Am. J. Trop. Med. Hyg., 75(1), 2006, pp. 21–25

SHORT REPORT: THE FIRST ISOLATION OF JAPANESE ENCEPHALITIS VIRUS FROM MOSQUITOES COLLECTED FROM MAINLAND AUSTRALIA

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Abstract. In response to an incursion of Japanese encephalitis virus (JEV) on Cape York Peninsula, Australia, in 2005, 23,144 *Culex* mosquitoes were processed for virus detection. A single isolate of JEV was obtained from a pool of *Culex sitiens* subgroup mosquitoes. This is the first reported mosquito isolate of JEV from the Australian mainland.

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus responsible for an estimated 30,000–50,000 cases of encephalitis annually.[1] In 1995, JEV emerged in northern Australia, with an outbreak in the Torres Strait.[2] In response to the outbreak, the human population was vaccinated, and a sentinel pig program was implemented to detect future incursions in the Torres Strait and on the Australian mainland.[3,4] With the exception of 1999, JEV activity has been reported annually in the Torres Strait, resulting in four human cases and two deaths.[5] The 1998 outbreak was the most widespread, with a human case and porcine seroconversion on Cape York Peninsula.[6] Mackenzie predicted that once JEV reached Cape York Peninsula, it would become established in natural transmission cycles, fueled by a convergence of wading birds, large populations of feral pigs, and *Culex* mosquitoes.[7]

Despite regular JEV activity in the Torres Strait, there is no evidence to suggest that JEV has become enzootic on the Australian mainland. Indeed, JEV had not been detected in sentinel pigs and mosquito collections from Cape York Peninsula since 1998. However, in late February 2004, sentinel pigs deployed on the Northern Peninsula Area (NPA) on the tip of Cape York Peninsula seroconverted to JEV. The NPA sentinel pigs began seroconverting on 23 February, and four of five pigs had seroconverted by 12 March, after which no more seroconversions were detected (I. L. Smith, unpublished data). No virus was detected in any sentinel pig serum samples. In response to the detection of antibodies to JEV, mosquitoes were collected for virus detection from the five communities that constitute the NPA. We report on the first Australian mainland isolation of JEV from these mosquitoes.

Mosquito collections were undertaken from March 22 to March 25, 2004, from the NPA (Figure 1). Mosquitoes were

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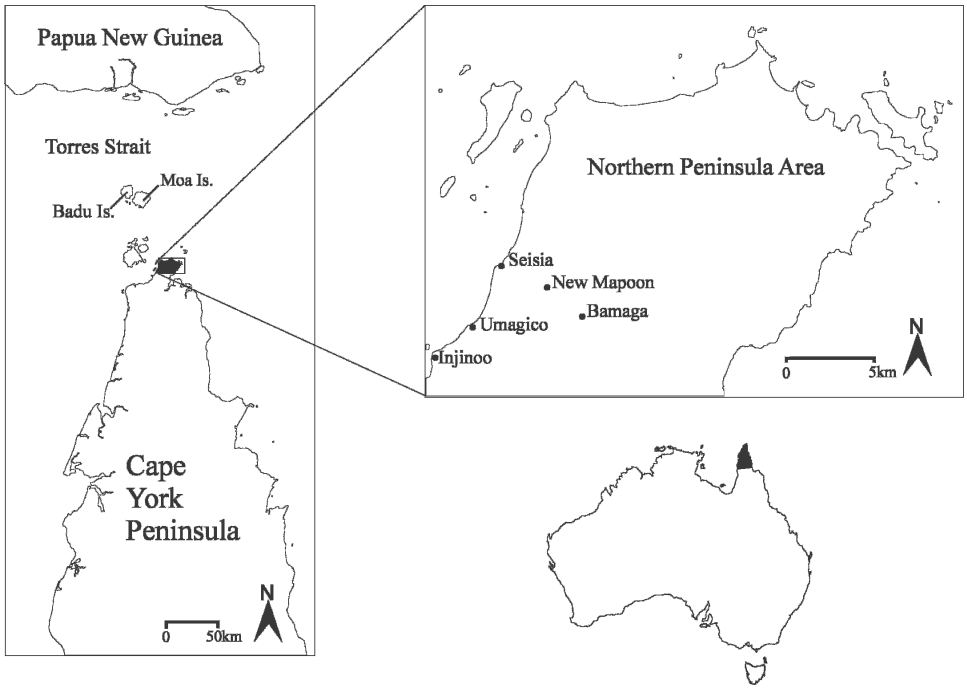

