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ISOLATION OF JAPANESE ENCEPHALITIS VIRUS FROM MOSQUITOES (DIPTERA: CULICIDAE) COLLECTED IN THE WESTERN PROVINCE OF PAPUA NEW GUINEA, 1997–1998

CHERYL A. JOHANSEN, ANDREW F. VAN DEN HURK, SCOTT A. RITCHIE, PAUL ZBOROWSKI, DEBRA J. NISBET, RAYMOND PARU, MOSES J. BOCKARIE, JOANNE MACDONALD, ALEXANDER C. DREW, TATIANA I. KHROMYKH, AND JOHN S. MACKENZIE

Department of Microbiology and Parasitology, The University of Queensland, St. Lucia, Brisbane, Queensland, Australia; Tropical Public Health Unit, Cairns, Queensland, Australia; Papua New Guinea Institute of Medical Research, Madang, Madang Province, Papua New Guinea

Abstract. After Japanese encephalitis (JE) virus emerged in the Torres Strait in Australia in 1995, investigations were initiated into the origin of the incursion. New Guinea was considered the most likely source, given its proximity to islands of the Torres Strait. Almost 400,000 adult mosquitoes were processed for virus isolation from 26 locations in the Western Province of Papua New Guinea (PNG) between February 1996 and February 1998, yielding three isolates of JE virus. Two isolates of Murray Valley encephalitis, 17 isolates of Sindbis, and 1 each of Sepik and Ross River viruses were also obtained.

Nucleic acid sequences of the PNG JE isolates were determined in the prM region, and in a region overlapping a part of the fifth nonstructural protein and the 3′ untranslated region. The PNG isolates belonged to genotype II, and shared $>$ 99.2% identity with isolates from humans and mosquitoes from the Torres Strait, suggesting that PNG is the source of incursions of JE virus into Australia.

INTRODUCTION

Japanese encephalitis (JE) virus is a member of the Japanese encephalitis serological complex of mosquito-borne viruses, within the family Flaviviridae. Japanese encephalitis virus is responsible for approximately 45,000 cases of disease each year in humans in eastern and southeastern Asia, and about 15,000 cases are fatal. However, most infections are asymptomatic.[1,2] Of symptomatic infections, about 25% are rapidly fatal, 50% of patients suffer from permanent neurologic sequelae, and only 25% of cases fully resolve; most cases occur in children. Symptoms range from mild fever to acute meningomyeloencephalitis.[1] The known geographic range of JE virus currently extends from Japan, Korea, and maritime Siberia, to China (all but two provinces), the Philippines, and throughout southeastern Asia from western Indonesia to Thailand, Vietnam, Laos, Burma, Nepal, India, Pakistan, and Sri Lanka.[2,3] Japanese encephalitis primarily exists as an enzootic cycle of transmission between waterbirds and/or pigs, and rice-field breeding mosquitoes, *Culex tritaeniorhynchus.*[1]

Japanese encephalitis virus activity occurred in Australia for the first time in March/April 1995, when three cases of encephalitis were diagnosed on Badu Island in the Torres Strait.[4] Two of the three cases were fatal. Serologic surveys revealed antibodies to JE virus were widespread in humans and domestic pigs residing on the outer Torres Strait Islands. Isolates of JE virus were obtained from subclinical infections in humans and from pools of *Culex annulirostris,* the likely vector of JE virus during the outbreak.[4,5] This outbreak was unprecedented, as the nearest known focus of JE virus was Bali in Indonesia, 3,000 km west of the Torres Strait, and the nearest site of isolation of JE virus was Flores, Indonesia, 2,200 km west of the Torres Strait.[6] Activity of JE virus has been detected in sentinel pigs on Saibai Island in the northern Torres Strait in all but one year since the 1995 outbreak (Lee J, Australian Quarantine Inspection Service and Phillips D, Queensland Health Scientific Services, unpublished data). Another two cases of disease occurred in humans in 1998. The first case occurred in an unvaccinated child on Badu Island, and the second in a fisherman working in the Mitchell River area of the Cape York Peninsula in northern Queensland. The latter case of JE is the first record of JE virus activity on mainland Australia.[7] Isolates of JE virus were obtained from mosquitoes collected on Badu Island, and sentinel pigs located on the northern Cape York Peninsula.[7]

Japanese encephalitis virus has been separated into four genotypes using phylogenetic analyses. Genotype I is found primarily in Laos, northern Thailand, Cambodia, and Malaysia; Genotype II consists of isolates from southern Thailand, Malaysia, and Indonesia; Genotype III appears to be the most widely distributed and includes isolates from Japan, Korea, China, Malaysia, Philippines, Indonesia, India, and Sri Lanka; and Genotype IV appears to be restricted to Indonesia.[8–10]

