FLAVIVIRUSES ISOLATED FROM MOSQUITOES COLLECTED DURING THE FIRST RECORD OF OUTBREAK OF JAPANESE ENCEPHALITIS VIRUS ON CAPE YORK PENINSULA, AUSTRALIA

ANDREW F. VAN DEN HURK, CHERYL A. JOHANSEN, PAUL ZBOROWSKI, DEBRA A. PHILLIPS, ALYSSA T. PYKE, JOHN S. MACKENZIE, AND SCOTT A. RITCHIE

Tropical Public Health Unit, Queensland Health, Cairns, Australia; Department of Microbiology and Parasitology, The University of Queensland, Brisbane, Australia; World Health Organization Collaborating Centre for Arbovirus Reference and Research, Queensland Health Scientific Services, Coopers Plains, Brisbane, Australia

Abstract. In response to an outbreak of Japanese encephalitis (JE) virus on Cape York Peninsula, Australia, in 1998, mosquitoes were collected using CO2 and octenol-baited Centers for Disease Control and Prevention light traps. A total of 35,235 adult mosquitoes, comprising 31 species, were processed for virus isolation. No isolates of JE virus were recovered from these mosquitoes. However, 18 isolates of Kokobera virus, another flavivirus were obtained from Culex annulirostris. Twelve isolates were from western Cape York (minimum infection rate (MIR) of 0.61:1,000 mosquitoes) and 6 were from the Northern Peninsula Area (MIR of 1.0:1,000). Potential explanations for the failure to detect JE virus in mosquitoes collected from Cape York Peninsula include the timing of collections, the presence of alternative bloodmeal hosts, differences in pig husbandry, asynchronous porcine seroconversion, and the presence of other flaviviruses.

INTRODUCTION

Japanese encephalitis (JE) virus is a mosquito-borne flavivirus of Southeast Asia responsible for over 50,000 clinical cases annually, with a 25% fatality rate, mainly in young children. The virus is maintained in a zoonotic cycle between rice field-breeding mosquitoes and domestic pigs and/or waterbirds, with humans as incidental hosts. Prior to 1995, the recorded geographical distribution of JE virus infection extended from India and Pakistan in the west, to Japan and the Philippines in the east, and from Korea and maritime Russia in the north, to Bali on the Indonesian archipelago in the south. However, in April 1995, an outbreak of JE occurred on Badu Island in the Torres Strait, Australia. There were 3 clinical cases of encephalitis, with 2 deaths, as well as evidence of widespread human and porcine infection on other islands. Entomological investigations during the Badu Island outbreak revealed that Culex annulirostris was the most likely vector, with 8 isolates of JE virus obtained at a carriage rate of 2.97:1,000. In response to the outbreak, an extensive vaccination program was undertaken on the outer islands of the Torres Strait, with 88% of the population receiving at least 2 doses. In addition, a sentinel pig surveillance system was established to monitor JE virus activity, both in the Torres Strait and the Northern Peninsula Area (NPA) of Cape York. Seroconversion to JE virus was detected in pigs in both 1996 and 1997, although the activity was restricted to the northern island of Saibai.

In late February 1998, a larger and more widespread outbreak of JE occurred in the Torres Strait, with one human case diagnosed in an unvaccinated child on Badu Island. By mid-April, many of the sentinel pigs on the outer islands, as well as on the inner island of Kiriri, had seroconverted to JE virus. During the 1998 outbreak on Badu Island, high numbers of JE virus isolates were again obtained from mosquitoes, with 42 isolated from Cx. annulirostris and one from a pool of Aedes vigilax.

Shortly after the Badu Island case, the first Australian-mainland clinical case of JE was diagnosed in a fisherman working at the mouth of the Mitchell River, on the western side of Cape York Peninsula. The presence of JE virus activity on western Cape York Peninsula was confirmed when 13 of 20 juvenile pigs at Baa’s Yard, a piggery located approximately 10 km from the mouth of the Mitchell River, had neutralizing antibodies to JE virus. In the NPA communities, seroconversions in sentinel pigs were detected from late March. The seroconversions were slow and protracted over a 3-month period, whereas in the Torres Strait, a more rapid synchronized rate of seroconversion was displayed.

Following the emergence of JE virus in the Australasian region, there has been much speculation on the risk of its establishment in enzootic cycles on the Australian mainland. Indeed, Mackenzie suggested that the convergence of high numbers of wild pigs and ardeid water birds, coupled with prolific mosquito breeding sites in the swamps and wetlands of western and south-western Cape York Peninsula, could provide an ideal milieu for JE virus to become rapidly amplified in the environment. However, the 1998 mainland outbreak was characterized by only one human case, the absence of subclinical JE virus infection in residents of nearby communities and low numbers of sentinel pig seroconversions. We report on the attempts to isolate JE virus from mosquitoes collected during investigations into the first Australian-mainland outbreak on Cape York Peninsula and the NPA.