FIGURE 1. The study sites on the NPA.

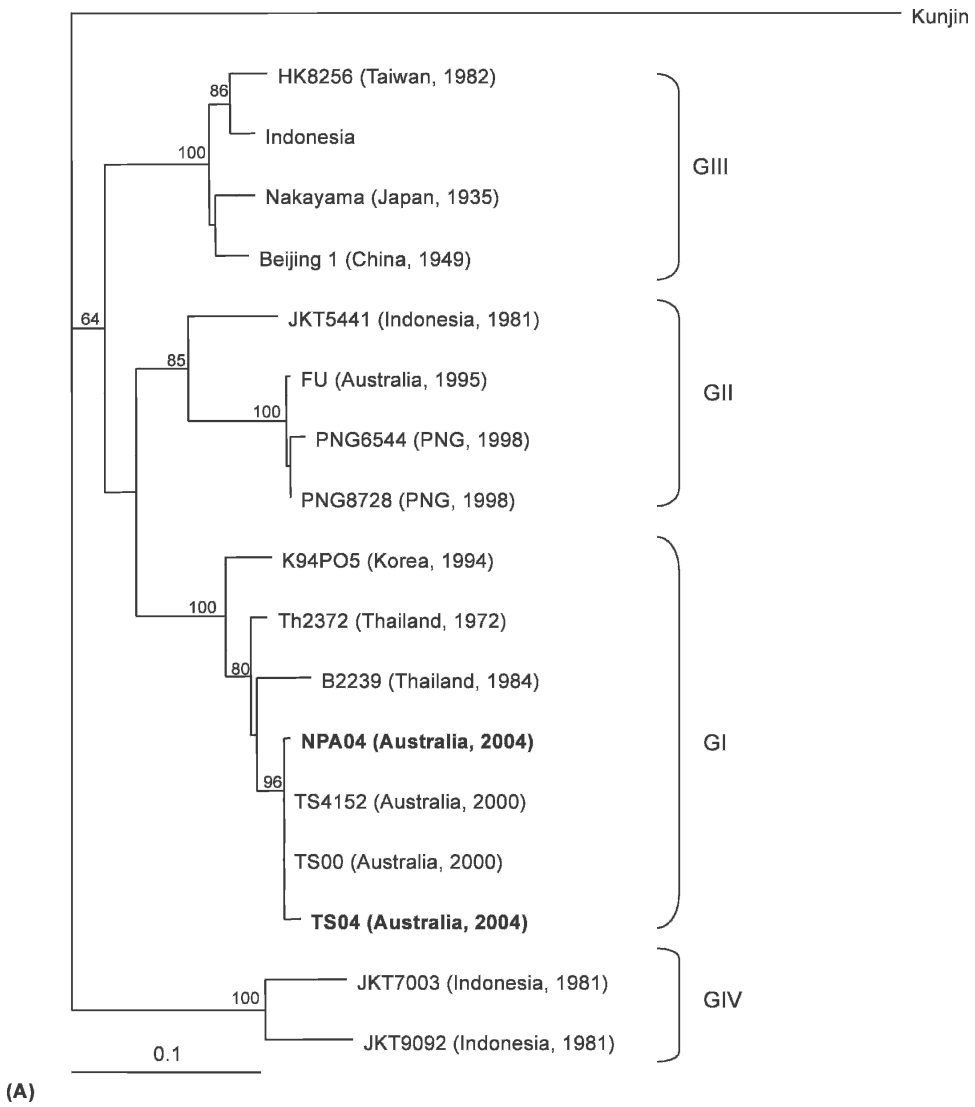

FIGURE 2. Phylogram of the Japanese encephalitis virus isolates from Australia in 2004 (shown in bold) for the (**A**) *prM-C* gene and (**B**) *partial E* gene, constructed using the neighbor-joining method. Bootstrap values are shown at the nodes. With the exception of NPA04, all Australian isolates have originated from the Torres Strait.