Comparison of the genetic sequence of the 1995 isolates of JE virus in the prM region revealed they probably had a common source and were most closely related to a 1970 isolate from Malaysia and a 1981 isolate from Indonesia, both of which belonged to Genotype II.[5] Sequence comparison of an overlapping region of the non-structural and 3′ untranslated region (NS5–3′ UTR) confirmed the relationship between the Australian, Indonesian, and Malaysian isolates, and also revealed the Australian isolates had a unique eleven base deletion immediately following the stop codon in the 3′ untranslated region.[11] Nevertheless, the Australian isolates of JE were still approximately 5% divergent from the most closely related isolates, and as such, the source could not be determined.[5] The most likely source was thought to be Papua New Guinea (PNG) or Irian Jaya, given their proximity to islands of the Torres Strait. However, there was little evidence to suggest that JE virus had occurred in PNG in the past. One early investigation showed the closely related flavivirus Murray Valley encephalitis (MVE) was

widespread in humans and birds in PNG, but the only region where JE virus may have been active was the region through which the Aramia River flowed, in the Western Province.[12] In another study in the late 1970s, no human sera from New Guinea had specific antibodies to JE virus, and more recently, there were no reports of JE virus activity in PNG.[13,14]

As a result of the outbreak of JE in the Torres Strait in 1995, a study was undertaken to investigate whether JE virus did exist in Papua New Guinea, by 1) attempting virus isolation from adult mosquitoes collected in the Western Province of PNG and 2) determining the prevalence of antibodies to JE virus in people living in PNG. This paper describes the isolation of JE virus and other arboviruses from mosquitoes collected in the Western Province of PNG during this study.

MATERIALS AND METHODS

Study sites. The Western Province (population estimated at 100,000 in 1990; area of 72,000 km^2) is the largest province in PNG. It shares borders with Irian Jaya in the west, West Sepik Province in the north, the Southern Highlands and Gulf Provinces in the north-west, and Australia (including the Torres Strait) to the south. The coastline is broken by a series of river deltas. Large expanses of flat, low-lying swamp (no more than 20 meters above sea level) run inland where they rise to foothills, and then to the mountains of the highlands. The lowlands and swamps are susceptible to flooding during two wet seasons, which are associated with the northwesterly monsoon between January and April and the southeasterly monsoon between September and December. The largest river in PNG, the Fly River, runs through the Western Province for its entire length. Lake Murray, located in the center of the Province, is the largest lake in PNG, although its size varies greatly between the wet and dry seasons.

Adult mosquito collections. Adult mosquitoes were collected in the Western Province of PNG towards the end of the early wet season, between February and May, when likely vector populations were most abundant. Mosquitoes were collected from 11 sites during two field trips in April and May in 1996, from 18 sites during two field trips in March and April, 1997, and from 7 sites on one field trip in February 1998 (see Figure 1). Some sites were sampled more than once. Ten to fifteen traps were set each night. Mosquitoes were collected using CDC-type light traps baited with approximately 0.5 kg of dry ice and 1-octen-3-ol (5 mg/hour).[5] Samples were transported on either dry ice or in liquid nitrogen dry shippers (Selby Scientific, Victoria, Australia) then stored at −70°C.

Virus isolation. Mosquitoes were sorted to species on a −15°C cold table (Lindner and May, Brisbane, Queensland, Australia) into pools of no more than 25 mosquitoes (1996–1997) or pools of up to 100 (1998). Blood-fed mosquitoes were not processed. Male and female mosquitoes were processed for virus isolation separately. Members of the *Culex sitiens* subgroup of mosquitoes, which includes *Cx. annulirostris, Cx. palpalis* and *Cx. sitiens,*[15] were processed separately. However, electrophoretic studies indicated that pools of mosquitoes identified as *Cx. annulirostris* were composed of variable proportions of *Cx. annulirostris, Cx. palpalis, Cx. sitiens* and other undescribed species,[16] and were subsequently analyzed together as the *Cx. sitiens* subgroup. Pools of up to 25 mosquitoes were homogenized in 5 mL sterile plastic tubes containing five glass beads (5 mm diameter) and 2.5 mL of M199 tissue culture medium (GIBCO BRL, Gaithersburg, MD) containing 2% fetal bovine serum, 2 mM L-glutamine, 50 units/mL Penicillin and 50 μg/mL Streptomycin (all from GIBCO BRL) using a SPEX 8000 mixer/mill (Spex Industries, Edison, NJ) as described previously.[5] Pools of 100 mosquitoes were processed similarly, with the exception that tubes contained 5 mL of media and 8 glass beads, and samples were homogenized for 3 min. Samples were centrifuged at 3,000 rpm for 15 minutes at 4°C and the supernatants were aliquoted and stored at −70°C. Mosquitoes were processed for virus isolation by the inoculation of homogenates onto cell lines using the method described by Lindsay and others,[17] with the exception that Vero cells were substituted with baby hamster kidney (BHK) cells. The BHK cells were cultured in growth media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/mL Peni-

