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Entomological Investigations of an Outbreak of Japanese Encephalitis Virus in the Torres Strait, Australia, in 1998

C. A. JOHANSEN,[1] A. F. VAN DEN HURK,[1] A. T. PYKE,[2] P. ZBOROWSKI,[1] D. A. PHILLIPS,[2] J. S. MACKENZIE,[1] AND S. A. RITCHIE[3]

Department of Microbiology and Parasitology, the University of Queensland, Brisbane, 4072, Australia

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ABSTRACT Japanese encephalitis (JE) virus first appeared in Australia in 1995, when three clinical cases (two fatal) were diagnosed in residents on Badu Island in the Torres Strait, northern Queensland. More recently, two confirmed human JE cases were reported in the Torres Strait Islands and Cape York Peninsula, in northern Queensland in 1998. Shortly after JE virus activity was detected in humans and sentinel pigs on Badu Island in 1998, adult mosquitoes were collected using CO_2 and octenol-baited CDC light traps; 43 isolates of JE virus were recovered. Although *Culex sitiens* group mosquitoes yielded the majority of JE isolates (42), one isolate was also obtained from *Ochlerotatus vigilax* (Skuse). Four isolates of Ross River virus and nine isolates of Sindbis (SIN) virus were also recovered from members of the *Culex sitiens* group collected on Badu Island in 1998. In addition, 3,240 mosquitoes were speciated and pooled after being anesthetized with triethylamine (TEA). There was no significant difference in the minimum infection rate of mosquitoes anesthetized with TEA compared with those sorted on refrigerated tables (2.8 and 1.6 per 1,000 mosquitoes, respectively). Nucleotide analysis of the premembrane region and an overlapping region of the fifth nonstructural protein and 3′ untranslated regions of representative 1998 Badu Island isolates of JE virus revealed they were identical to each other. Between 99.1% and 100% identity was observed between 1995 and 1998 isolates of JE from Badu Island, as well as isolates of JE from mosquitoes collected in Papua New Guinea (PNG) in 1997 and 1998. This suggests that the New Guinea mainland is the likely source of incursions of JE virus in Australia.

KEY WORDS Japanese encephalitis virus, *Culex sitiens* group, *Ochlerotatus vigilax*, triethylamine, Australia, emerging diseases

JAPANESE ENCEPHALITIS (JE) virus is a mosquito-borne flavivirus responsible for ≈45,000 clinical cases, mostly affecting children in eastern and southeastern Asia each year. About 25% of cases are fatal (Vaughn and Hoke 1992). JE primarily exists in a zoonotic transmission cycle between rice-field breeding mosquitoes and domestic pigs or ardeid waterbirds. Humans are generally considered to be dead-end hosts (Vaughn and Hoke 1992).

Recently, JE has emerged as a disease entity in northern Australia, with the first recognized outbreak in the Torres Strait Islands in 1995 (Hanna et al. 1995, 1996b). Three human cases, with two deaths, occurred on Badu Island. *Culex annulirostris* Skuse, a member of the *Culex sitiens* group (Lee et al. 1989), was implicated as the vector (Ritchie et al. 1997b). Subsequently, sentinel pigs were established on the Torres Strait Islands and the northern Cape York Peninsula. Blood samples were collected regularly during the wet season to investigate seroconversions to JE. Activity of JE virus was detected in sentinel pigs in both 1996 and 1997 (Shield et al. 1996, Hanna et al. 1999). However, the activity appeared to be restricted to Saibai Island, located only 5 km from Papua New Guinea (PNG). A larger outbreak of JE virus was recorded in the Torres Strait and Cape York Peninsula in 1998. One human case on Badu Island was serologically confirmed, despite vaccination of ≈93% of residents on northern Torres Strait islands after the 1995 outbreak of JE (Hanna et al. 1996a). Seroconversions to JE virus were detected in sentinel pigs on numerous Torres Strait Islands (Hanna et al. 1999). In addition, JE virus activity was detected on the Australian mainland, when JE was diagnosed in a fisherman working at the mouth of the Mitchell River, on western Cape York Peninsula (Hanna et al. 1999). Seroconversions to JE virus were detected in sentinel pigs on Cape York Peninsula, and antibodies to JE were detected in juvenile domestic pigs near the mouth of the Mitchell River. This was the second time JE activity has been detected on Badu Island, and the first evidence of JE activity on the Australian mainland (Hanna et al. 1999).