collected with Model 512 CDC light traps (John W. Hock, Gainesville, FL) baited with either CO_2 alone or in combination with 1-octen-3-ol, set from 4:00 PM to 6:30 AM. Mosquitoes were killed by freezing and stored in liquid N_2 dry shippers for transport to the Tropical Public Health Unit, Cairns, Queensland, for sorting. Because they are the primary JEV vectors, only *Culex* spp. were submitted for virus detection. In addition, as some members of the *Cx. sitiens* subgroup share overlapping morphology, mosquitoes belonging to this group were pooled together for virus detection.

At the University of Queensland, Brisbane, mosquito pools of $\leq$ 200 were homogenized in 5 mL of cell culture media using a SPEX 8000 mixer/mill (Spex Industries, Edison, NJ), centrifuged, and filtered using a combined 0.8/0.2-μm filter. Viral RNA was extracted from 140 μL of the filtered supernatant using the QIAamp Viral RNA kit (Qiagen, Clifton Hill, Australia). At Queensland Health Scientific Services, Brisbane, samples were initially screened for JEV RNA using a TaqMan reverse transcriptase-polymerase chain reaction (RT-PCR) assay.[8]

A total of 23,144 *Culex* spp. from 56 trap collections were tested for the presence of JEV antigen. *Cx. sitiens* subgroup mosquitoes comprised 22,833 (98.7%) of mosquitoes processed. The remaining mosquitoes processed were 228 (1.0%) *Cx. pullus*, 13 (< 0.1%) *Cx. gelidus*, 33 (≈0.1%) *Cx. quinquefasciatus*, 29 (≈0.1%) *Cx. cubiculi*, and 8 (< 0.1%) *Cx. bitaeniorhynchus*.

JEV RNA was detected in a single pool of *Cx. sitiens* subgroup mosquitoes collected from the Bamaga rubbish tip on 23 March 2004. Maximum likelihood estimation of mosquito infection rate (MLE-IR) with 95% confidence limits in mosquitoes collected from the NPA was 0.04 per 1,000 using the PooledInfRate statistical software package.[9] The species composition of the virus positive pool was established retrospectively using an allele-specific PCR assay based on the ribosomal internal transcribed spacer 1 (N.W. Beebe, unpub-