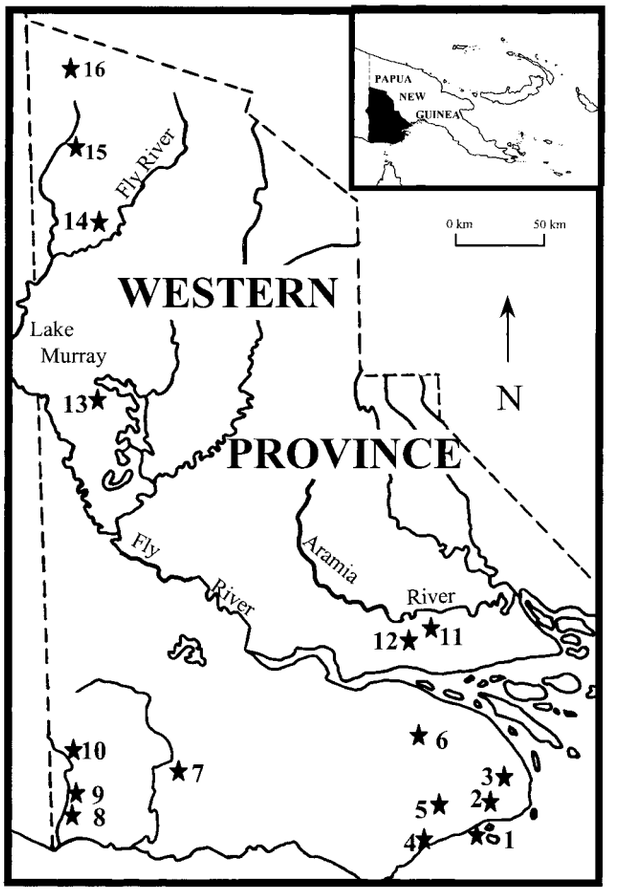

FIGURE 1. Location of adult mosquito collection sites in the Western Province of Papua New Guinea from 1996 to 1998. 1) Daru, including the coastal villages of Dorogori, Kadawa and Katatai; 2) Abam, includes Woigi and a temporary camp; 3) Peawa; 4) Kynini; 5) Boze; 6) Upiara; 7) Morehead; 8) Wando, Balamuk, and Korombo; 9) Bensbach, includes Jangari; 10) Weam and Wereave; 11) Balimo, includes Sawito; 12) Dagono; 13) Lake Murray Patrol Post; 14) Kiunga; 15) Rumginae; and 16) Tabubil.

cillin and 50 μg/mL Streptomycin in 5% CO_2 at 37°C. Pools of 100 mosquitoes were filtered through 0.2 μ syringe filters (Acrodisc, Gelman Sciences, Ann Arbour, MI) prior to inoculation on C6/36 96-well monolayers.

Virus identification. Viruses were identified using the tissue culture enzyme immunoassay (TCEIA) described elsewhere.[18] Confluent 96-well monolayers of C6/36 cells were inoculated with stock virus supernatant and incubated for up to five days. Cell monolayers were fixed with PBS/BSA (0.2% w/v) containing acetone (20% v/v). Monoclonal antibodies used in the assay included 4G2 (flavivirus group reactive), 995 (JE), 8C4 (Murray Valley encephalitis and Alfuy), 2B2 (Kunjin and West Nile), 2E5 (Kokobera and Stratford), 6F7 (Edge Hill and Sepik), 11F4 (alphavirus group reactive), B82A2 (Ross River), 9E8 (Barmah Forest), and 2F2 (Sindbis).[18–23] Monoclonal antibodies 11F4, 9E8, and 2F2 were obtained from James Cook University Tropical Biotechnology Pty Ltd (Townsville, Australia). Microtiter plates containing monolayers of C6/36 cells were also infected with reference viruses; these and uninfected C6/36 cell monolayers were included in the TC/EIA as controls. The reference viruses used as controls were JE (Nakayama), Murray Valley encephalitis (OR2), Alfuy (MRM3929), Kunjin (OR354), West Nile (Sarafend), Kokobera (OR408), Edge Hill (24880), Stratford (C338), Ross River (T48), Barmah Forest (BH2193), Sindbis (SW33130), Getah (AM2021), and Semliki Forest. The final absorbance was measured using a Titertek Multiskan MCC/340 plate reader (ICN Biomedicals Ltd., High Wycombe, Buckinghamshire, UK) at the single wavelength of 490 nm. Isolates of Murray Valley encephalitis (MVE) viruses were confirmed by TC/EIA using the specific monoclonal antibody 10C6 (MVE), and monoclonal antibodies 6F7 and 3G1 (Edge Hill virus specific) were used to tentatively identify one isolate of a Sepik-like virus. Minimum infection rates were calculated using methods described elsewhere.[24]