In response to JE virus activity, mosquitoes were collected from Badu Island and various locations on Cape York Peninsula. Mosquito pools were processed

[1] Department of Microbiology and Parasitology, The University of Queensland, Brisbane, Queensland, Australia 4072.

[2] World Health Organization Collaborating Center for Arbovirus Reference and Research, Queensland Health Scientific Services, Brisbane, Queensland, Australia 4108.

[3] Tropical Public Health Unit, PO Box 1103, Cairns, Queensland, Australia 4870.

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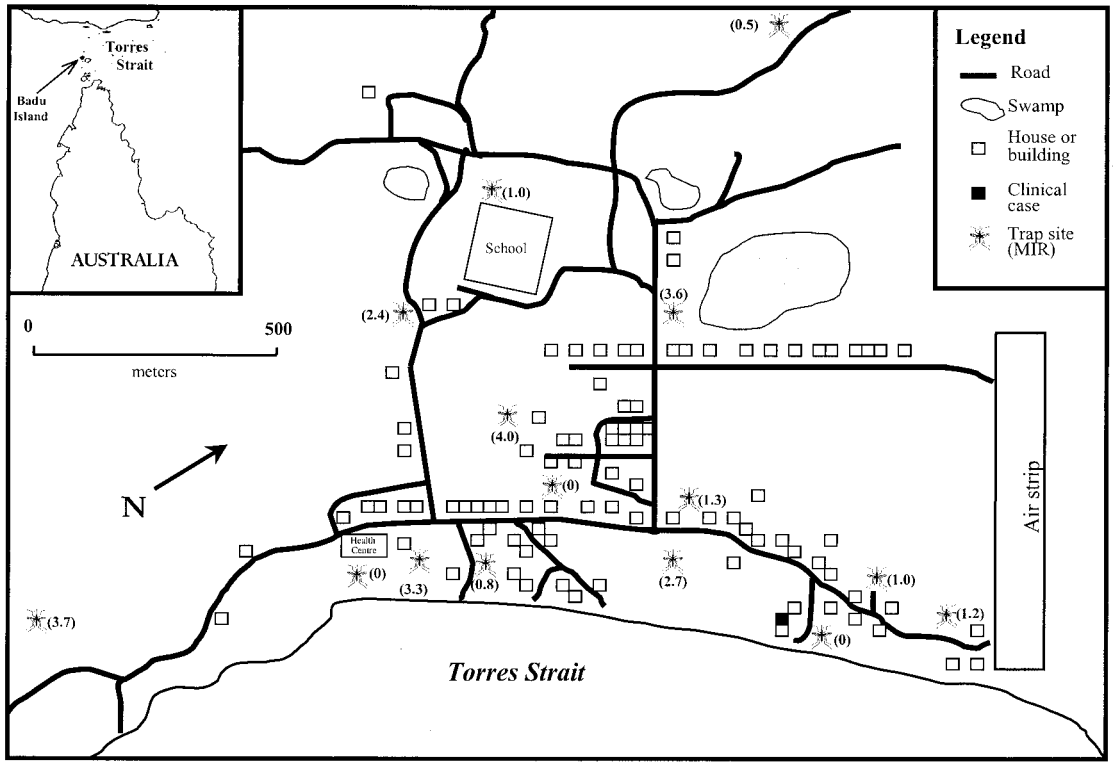

Fig. 1. Locations of adult mosquito collections and minimum infection rates at each mosquito collection site on Badu Island following Japanese encephalitis virus activity in 1998.

for virus isolation to continue investigations into the role of members of the *Culex sitiens* group in JE virus transmission, to examine the possible role of other mosquito species in JE virus transmission, and to determine mosquito infection rates. Additionally, some mosquitoes were sorted to species at the field site using triethylamine (TEA) as an anesthetizing agent as described by Kramer et al. (1990) to investigate differences in infection rates of mosquitoes sorted to species at ambient temperature at the field site versus those sorted on refrigerated tables in the laboratory. Results pertaining to investigations on mainland Australia are presented elsewhere (van den Hurk et al. 2000).