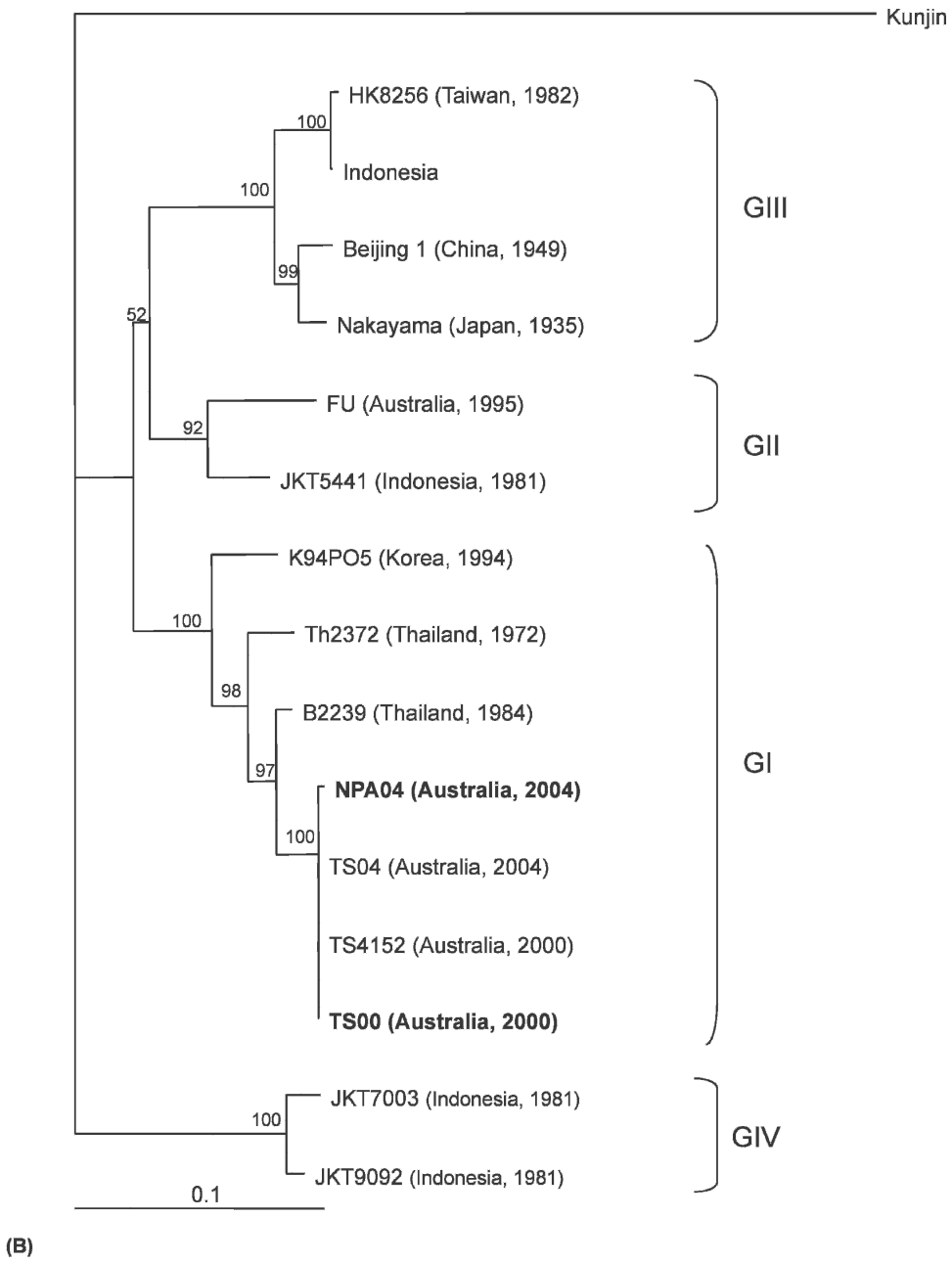

FIGURE 2. Continued.

lished data). The results revealed that the majority of the mosquitoes were *Cx. annulirostris* and a small number of *Cx. palpalis*.

To confirm the TaqMan identification of JEV, C6/36 cells were inoculated with a different aliquot of the original suspension, and the resultant virus isolate (designated NPA04) identified using a panel of monoclonal antibodies in an immunofluorescence assay (IFA). Genotyping of NPA04 and a 2004 Badu Island sentinel pig isolate (designated TS04) was undertaken on the core to pre-membrane (C-prM) (GenBank accession numbers DQ267756 and DQ267757) and partial envelope (E) genes (accession numbers DQ267758 and DQ267759) using the methods of Pyke and others.[10] Virtually identical phylogenetic trees were generated for the *C-prM* and *E* genes by the neighbor-joining method (Figure 2, A and B).[11] The 2004 Australian isolates clustered with the Australian isolates (TS00 and TS4152) in genotype I from 2000. The NPA04 isolate displayed nucleotide identities of 99.6% and 99.8%, respectively, with TS00 in the *C-prM* and *E* genes, whereas the TS04 isolate displayed identities of 99.2% and 100%, respectively, with TS00. Both isolates had the nucleotide deletion in the NS5-3′UTR region, previously reported for the 2000 isolates.[10]

To detect the presence of any co-circulating enzootic flaviviruses, a general flavivirus-specific hemi-nested RT-PCR was used.[12] No other flaviviruses were detected in the remaining pools.