Reverse transcriptase-polymerase chain reaction and sequencing. Confluent 25 cm^2 flasks of porcine squamous equine kidney (PSEK) cells were infected with 50 μL of JE virus supernatant. When approximately 50% of the monolayer showed evidence of cytopathic effect, PSEK cells were removed, and total RNA was isolated using Total RNA isolation reagent (Advanced Biotechnologies, Epsom, Surrey, UK). Primers were used to amplify the prM gene, and an overlapping region of the NS5–3′ UTR of the genome.[5] These regions of the genome were chosen for analysis to: 1) identify the genotype;[8,9] 2) compare isolates of JE virus from PNG with others from the Torres Strait; and 3) determine whether PNG isolates of JE had the unique eleven base deletion in the NS5–3′ UTR as observed in isolates from the Torres Strait.[5] cDNA was obtained by reverse transcription of 2 μL of the total RNA using the antisense primer of each primer pair. The reverse transcription was carried out with 10 units of avian myeloblastosis virus reverse transcriptase (Promega Corporation, Madison, WI) in a 20 μL volume of the manufacturer's buffer, supplemented with 100 mM of each dNTP, 120 units of RNasin (Promega) and 20 pM of the antisense primer. The reverse transcription was incubated at 42°C for 1 hour. Two μl of the cDNA was amplified by the polymerase chain reaction (PCR). The PCR amplification was carried out with 1 unit of *Taq* polymerase (Red Hot Taq, Advanced Biotechnologies) in a 25 μL volume of the manufacturer's buffer supplemented with 100 mM of each dNTP, 2 mM $MgCl_2$ and 20 pM each primer. The PCR involved a touchdown procedure entailing denaturation for 5 min at 94°C, followed by 3 cycles of denaturation at 94°C for 15 sec, annealing at 53°C for 30 sec, and elongation at 72°C for 3.5 min. The annealing temperature was then varied to 51°C, 49°C, 47°C, and 45°C, with 3 cycles of PCR at each annealing temperature. Finally, 20 cycles of PCR were carried out with a denaturing temperature of 94°C for 15 sec, an annealing temperature of 42°C for 30 sec, and elongation at 72°C for 1 min.[25] The resulting PCR products were purified after agarose gel electrophoresis (QIAquick Gel Extraction Kit, Qiagen Pty., Ltd., Clifton Hill, Victoria, Australia) and sequenced using the BigDye system (Perkin Elmer Biosystems, Foster City, CA). The prM and NS5–3′UTR sequences of PNG4837 (GenBank accession numbers AF092551 and AF092550 respectively), PNG6544 (accession numbers AF139530 and AF139531 respectively) and PNG8728 (accession numbers AF218067 and AF218068 respectively) were deposited in GenBank, and compared with other isolates of JE virus. All computer analysis was performed on the Australian Genomic Information Centre (ANGIS) (Sydney, Australia) computer system and on BioNavigator.com provided by eBioinformatics (Sydney, Australia). Multiple nucleotide sequence alignments were performed in the CLUSTAL W program.[26] Aligned sequences were subjected to phylogenetic analyses using programs from the PHYLIP package.[27] Distance matrices and phylograms were constructed using the neighbor-joining method.[28] Bootstrap analysis of 100 replicates were used to place confidence values on groupings within phylogenetic trees. Two hundred and nineteen bases were used in the construction of the prM phylogram, and 424 nucleotides were used to construct a phylogram of the NS5–3′ UTR.

RESULTS

Virus isolation and identification. Forty-six species of female mosquitoes were collected between April 1996 and February 1998 (Table 1) and subsequently processed for virus isolation. In addition, 118 males of six species were processed in 1996, and 293 males of two species were processed in 1997; no viruses were obtained from male mosquitoes. The most common species processed for virus isolation in 1996 included the *Culex sitiens* subgroup (33%), *Anopheles farauti* sensu lato (s.l.) (14%), *Aedes funereus* (12%) and *Ae. lineatus* (9%). In 1997, the most abundant species were the *Cx. sitiens* subgroup (44%), *An. farauti* s.l. (25%) and *Cx. vicinus* (9%), while in 1998, the *Cx. sitiens* subgroup (74%) and *Ae. funereus* (11%) were the most common species processed for virus isolation. Twenty-four isolates were obtained from mosquitoes processed for virus isolation (Table 2). One isolate of JE virus (PNG4837), with a minimum infection rate (MIR) of 0.09 per 1,000 mosquitoes, was obtained from the *Cx. sitiens* subgroup collected in one trap (of ten) at Lake Murray Patrol Post in April 1997. Two isolates of JE virus were obtained from mosquitoes collected in 1998; the first (PNG6544, MIR of 0.02 per 1,000 mosquitoes) was isolated from the *Cx. sitiens* subgroup collected in one trap (of ten) at Balimo in February 1998; the second

TABLE 1
Number of mosquitoes processed from the Western Province of Papua New Guinea, 1996–1998.