MATERIALS AND METHODS

Study sites. Cape York Peninsula is located in far north Queensland, Australia, extending from 16°S to 11°S and 141°30’E to 146°E (Figure 1). It is bordered by the Gulf of Carpentaria to the west and the Coral sea to the east and extends over an area of approximately 137,200 km². The 2 regions that were included in our investigations were the Mitchell River area of western Cape York Peninsula and the NPA. The vegetation of both of these regions is characterized by open grassland and mixed woodland, with salt flats and mangroves confined to the coast and some river systems.
The climate of both of the regions is monsoonal, with the average annual rainfall ranging from 1,200 mm on western Cape York Peninsula to 1,600 mm in the NPA. More than 80% of the annual rainfall occurs during the wet season between December and March. Temperatures are generally warm to hot with maximum temperatures of over 40°C recorded in the summer. Temperatures below 10°C are rare. The estimated population of the communities located on
Mosquito collections. Adult mosquitoes were collected using Centers for Disease Control and Prevention (CDC) (John W. Hock Company, Gainesville, FL) light traps baited with CO₂ (1 kg dry ice) and 1-octen-3-ol (octenol; release rate of 4.5 mg/h).\(^{12}\) Mosquitoes were collected from the western side of Cape York Peninsula approximately 3 weeks after the fisherman at the mouth of the Mitchell River became ill. Between March 26–27, 1998, a total of 29 CDC traps were set, with 13, 14, and 2 set at Kowanyama, Pormpuraaw, and Baa’s Yard, respectively. Mosquito trapping on the NPA was undertaken approximately 2 weeks after the beginning of seroconversions in sentinel pigs. Between April 7–8, 1998, 29 CDC traps were set at Bamaga, Injinoo, New Mapoon, Seisia, Umagico, and Woombah Station.

Mosquitoes were killed on dry ice and stored in liquid nitrogen dry shippers before being transported to Cairns for storage at \(-70°C\). On a refrigerated cold table, mosquitoes were identified to species using the taxonomic keys of Lee and others and sorted into pools of 100 or less.\(^{13}\) Due to the large numbers of mosquitoes collected from the western Cape, all *Culex* spp. were identified and processed, while only an aliquot of the remaining mosquitoes was identified and processed. Mosquito pools were sent on dry ice to the Department of Microbiology at the University of Queensland, Brisbane, and the World Health Organization Collaborating Center for Arbovirus Reference and Research, Queensland Health Scientific Services, Brisbane, for virus isolation.

**Virus isolation.** Mosquitoes were processed using the methods described by Ritchie and others and Broom and others.\(^{3,14}\) At Queensland Health, mosquito pools were examined for both alphaviruses and flaviviruses after inoculation of homogenized mosquito pools onto monolayers of C6/36 cells. After 10 days incubation, cells were harvested and viruses identified by immunofluorescent staining using a panel of monoclonal antibodies. Virus isolates were confirmed by RT-PCR and sequencing of the NS5-3'UTR region. At the University of Queensland, mosquito samples were examined only for the presence of flaviviruses. After inoculation of homogenized mosquitoes onto monolayers of C6/36 cells, monolayers were subsequently fixed and screened using a panel of monoclonal antibodies in a tissue culture enzyme immunoassay.\(^{14}\) All virus isolations were confirmed by re-isolation from original suspensions. The minimum infection rate (MIR) for pools of infected mosquitoes was calculated using a formula derived from Chiang and Reeves.\(^{15}\)

**RESULTS**

A total of 35,235 adult mosquitoes were processed for virus isolation, with 28,475 (80.8%) collected from western Cape York Peninsula and 6,760 (19.2%) from the NPA (Table 1). A total of 31 mosquito species, representing 7 genera, were processed, with the majority being *Cx. annulirostris* (71.9%), followed by *Aedes normanensis* (18.2%) and *Ae. vigilax* (5.0%).

Japanese encephalitis virus was not isolated from mosquitoes collected from Cape York Peninsula. However, 18 isolates of Kokobera (KOK) virus were recovered from *Cx. annulirostris*, of which 12 were isolated from the western Cape and 6 from the NPA. The minimum infection rate (MIR) was 0.62 per 1,000 mosquitoes on the western Cape and 1.0 per 1,000 on the NPA.

