despite an infection rate of 57%, only 17% of the wild-caught *Oc. vigilax* transmitted the virus, indicating that the Cairns strain used in these experiments was a relatively inefficient vector. Transmission for the colony strain of *Oc. vigilax* was not demonstrated, as only four females had fed on recipient mice and none of these mosquitoes were infected. All of the infected females of the southeast Queensland colony strain developed a disseminated infection, indicating that there was potentially a salivary gland infection barrier that limited virus transmission by these mosquitoes (Hardy et al. 1983). We did not demonstrate vertical transmission of JE by *Oc. vigilax*, because only low numbers of progeny were processed from infected parents. Further experiments are required with *Ochlerotatus* spp., to determine whether vertical transmission is a viable overwintering mechanism for JE in Australia.

Our study has demonstrated that *Oc. notoscriptus* is capable of being infected with and transmitting JE. The status of *Oc. notoscriptus* as a potential vector of arboviruses in Australia recently has been extended when this species was shown to be a laboratory vector of Rift Valley fever, Ross River and Barmah Forest viruses (Turell and Kay 1998, Watson and Kay 1998, 1999). *Ochlerotatus notoscriptus* is a common peridomestic species, so the close association between humans, its propensity to feed on mammals including pigs and humans (Kay et al. 1979) and its ability to transmit JE indicate that it could play a role in transmission in Australia.

The potential role of other native Australian mosquito species as vectors of JE in northern Australia also was investigated. Australian *Mansonia* could be potential vectors of JE because they have been implicated in India (Gajanana 1998), Sri Lanka (Peiris et al. 1994), and Sarawak (Simpson et al. 1970). Transmis-

sion experiments using *Ma. uniformis* and *Ma. septempunctata* are required to determine whether these species could play a role in transmission cycles in Australia. The low infection and/or transmission rates observed for several species, including *Ae. aegypti*, *Ve. funerea*, and *Oc. kochi*, in conjunction with a paucity of field isolates, indicate that these species probably would not play a significant role in JE transmission.

The pattern of virus growth in *Cx. sitiens* is similar to that observed for *Ochlerotatus japonicus* Theobald, *Culex pipiens molestus* Forskal, and *Cx. tritaeniorhynchus* in Southeast Asia (Takashima and Rosen 1989, Weng et al. 2000). After an initial drop in virus titer, the titer increased to a similar level to that which was ingested (i.e., $10^{4.4\pm0.2}$ $CCID_{50}$/mosquito) before peaking at $10^{5.0\pm0.1}$ $CCID_{50}$/mosquito on day 11. The titer of $10^{5.5\pm0.3}$ $CCID_{50}$/mosquito in *Cx. annulirostris* was similar to the titer of $10^{5.18\pm0.04}$ plaque forming units/mosquito reported for a Taiwan strain of *Cx. tritaeniorhynchus* (Weng et al. 2000).

Interestingly, virus transmission was demonstrated in the colony mosquitoes that did not have a detectable disseminated infection (i.e., virus recovered from the legs). Consequently, wild-caught mosquitoes that had fed on recipient mice were not tested for a disseminated infection. It is likely that that JE did not readily disseminate through the legs of some of the infected mosquitoes in titers high enough to be detected in the CCEIA assay used in these experiments. Whenever possible, mosquitoes should be encouraged to feed on susceptible vertebrate hosts to demonstrate virus transmission and assess the vector status of a particular species. However, this is sometimes difficult to achieve in the laboratory, as mosquitoes may be reluctant to feed on the recipient animal.

With the exception of 1999, JE activity has been recorded every year in the Torres Strait since 1995, and on Cape York Peninsula in 1998 (Hanna et al. 1996, Shield et al. 1996, Hanna et al. 1999, Pyke et al. 2001; A. Pyke, Queensland Health Scientific Services, unpublished results). The high number of isolates, coupled with our vector competence results demonstrated that specific strategies for controlling *Cx. annulirostris* need to be developed, especially should JE became established on the Australian mainland. However, implementation of large scale vector control in areas such as Cape York Peninsula or the Gulf country of northern Queensland, may not be feasible, because *Cx. annulirostris* larval habitats are numerous and widespread. Therefore, vaccination and removal of domestic pigs from human habitation would appear to be the most viable option of preventing JE transmission to humans in these areas.

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