Since JEV was first recognized in the Australasian zoogeographical region in 1995, there have been two reported instances where virus incursions have extended onto mainland

Australia.[6] During the 1998 Cape York Peninsula outbreak, no JEV isolates were obtained from > 6,000 *Culex* spp., comprising primarily *Cx. annulirostris*, processed for virus isolation from the NPA.[13] Additional collections of > 400,000 mosquitoes from western Cape York Peninsula as far south as Croydon in the Gulf Plains region have failed to yield any JEV isolates.[13–15] Therefore, the isolate we obtained from the 2004 collections is the first JEV isolate from mosquitoes collected from the Australian mainland.

The sudden appearance of JEV on Cape York Peninsula in 2004, 6 years after the 1998 outbreak suggests that it either dispersed into the area from infected areas to the north or that JEV has been present in cryptic cycles since 1998. However, the 1998 and 2004 viruses have been shown by RNA sequencing to be distinct, with the 1998 virus belonging to genotype II, and all isolates since 2000 belonging to genotype I.[10] Thus, the 2004 event represents a relatively new incursion of JEV into the mainland of Australia. Furthermore, sequence similarity between the 2004 NPA and Badu Island isolates suggest a possible common source of the virus.

Several mechanisms have been suggested for the incursion of JEV into Australia. Perhaps the strongest circumstantial evidence is provided by Ritchie and Rochester,[16] whose trajectory models indicate that deep low pressure systems in the Gulf of Carpentaria can create northerly winds capable of transporting mosquitoes from southern New Guinea into the Torres Strait and Cape York Peninsula. A large low pressure system was present on this area from January 13 to January 15, 2004, that was potentially capable of transporting mosquitoes from southern New Guinea to Badu and Moa islands and to the NPA of Cape York Peninsula (S. Ritchie, unpublished data), or even from Badu and Moa islands onto Cape York Peninsula. In support of the mosquito incursion mechanism, Chapman and others[17] used isozyme analysis to show that *Cx. annulirostris* populations from southern PNG, the Torres Strait, and Cape York Peninsula are panmictic, indicating dispersal across the Torres Strait. Alternatively, migrating birds or flying foxes are also suspected of having introduced JEV into northern Australia.[5]

Despite virus activity occurring in the Torres Strait, the sentinel pigs and weekly mosquito trap collections failed to detect JEV on the NPA in 2005. This suggests that the virus has not become established in natural transmission cycles on mainland Australia or, if it has become endemic, it is circulating at levels that cannot be detected using the current sentinel systems. The apparent low-level JEV transmission on Cape York Peninsula has been linked to the presence of alternative blood meal hosts, such as macropods, and competition with antigenically related flaviviruses for susceptible vertebrate hosts.[6] Interestingly, at the Bamaga Rubbish tip, there was evidence of nightly feral pig activity, such as hoof prints and feces, demonstrated pig feeding by *Cx. annulirostris*, and high larval populations of this species (A. F. van den Hurk and W. Y. Cheah, unpublished data). Therefore, small foci of JEV activity could occur where feral pigs and *Cx. annulirostris* congregate and proliferate.

Received January 30, 2006. Accepted for publication March 13, 2006.

Acknowledgments: The authors thank the Northern Peninsula Area communities for supporting these studies. Harry Seriat and Ronald Williams were invaluable in assisting with the mosquito collections. We also thank Debra Nisbet, Nigel Beebe, and the staff of Public Health Virology, Queensland Health Scientific Services, for assisting in the processing of samples and Tony Sweeney for providing the map.

Financial support: This study was funded by the Australian Health Minister's Advisory Council (AHMAC) Priority Driven Research Program and Queensland Health.

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