Materials and Methods

Study Sites. Badu Island is a small, granitic island in the Torres Strait, midway between the Australian mainland and coast of PNG (latitude 10° 00′ S, longitude 142° 30′ E) (Fig. 1). Badu Island has a tropical climate characterized by a wet season (December to April) with northwest monsoon winds, and a dry season (May to November), dominated by southeast trade winds. It has a population of ≈950 people (local census, Badu Island Health Clinic 1999). For a more detailed description of Badu Island refer to Ritchie et al. (1997a).

Adult Mosquito Collections. Adult mosquitoes were collected on Badu Island approximately 1 wk after the admission of a human JE case to the hospital, and 2 wk after seroconversions to JE virus occurred in sentinel pigs. Centers for Disease Control (CDC) light traps baited with 1 kg dry ice and 1-octen-3-ol (octenol, release rate approximately 5 mg/h) (van Essen et al. 1994) were set at 15 trap sites around the Badu Island community (Fig. 1). In total, 25 mosquito traps were set over two consecutive nights (5–6 March 1998). With the exception of three mosquito collections sorted using TEA as an anesthetizing agent (see below), the majority of mosquitoes were killed on dry ice and immediately placed in liquid N_2 dry shippers (Forma Scientific, USA) for transport. Mosquitoes were stored at –70°C at the Tropical Public Health Unit in Cairns.

Culex mosquitoes from three CDC traps were anesthetized with TEA (Ajax Chemicals, Sydney, Australia) and then sorted at room temperature into pools of up to 25, before storage in a liquid N_2 dry shipper. Mosquitoes were anesthetized by applying undiluted TEA to cotton wool, which was placed into a sealed plastic bag with the mosquito collection container until mosquitoes were incapacitated.

The remaining mosquitoes were sorted into pools of 25 or 100 on a refrigerated table (Johansen et al. 2000). Members of the morphologically similar *Cx. sitiens*

Table 1. Details of virus strains used in nucleotide sequence analyses

Virus strain	Genbank accession number	Location of origin	Year of isolation
FU	L48968, L43565	Torres Strait	1995
NO	L48967, L43566	Torres Strait	1995
M40	L47350	Torres Strait	1995
M164	L54068	Torres Strait	1995
TS3306	AF092553, AF092939	Torres Strait	1998
TS3503	AF092552, AF092938	Torres Strait	1998
TSMos1998	AF148902	Torres Strait	1998
TSPig1998	AF148900	Torres Strait	1998
PNG4837	AF092550, AF092551	Papua New Guinea	1997
PNG6544	AF139530, AF139531	Papua New Guinea	1998
PNG8728	AF218067, AF218068	Papua New Guinea	1998
K94P05	AF045551	Korea	1994
Beijing-1	L48961	China	1949
p3	JEU47032, JEU03695	China	1949
SA-14	JEU14163	China	1954
SA-14-2-8	JEU15763, JEU02367	China	1954
P20778	AF0802051	India	1958
GP78	AF075723	India	1978
JaGAr01	AF069076	Japan	1959
JaOArS982	M18370	Japan	1982
HV1	AF098735	Taiwan	?
TC	AF098736	Taiwan	?
RP-9	AF014161	Taiwan	1985
CH2195	AF030550	Taiwan	1994
ThCMAr4834	D76422	Thailand	?
B1065	D00996	Thailand	1983
WTP/70/22	D00998	Malaysia	1970
MaKAr60092	D83931	Malaysia	1992
MaKAr60192	D83932	Malaysia	1992
MaKAr158793	D83926	Malaysia	1993
MAPAV294	D83922	Malaysia	1994
MaSAr03594	D83942	Malaysia	1994
Indonesia	JEU03692	Indonesia	?
JKT1749	L42166	Indonesia	1979
JKT2219	L42165	Indonesia	1979
JKT2363	L42163	Indonesia	1979
JKT7003	L42161	Indonesia	1981
MVE-1-51	AF161266	Southeastern Australia	1951