Species	Year		
	1996 No. processed	1997 No. processed	1998 No. processed
Aedes aurantius aurantius	20	66	113
Ae. carmenti	6,534	237	365
Ae. culiciformis	1	0	0
Ae. foliformis	372	0	0
Ae. funereus	9,544	459	29,606
*Ae. kochi**	1,380	2,028	360
Ae. lineatus	7,237	326	2,008
Ae. littlechildi	1	6	0
Ae. notoscriptus	58	6	3
Ae. palmarum	1	0	0
Ae. scutellaris	7	0	0
Ae. stoneorum	0	0	20
Ae. tremulus	1	0	0
Ae. vigilax	16	0	1,721
Ae. (*Macleaya*) E.N. Marks species No. 170§	2	0	0
Ae. (*Verrallina*) species†	869	19	1,067
Aedeomyia catasticta	0	178	0
Anopheles annulipes	11	0	0
An. bancrofti‡	463	601	0
An. farauti s.l.	11,375	15,082	14,180
An. novaguinensis	3	0	0
Armigeres papuensis	163	56	201
Bironella simmondsi	33	98	0
Coquilletidia crassipes	2,322	1,137	2
Cq. xanthogaster	1,115	0	0
Culex. australicus	0	186	0
Cx. bitaeniorhynchus	14	1,212	0
Cx. hilli	6	293	3
Cx. pullus	176	56	31
Cx. quinquefasciatus	37	918	680
Cx. sitiens subgroup**	25,963	26,804	192,716
Cx. starckeae	245	0	0
Cx. vicinus	4,158	5,569	16,770
Ficalbia minima	9	535	0
Mansonia papuensis	3,863	1,948	44
Man. septempunctata	1,214	1,024	20
Man. uniformis	1,033	1,636	0
Tripteroides atripes	0	3	0
Trip. alboscutellatus	0	0	6
Trip. magnesianus	4	0	1
Uranotaenia albescens	7	0	0
Ur. amiensis	108	0	0
Ur. lateralis	1	0	0
Ur. nivipes	3	0	0
Unidentified	312	345	403
TOTAL	78,681	60,828	260,320

* Members of the *kochi* subgroup with profusely spotted wings, femora, and tibia were classified as *Ae. kochi*.
§ Currently unnamed, but recognized as a separate species by leading Australian Culicid taxonomist, Dr. Elizabeth N. Marks.
† Mosquitoes belonging to the Verrallina subgenus, but unable to be identified to species.
‡ May include *An. pseudobarbirostris*.
** Originally identified as *Cx. annulirostris*, genetic studies indicate it was composed of several species including *Cx. annulirostris*, *Cx. palpalis*, *Cx. sitiens*, and other undescribed species.[15]

(PNG8728, MIR 0.01 per 1,000 mosquitoes) from the *Cx. sitiens* subgroup collected at Abam, also in February 1998. Two isolates of Murray Valley encephalitis (MVE) virus were also obtained from the *Cx. sitiens* subgroup (MIR 0.03 per 1,000 mosquitoes) trapped at Balimo in February 1998, in addition to one isolate of Sepik virus (MIR of 0.02 per 1,000 mosquitoes) and twelve isolates of Sindbis (SIN) virus. Isolates of SIN virus were also acquired from the *Cx. sitiens* subgroup collected at Abam (2 isolates) and Peawa (3 isolates) in February 1998. *An. farauti* s.l. collected at Wando Village in April 1997 yielded one isolate of Ross River virus.

Sequence analysis. Comparison of a 219 nucleotide region of the prM gene indicated that isolate PNG4837 was identical in this region to the 1995 Torres Strait human isolates FU, NO and M40. Isolates PNG6544, PNG8728, and the 1998 Torres Strait mosquito isolate (TS3306) were identical and differed from the PNG4837 sequence by only one nucleotide (99.5% identity). A phylogenetic comparison between these and other JE virus sequences (Figure 2) indicated that these PNG and Torres Strait isolates were most closely related to viruses belonging to Genotype II from Malaysia (MaKAr158793) and Indonesia (JKT2219), sharing approximately 95% nucleic acid similarity.