?, Year of isolation unknown.

group, which includes *Cx. annulirostris*, *Cx. palpalis* (Taylor), and *Cx. sitiens* (Wiedemann), were not separated. Pools of 25 mosquitoes were sent to the Department of Microbiology at the University of Queensland, and pools of 100 mosquitoes were sent to the World Health Organization Collaborating Center for Arbovirus Reference and Research, Queensland Health Scientific Services in Brisbane, for virus isolation.

Virus Isolation and Characterization. The methods of virus isolation and identification have been described elsewhere (Ritchie et al. 1997b, Johansen et al. 2000). Briefly, samples from six trap collections were processed for virus isolation at Queensland Health Scientific Services, and examined for the presence of both flaviviruses and alphaviruses after inoculation of homogenized pools of mosquitoes onto confluent monolayers of C6/36 cells. Monolayers were incubated for 7–10 d before cells were fixed onto slides. Viruses were identified by immunofluorescence using a panel of monoclonal antibodies.

Mosquitoes from the remaining 19 trap collections were processed at the Department of Microbiology and Parasitology, University of Queensland, and examined for the presence of flaviviruses only. Homogenized pools of mosquitoes were inoculated onto confluent monolayers of C6/36 cells. After 6 d incubation, monolayers were subsequently fixed and screened using a panel of monoclonal antibodies in a tissue culture enzyme immunoassay (Broom et al. 1998, Johansen et al. 2000). All isolates obtained at Queensland Health Scientific Services and the University of Queensland were confirmed by reisolation. Minimum infection rates (MIR's) were calculated using $[MIR = [1 - (X - Y/X)^{1/(Z/X)}] \times 100]$, where X is the number of pools of mosquitoes processed for virus isolation, Y is the number of pools yielding virus and Z is the total number of mosquitoes processed for virus isolation (Chiang and Reeves 1962). Infection rates of mosquitoes sorted using TEA versus refrigerated tables were compared using the two-tail Fisher exact test, SYSTAT for Windows: Statistics, version 5 (Wilkinson et al. 1992). Selected isolates of JE virus were analyzed further by RT-PCR and sequenced in the premembrane (prM) region, and an overlapping region in the fifth nonstructural protein and 3′ (NS5–3′) untranslated region of the genome as described elsewhere (Johansen et al. 2000). Nucleotide sequences were

Table 2. Mosquitoes processed for virus isolation from the Torres Strait in 1998

Species[a]	No. processed	No. viruses and identification[b]	MIR[c]
Ae. katherinensis[d]	2		
Ae. scutellaris	1		
Anopheles farauti s.l.	71		
An. hilli	28		
Cx. sitiens group[e]	25,292	42 JE	1.7
		4 RR	0.6
		9 SIN	1.4
Cx. vicinus	866		
Ochlerotatus culiciformis	653		
Oc. kochi[f]	338		
Oc. littlechildi	25		
Oc. notoscriptus	7		
Oc. stoneorum	84		
Oc. tremulus	36		
Oc. vigilax	3,073	1 JE	0.3
Verrallina carmenti[g]	716		
Ve. funerea	696		
TOTAL	31,898	43 JE, 4 RR, 9 SIN	

[a] All mosquitoes processed were female.
[b] Only 6,714 *Cx. sitiens* group mosquitoes and 244 *Cx. vicinus* from six collection sites were processed for both flavivirus and alphavirus isolation. JE, Japanese encephalitis virus. RR, Ross River virus, includes one pool of mosquitoes with dual RR and JE virus infections. SIN, Sindbis virus, includes five pools of mosquitoes with dual SIN and JE virus infections.
[c] MIR is minimum infection rate per 1,000 mosquitoes based on Chiang and Reeves (1962).
[d] The genus *Aedes* has recently been separated into the two genera *Aedes* and *Ochlerotatus* (Reinert 2000).
[e] Consists of the morphologically similar species *Cx. annulirostris*, *Cx. palpalis* and *Cx. sitiens.*
[f] Mosquitoes with profusely spotted wings, femora and tibia belonging to the *kochi* subgroup were classified as *Oc. kochi.*
[g] The subgenus *Verrallina* has recently been restored to genera rank (Reinert 1999).

submitted to GenBank and compared with other isolates of JE virus in the same regions of the genome. Genbank accession numbers, location and year of isolation for each virus strain used in the analysis are shown in Table 1. Bootstrap analysis of 100 replicates were used to place confidence values on groupings within phylogenetic trees.