The three PNG isolates were also sequenced in a 424 base region spanning the NS5 and 3′UTR. A phylogram (Figure

TABLE 2
Minimum infection rates (MIR) of mosquitoes collected in the Western Province of Papua New Guinea, 1997–1998

Year of collection	Locality	Species	Virus isolates (No.)	MIR/ 1000
1997	Bensbach	*Anopheles farauti* s.1.	RR* (1)	0.84
	Lake Murray	*Culex sitiens* subgroup§	JE (1)	0.09
1998	Abam	*Culex sitiens* subgroup	JE (1)	0.01
	Abam	*Culex sitiens* subgroup	SIN (2)	0.03
	Balimo	*Culex sitiens* subgroup	JE (1)	0.02
	Balimo	*Culex sitiens* subgroup	MVE (2)	0.03
	Balimo	*Culex sitiens* subgroup	SEP (1)	0.02
	Balimo	*Culex sitiens* subgroup	SIN (12)	0.21
	Peawa	*Culex sitiens* subgroup	SIN (3)	0.35

* RR = Ross River virus; JE = Japanese encephalitis virus; SIN = Sindbis virus; MVE = Murray Valley encephalitis virus; SEP = Sepik virus.
§ *Cx. sitiens* subgroup mosquitoes from Lake Murray and Abam were initially identified as *Cx. annulirostris*, and those from Balimo were initially identified as *Cx. palpalis*.

3) showing the close relationship between the PNG and Torres Strait isolates in this region is seen to be similar to the prM phylogram shown in Figure 2. All PNG and Australian isolates of JE included in the analysis shared > 99.2 nucleic acid identity and had identical amino acid sequences except for PNG8728, which had a single amino acid substitution. These isolates were all observed to have the unique eleven base deletion first described for the 1995 Torres Strait strains by Poidinger and others.[11]

DISCUSSION

The isolate of Ross River virus is the first from mosquitoes collected in PNG. It is also the first isolate from *An. farauti* s.l., a complex of seven species, of which six have been collected from PNG.[29] Studies into sibling species distribution suggest that *An. farauti* No. 2 was the species present in this area.[30] Cases of Ross River virus disease have been confirmed in patients in PNG in the past.[31] It is possible that *An. farauti* s.l. is involved in Ross River virus transmission cycles in PNG.

This isolation of SIN virus is only the second reported isolation of this virus from mosquitoes collected in PNG.[32] SIN virus is the most widely distributed of all arboviruses; it is also the most common arbovirus isolated from mosquitoes in Australia.[33] However, disease caused by SIN virus infection is rare in Australia, with few confirmed human cases.[34,35] Nucleotide sequence analysis of the E2 and C genes of an isolate of a 1966 isolate of SIN virus from northern PNG was shown to be more closely related to the Oriental/Australasian genotype of SIN virus than to the Paleoarctic/Ethiopian genotype.[36] If nucleotide sequence analysis reveals the strain of SIN virus circulating in transmission cycles in PNG is similarly related to the Oriental/Australasian genotype, it is unlikely to pose a risk for people living in PNG.

Sepik virus was initially isolated from a mixed pool of mosquitoes collected in the Sepik district of PNG in 1966.[32] The role of Sepik virus in human disease is uncertain. The recently acquired Sepik virus isolate from Balimo is currently being characterized further by nucleotide sequencing to clarify its relationship with the original isolate of Sepik virus and other flaviviruses. MVE virus has also been isolated in PNG in the past, albeit on only two occasions.[37,38] Despite few isolations and rarely recorded clinical cases, historic serologic investigations suggested that MVE does infect people living in some areas of PNG, including those living along the Aramia River floodplain in the Western Province of PNG.[12] The isolation of MVE and other flaviviruses from mosquitoes collected at Balimo in 1998 provides further evidence that the Aramia River floodplain is an important area of flavivirus activity.

The isolation of JE virus from mosquitoes collected from different locations in the Western Province in 1997 and 1998 provides the first definitive evidence that JE virus is circulating in mosquito and vertebrate host transmission cycles in PNG. Isolates of JE were acquired from mosquitoes initially identified as *Cx. annulirostris* collected from Lake Murray in 1997 and Abam in 1998, and *Cx. palpalis* collected at

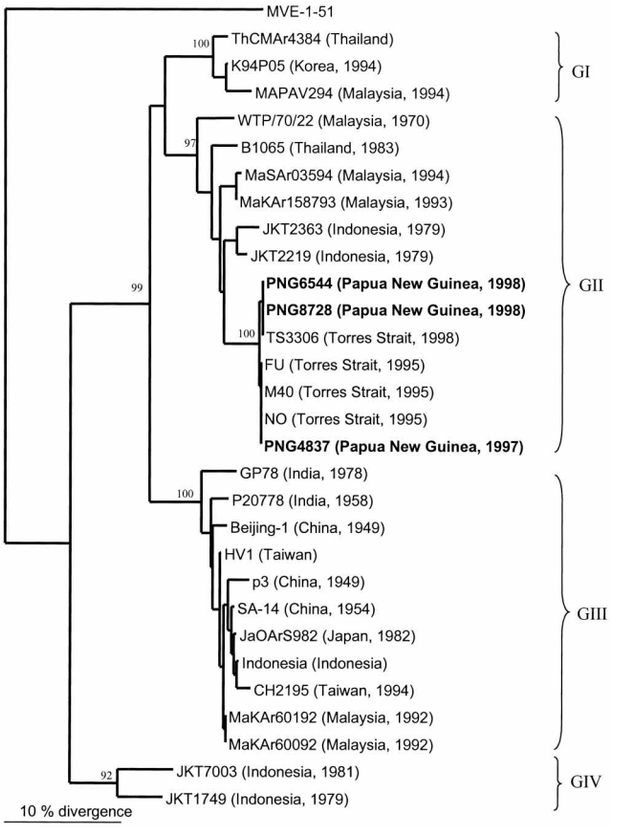

FIGURE 2. Phylogram representing the nucleotide sequence homology of the pre-membrane (prM) region of the genome of isolates of Japanese encephalitis virus. All four genotypes are represented.