Results

Virus Isolations and Infection Rates. A total of 31,898 mosquitoes, comprising 16 species, was processed for virus isolation from Badu Island (Table 2). Members of the *Cx. sitiens* group were the most abundant species processed (79.3%), followed by *Ochlerotatus vigilax* (Skuse) (9.6%) (formerly classified as *Aedes vigilax* [Reinert 2000]). Fifty-six arbovirus isolates were recovered, including 43 isolates of JE virus, and nine and four isolates of the alphaviruses Sindbis (SIN) and Ross River (RR), respectively. The overall minimum infection rate (MIR) of the *Cx. sitiens* group was 1.7, 1.4, and 0.6 per 1,000 mosquitoes for JE, SIN, and RR viruses, respectively. Isolates of JE virus were recovered from all but three trap sites (Fig. 1). Members of the *Cx. sitiens* group yielded 97.7% of JE isolates. The MIR's for the *Cx. sitiens* group with JE virus at individual collection sites ranged from 0–4.0 per 1,000 mosquitoes (Fig. 1). A single isolate of JE virus was obtained from *Oc. vigilax* (overall MIR 0.3 per 1,000 mosquitoes). One pool of *Cx. sitiens* group mosquitoes yielded isolates of both JE and RR viruses, and five pools yielded concurrent JE and SIN virus isolates.

Triethylamine. Seven isolates of JE virus (MIR of 2.8 per 1,000 mosquitoes) were obtained from 2,615 *Cx. sitiens* group mosquitoes sorted to species after anesthetizing with TEA. There was no significant difference ($P = 0.201$) between infection rates of members of the *Cx. sitiens* group sorted using TEA and those sorted on refrigerated tables (MIR of 1.6 per 1,000, from 22,677 mosquitoes).

Nucleotide Sequence Analysis. Two isolates of JE virus (TS3306 and TS3503) from Badu Island in 1998 were sequenced in the prM (219 nucleotides) and NS5–3′ untranslated region (344 nucleotides) and compared with previously published sequences of JE virus. The isolates were 100% similar to each other at the nucleic acid level in both regions of the genome. A high degree of nucleic acid identity (>99%) was observed between the 1998 isolates and representative isolates from the Torres Strait in 1995. Badu Island isolates TS3306 and TS3503 also shared 100% identity in the prM region (Fig. 2) with two isolates of JE (PNG6544 and PNG8728) from *Cx. sitiens* group mosquitoes collected in the Western Province of PNG in 1998, and were 99.5% similar to PNG4837 and three isolates of JE virus (NO, M40, and FU) recovered from the Western Province of PNG in 1997 and from Badu Island in 1995, respectively (Ritchie et al. 1997b, Johansen et al. 2000). All Torres Strait and PNG isolates of JE virus were 100% identical at the amino acid level in the prM region of the genome. Isolates TS3306, TS3503 and TSMos98 shared 100% identity with two isolates of JE virus (M164 and NO) from Badu Island in 1995 in the NS5–3′UTR (Fig. 3). Less than 0.9% difference was observed between any of ten isolates of JE virus from the Torres Strait and PNG in the NS5–3′UTR. With the exception of one isolate of JE virus from a sentinel pig (TSPig98) in the Torres Strait in 1998 (Hanna et al. 1999), which had a single amino acid substitution, isolates were identical at the amino acid level in the NS5 coding region of the genome.