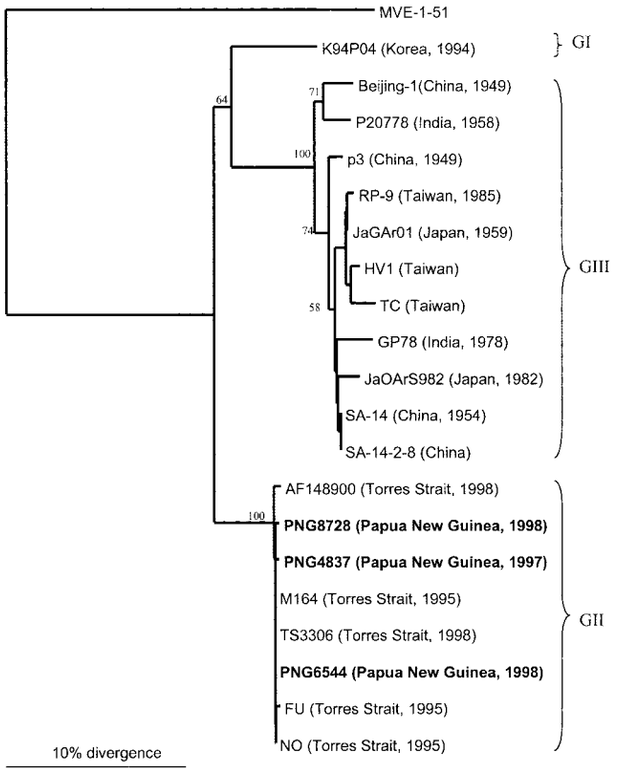

FIGURE 3. Phylogram representing the nucleotide sequence homology of the non-structural protein 5 to 3′ untranslated region (NS5–3′UTR) of the genome of isolates of Japanese encephalitis virus. All Papua New Guinea and Torres Strait isolates, in addition to the Korean isolate K94P05, had a deletion immediately downstream of the stop codon in the 3′ UTR. Papua New Guinea and Torres Strait isolates belong to Genotype II, K94P05 belongs to Genotype I, and all remaining isolates belong to Genotype III; Genotype IV is not represented.

Balimo in 1998. However, electrophoretic studies of a subsample of mosquitoes identified as *Cx. annulirostris* at Lake Murray revealed that 87.5% of the sample was *Cx. palpalis*, 5.0% was *Cx. annulirostris*, and 7.5 % undefined species.[16] Similarly, the same study showed that 94.9% of a sample of mosquitoes identified as *Cx. annulirostris* collected at Balimo in 1997 was *Cx. palpalis*. Thus, due to the difficulty separating *Cx. annulirostris* and *Cx. palpalis* using morphologic characteristics, it was considered most appropriate to pool results pertaining to members of the *Cx. sitiens* subgroup of mosquitoes in the final analysis. The infection rate of mosquitoes infected with JE virus (< 0.1:1,000) at Lake Murray and Abam was lower than that of *Cx. annulirostris* collected in the Torres Strait in 1995 (3.3:1,000) and 1998 (approximately 2.0:1,000).[5,7] Nevertheless, the relative abundance of members of the *Cx. sitiens* group of mosquitoes in the Western Province suggests that these species may be important in JE virus transmission cycles.

The high level of nucleotide sequence similarity between PNG and Torres Strait isolates of JE virus in the prM (> 99.5%) and NS5–3′UTR (> 99.2%) regions of the genome suggests a common source. Indeed all Australian and PNG isolates share an eleven base deletion in the 3′ UTR immediately downstream of the open reading frame stop codon. Thought previously to be the only isolates with this unique deletion,[6,11] the Korean isolate of JE (K94PO5) has a similar deletion in the NS5–3′ UTR but shares only 91% nucleic acid sequence similarity with the PNG and Australian isolates. However, the Korean isolate is classified by sequence analysis in the prM gene as a member of Genotype I whereas the isolates described in this paper belong to Genotype II, so the biological significance of this finding is not known.