Discussion

The high isolation rate of JE virus from *Cx. sitiens* group mosquitoes collected on Badu Island in 1998 provides further evidence that these mosquitoes may be vectors of JE virus in Australia. The MIR of the *Cx. sitiens* group was lower in 1998 (1.7 per 1,000 mosquitoes) than 1995 (2.9 per 1,000 mosquitoes morphologically identified as *Culex annulirostris*) (Ritchie et al. 1997b). However, trap collections of *Cx. sitiens* group mosquitoes were significantly greater in 1998 than 1995 (mean = 126 and 991 for 1995 and 1998, respectively) (Mann–Whitney rank sum test; $T = 971$; $n = 30$ and 25 for 1995 and 1998, respectively; $P < 0.001$), and yielded approximately five times (42 versus 8) the number of isolates of JE virus in 1998 as in 1995. Activity of JE virus was widespread throughout the

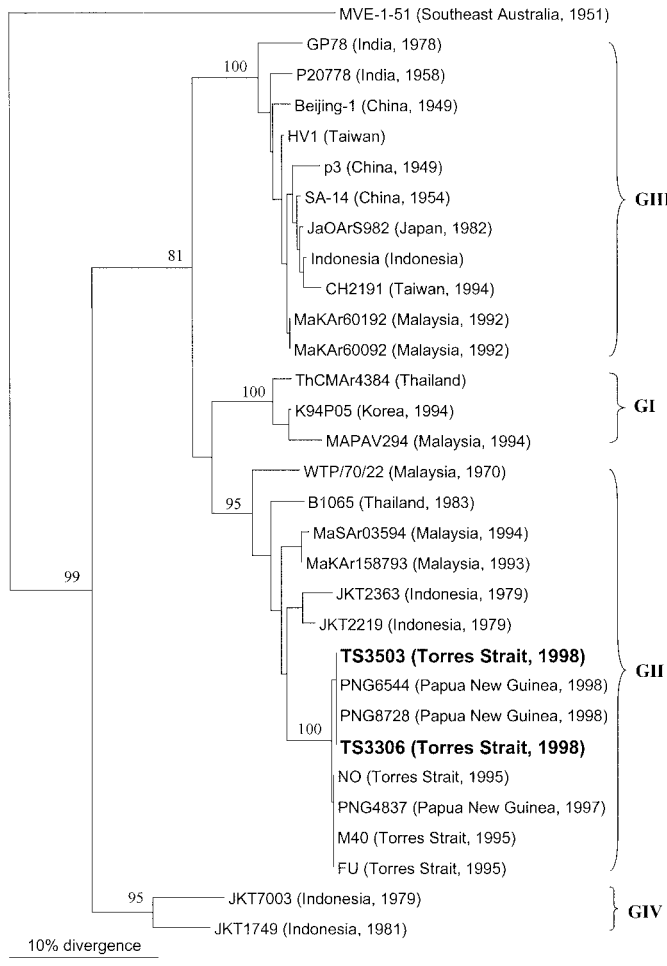

Fig. 2. Phylogram representing the nucleotide sequence homology of the premembrane (prM) region of the genome of isolates of Japanese encephalitis virus. All four known genotypes are represented.

community; only three of 15 collection sites failed to yield isolates of JE virus (Fig. 1).

Allozyme analyses of populations of *Cx. sitiens* group mosquitoes from Badu Island in 1995 and 1998 suggests that *Cx. annulirostris* is the most abundant species present (100 and 80.5%, respectively); a small proportion of mosquitoes identified as *Cx. annulirostris* in 1998 were actually *Cx. sitiens* (14.6%) and other undefined species (4.9%) (Chapman et al. 2000). Thus, although it is apparent that multiple members of the *Cx. sitiens* species complex are present on Badu Island, *Cx. annulirostris* is the most abundant species, and is the most likely vector of JE virus in the region.

There are a number of possible reasons why JE activity occurs on Badu Island (Hanna et al. 1996b, Ritchie et al. 1997a). The community, located in a flat area on the west coast of the island, has a perched water table that creates numerous swampy areas in the wet season. A roaming herd of horses defecate along swamp edges, creating ideal breeding sites for members of the *Cx. sitiens* group such as *Cx. annulirostris.* Furthermore, drains and waterways are often blocked by litter and grass, septic tanks are often defective, and a large domestic pig population was, until recently, maintained in small groups in backyard pens that were often filthy and flooded (Hanna et al. 1996b, Ritchie et al. 1997a). The blocked drains, defective septic tanks, and poorly maintained pigpens created additional breeding sites for *Cx. annulirostris.* Consequently, vectors and amplifying hosts were in close proximity to each other resulting in intense JE transmission. It is for these reasons that Badu Island was calculated to have the highest relative risk of an outbreak of JE, compared with other islands in the Torres Strait (Ritchie et al. 1997a).

Recurrence of JE virus activity in the Torres Strait supports the continuation of the vaccination campaign that commenced on northern Torres Strait islands following the initial outbreak of JE in 1995 (Hanna et al. 1996a). It also highlights the need for removal of all domestic pigs into a communal piggery at a location several kilometers from the human population to reduce the risk of JE transmission to humans.

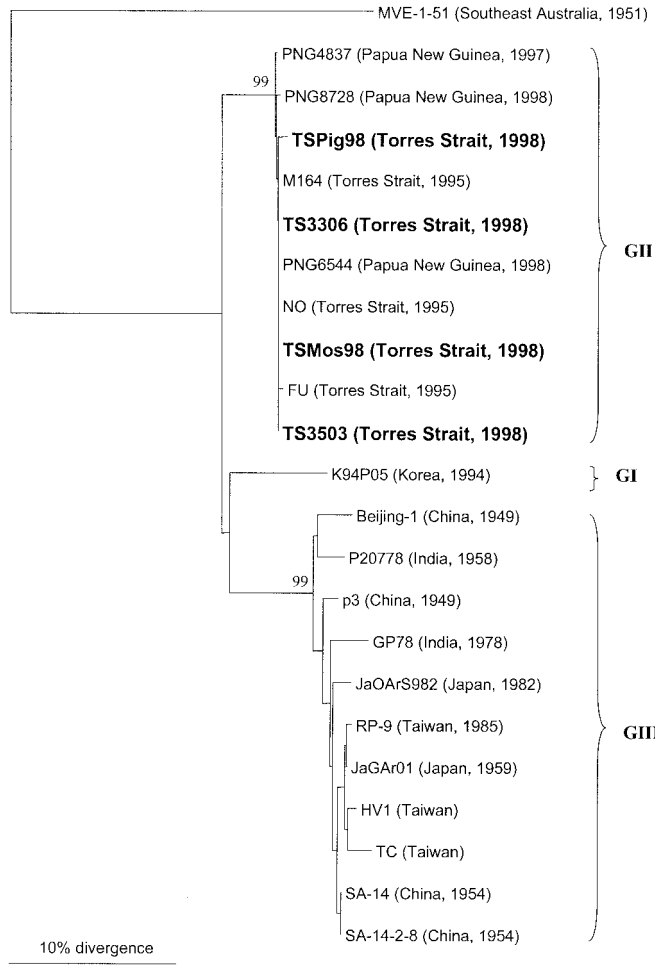

Fig. 3. Phylogram representing the nucleotide sequence homology of the nonstructural protein 5–3′ untranslated region (NS5–3′UTR) of the genome of isolates of Japanese encephalitis virus. Genotypes I, II and III are represented.