These results provide solid evidence that PNG is the source of incursions of JE virus in Australia. The mechanism by which JE virus is being introduced into Australia has yet to be defined. One possible explanation is that JE virus is being introduced in infected wind-blown mosquitoes. Allozyme analysis of populations of *Cx. annulirostris* from the Western Province of PNG, the Torres Strait, and Cape York (north Queensland) has revealed a substantial amount of genetic mixing between these regions, suggesting that there is movement of mosquitoes between PNG and Australia, probably during cyclonic or monsoonal weather events.[39] There is evidence that the major vector of JE virus in Asia, *Cx. tritaeniorhynchus*, moves in a similar fashion in China and India.[40,41] Another possible mechanism of introduction involves migratory water birds. Many species of migratory water birds move south from PNG into northern Australia. The role of migratory birds in the introduction of JE virus into northern Australia needs to be investigated.

It is not known when JE virus was introduced into PNG. There is serologic evidence that JE virus has occurred in some areas of the Western Province since at least 1989, and the incidence may be increasing.[42] Human cases of disease caused by infection with JE virus have now been confirmed in the upper Fly River region of the Western Province of PNG (Oakley J, Rumginae Health Centre and Flew S, Ok Tedi Mining Limited, unpublished data). More recently, there are indications that the distribution of JE virus in PNG is increasing, with suspected outbreaks in Milne Bay Province [43] and the detection of antibodies to JE virus in pigs in West Sepik Province (Lee J, Australian Quarantine Inspection Service, unpublished data). Analysis of nucleotide sequences of the prM gene has revealed that the PNG and Torres Strait isolates of JE virus are members of Genotype II and appear to be most similar to isolates from Malaysia and Indonesia, with a 95% nucleic acid similarity. It seems likely that the virus was introduced into PNG from Indonesia by its gradual movement in mosquito and vertebrate host transmission cycles through the Indonesian archipelago and Irian Jaya.

A number of flaviviruses other than JE are known to exist in transmission cycles in PNG, including MVE, Kunjin, Kokobera, and Sepik viruses.[39,44] There is also serologic evidence suggesting dengue serotypes 1, 2, and possibly 3 have caused outbreaks of disease in PNG.[14] It is not known what effect the occurrence of multiple flaviviruses in the one region will have on the ability of these viruses to co-circulate. Given that isolates of JE, MVE, and a Sepik virus were obtained from mosquitoes collected from the same location in the Western Province in 1998, it appears that at least some of these viruses can effectively circulate between the same species of mosquitoes and vertebrate hosts in the one region. This finding has important implications for Australia, where numerous flaviviruses are enzootic, particularly in the north-

ern regions that are at risk of incursions and possible establishment of JE virus in enzootic transmission cycles. Given the results in PNG, it seems unlikely that active transmission of flaviviruses in northern regions of Australia will impede the establishment of enzootic transmission cycles of JE virus.

It is apparent that JE virus is emerging as a serious threat to human health in PNG. Proposed logging in areas such as Kawito (near Balimo) on the Aramia River are likely to increase available mosquito breeding sites, vector abundance, and incidence of mosquito-borne disease. Thus, efforts to define the distribution and seasonality of JE virus in PNG need to continue. The method by which JE virus is entering Australia should be investigated to determine risk periods of introduction and develop appropriate programs for control and prevention. Finally, the role of members of the *Cx. sitiens* subgroup in transmission of JE needs to be studied further.

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Authors' addresses: Cheryl A. Johansen (cheryl_johansen@health.qld.gov.au), Department of Microbiology and Parasitology, University of Queensland, C/- Tropical Public Health Unit, P.O. Box 1103, Cairns, Queensland, 4870. Scott A. Ritchie (ritchies@health.qld.gov.au) and Paul Zborowski (closeup@ozemail.com.au), Tropical Public Health Unit, P.O. Box 1103, Cairns, Queensland, 4870, Australia. Andrew F. van den Hurk (hurkynjules@ozemail.com.au), Debra J. Nisbet (nisbet@biosci.uq.edu.au), Joanne Macdonald (peake@biosci.uq.edu.au), Tatiana I. Khromykh (T.Khromykh@cmcb.uq.edu.au) and John S. Mackenzie (jmac@biosci.uq.edu.au), Department of Microbiology and Parasitology, University of Queensland, St. Lucia, Queensland, 4072, Australia (telephone: 61 7 3365 4648; fax: 61 7 3365 6265). Raymond Paru and Moses J. Bockarie (mbockarie@datec.com.pg), Papua New Guinea Institute of Medical Research, PO Box 378, Madang, Madang Province, Papua New Guinea. Alexander C. Drew (A.Drew@nhm.ac.uk), Department of Zoology, The Natural History Museum, Cromwell Road, South Kensington, London, SW7 5BD, United Kingdom.

Address for reprints: John S. Mackenzie, Department of Microbiology and Parasitology, The University of Queensland, St. Lucia, Queensland, 4072, Australia (email: jmac@biosci.uq.edu.au).

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