Despite care being taken to identify mosquito species and remove blood fed females, the isolate of JE virus from *Oc. vigilax* may be from an infected blood meal or contamination via infected hemolymph of *Cx. sitiens* group mosquitoes. However, if the isolate is not spurious, there are some important implications. *Oc. vigilax* is a saltmarsh mosquito, which lays desiccation resistant eggs that can survive for many months; it is also autogenous (Lee et al. 1984). It is possible that vertical transmission could occur with JE virus, allowing the virus to be rapidly activated when conditions for mosquito breeding become optimal. Certainly, vertical transmission of JE virus is one of the few mechanisms of virus maintenance that could feasibly occur on a small island, with a limited number of susceptible vertebrate hosts. Vertical transmission of JE virus has been demonstrated in a number of other mosquito species including *Cx. tritaeniorhynchus* Giles, *Cx. annulus* Theobald, *Cx. quinquefasciatus* Say, *Armigeres subalbatus* (Coquillett) (Rosen et al. 1989), *Oc. japonicus* (Theobald) (Takashima and Rosen 1989), and *Ae. albopictus* (Skuse) (Rosen 1988). Although vertical transmission of JE virus could occur in members of the *Cx. sitiens* group, aestivating *Ochlerotatus* eggs may also be an effective "overwintering" mechanism for maintenance of JE virus on Badu Island during the dry season. The ability of *Oc. vigilax* and other species to transmit JE virus in this manner needs to be investigated in laboratory transmission studies.

The high level (>99%) of nucleic acid sequence identity between isolates obtained from mosquitoes collected on Badu Island and PNG in 1998 suggests that the New Guinea mainland is a source of incursions of JE virus into Australia (Johansen et al. 2000). Circumstantial evidence suggests that mosquitoes are blown into the Torres Strait islands and northern Queensland during extreme weather events, such as tropical monsoonal lows or cyclones (Hanna et al. 1999). Viremic migratory waterbirds may also be involved in the introduction of JE virus into Australia, in a similar way that migrant birds may have been in-

volved in the introduction of West Nile virus into North America in 1999 (Rappole et al. 2000).

The isolations of SIN virus from mosquitoes collected on Badu Island is not surprising. SIN virus is the most common arbovirus isolated from mosquitoes in Australia (Mackenzie et al. 1994). Although *Cx. annulirostris* yields most isolates of SIN virus in Australia (Mackenzie et al. 1994), it has also been isolated from 26 other species, including *Cx. sitiens* in northern New South Wales and north Western Australia (Russell 1995). Human infections with SIN virus have been reported in the past, including residents in northern Queensland (Doherty 1973). However, disease caused by SIN virus infection is rare in Australia, with few confirmed human cases (Doherty et al. 1969, Guard et al. 1982).

This is the first reported isolation of RR virus from mosquitoes in the Torres Strait, although it was isolated from *Cx. annulirostris* collected at Bamaga (Fig. 1) in northern Cape York in 1996 (Johansen et al. 1997). RR virus is a common cause of human disease in Australia, particularly in Queensland; an average of 4,844 cases of RR virus disease were notified per year between 1991 and 1998 in Australia, and more than half of those (mean = 2,844 per year) were from Queensland (Harley 2000). However, there were no notifications of RR virus disease from the Torres Strait Islands in the 1998 calendar year (Notifiable Diseases Surveillance System, Communicable Diseases Center, Queensland Health, Brisbane).

The number of pools of *Cx. sitiens* group mosquitoes dually infected with JE, SIN, and RR viruses was surprising. Given that all arboviruses isolated during this study were confirmed by reisolation, it is unlikely that pools containing more than one virus were due to contamination from other pools of infected mosquitoes. For the collection sites where mosquitoes were examined for the presence of both alpha and flaviviruses, there was no significant difference in the numbers of *Cx. sitiens* group mosquitoes in traps yielding isolates of JE, SIN, and RR viruses and those that did not (t-test; $P = 0.445$). However, the three collection sites yielding JE, SIN, and RR viruses (including the dual infections) were peripheral to the Badu Island community, and closer to habitats for potential vertebrate hosts of SIN virus such as birds or mammals (Doherty et al. 1971, Liehne et al. 1976, Marshall et al. 1982), yet still within reach of pigpens in the community that were likely to be involved in JE virus transmission. Thus, if not coincidental, the proximity of collection sites from which alphaviruses were obtained to potential vertebrate hosts may explain the unexpected number of pools of *Cx. sitiens* group mosquitoes with multiple infections of JE, SIN, and RR viruses.

Finally, the results presented in this article confirm those of Kramer et al. (1990) who found that TEA had no deleterious effects on the ability to isolate arboviruses from mosquitoes. TEA is a useful tool to process mosquitoes in the field without the necessity of cumbersome refrigerated tables.

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