**DISCUSSION**

KOK virus belongs to the JE serological subgroup of flaviviruses. KOK virus as well as Murray Valley encephalitis (MVE) and Kunjin (KUN) viruses were originally isolated from *Cx. annulirostris* collected at Kowanyama.\(^{16}\) KOK virus has also been isolated from other mosquito species, including *Ae. vigilax*, *Aedes camptorhynchus*, and *Ae. normanensis*.\(^{17–19}\) Seroepidemiological studies have revealed that KOK virus infects humans in northern Queensland and coastal and inland regions of New South Wales.\(^{20,21}\) Infection with KOK was responsible for cases of polyarticular illness recorded in New South Wales, Victoria, and more recently in Central Queensland.\(^{20,23}\) Evidence from serological surveys suggests that the vertebrate hosts of KOK virus are macropods, with horses possibly involved.\(^{18,20,24}\)

Despite the presence of JE virus infection in both humans and pigs, JE virus was not isolated from mosquitoes collected from Cape York Peninsula. Interestingly, similar numbers of *Cx. annulirostris* were processed from Badu Island (25,622 mosquitoes), and 42 isolates of JE virus were obtained.\(^{8}\)

There are several possible reasons for the differences in mosquito isolation rates between the 2 foci of JE virus transmission. On Cape York Peninsula, there may not have been sufficient numbers of viremic pigs or significant levels of viremia in pigs to infect mosquito populations. Furthermore, the potential mosquito breeding sites were extensive and, in most cases, distant from human habitation. On Badu Island, in both 1995 and 1998, intense JE virus activity was characterized by complete porcine seroconversion and high numbers of mosquito isolates over a short period of several weeks in a very confined community area.\(^{14}\) However, in the NPA, seroconversions in sentinel pigs were slow and protracted over a 3-month period, with none of the 5 sentinel herds completely seroconverting.\(^{8}\) The extended period of seroconversion observed in pigs in the NPA may be similar to the pattern of transmission in some parts of Sri Lanka, where asynchronous infections occur in pigs, and transmission to humans is rare, compared to other regions where synchronous infections in pigs lead to epidemics of JE in humans.\(^{25}\) Additionally, Ritchie and others suggested that it was the proximity of domestic pigs, people and mosquitoes that contributed to the intense JE virus activity on Badu Island in 1995.\(^{26}\) On Badu Island, domestic pigs were kept by approximately 50% of residences, usually within 50 m of houses.\(^{6}\) However in the NPA, only approximately 10% of premises kept pigs, which were generally located away from the community.\(^{8}\) Even fewer domestic pigs were kept in communities on western Cape York Peninsula.

The presence of alternative bloodmeal hosts could divert host-seeking mosquitoes away from viremic pigs. In southern India, the difference in availability of pigs and alternative hosts was the main factor in epidemiologic differences in JE transmission between study sites.\(^{27}\) Host preference...
It is not known whether the presence of other flaviviruses, such as KOK and KUN viruses, would enhance or reduce the ability of JE virus to become established in north Queensland. A number of recent flavivirus infections in both humans and feral pigs in the NPA and western Cape York Peninsula could not be characterized due to cross-reacting flavivirus antibodies. This not only makes serological identification of infection difficult, but it may also affect the ability of JE virus to become established in natural vertebrate-mosquito transmission cycles. However, the isolation of both JE and MVE viruses from *Culex sitiens* group mosquitoes collected at Balimo, in the Western Province of Papua New Guinea (PNG), in 1998, suggests that 2 closely related flaviviruses can effectively circulate in the same area (Johansen CA and others, unpublished data). A similar situation exists in some areas of India and Pakistan, where both West Nile and JE viruses infect humans.

The potential exists for JE virus to become established in enzootic cycles on Cape York Peninsula, since there are extensive swamps and wetlands that possess significant populations of wild pigs, ardeid waterbirds, and *Culex annulirostris* mosquitoes. However, despite JE virus appearing on the Australian mainland for the first time in 1998, there is no evidence to suggest that transmission occurred in 1999. If the virus has not become established in an enzootic cycle on Cape York Peninsula, future transmission would depend on the re-introduction of JE virus from an enzootic focus.

Incursions of JE virus into northern Australia in 1995 and 1999 were not unexpected. JE virus infects a wide range of wild and domestic animals and recent evidence indicates that the potential for JE virus to become established in areas outside its geographical range exists. The development of diagnostic tests and the recognition of flaviviruses in tropical regions worldwide has increased dramatically in recent years. The potential for JE virus to become established in southern Australia will depend on its ability to adapt to new hosts and environments.
1998 have been circumstantially linked to mosquitoes being transported by winds associated with monsoonal weather systems from PNG, where JE virus has recently been isolated from Cx. sitiens group mosquitoes (Johansen CA and others, unpublished data). Research is currently being undertaken to determine the mechanisms of incursion of JE virus, as well as the role of vertebrate hosts and mosquito species in potential Australian JE virus transmission cycles.

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Authors addresses: Andrew F. van den Hurk, and John S. Mackenzie, Department of Microbiology and Parasitology, The University of Queensland, C/- Public Health Virology, Queensland Health Scientific Services, 39 Kessels Rd, Coopers Plains, Queensland, 4108, Australia. Cheryl A. Johansen, Paul Zborowski, and Scott A. Ritchie, Tropical Public Health Unit, Queensland Health, PO Box 1103, Cairns Queensland, 4870, Australia. Debra A. Phillips, C/- Immunology Department, Queensland Medical Laboratory (QML), 60 Ferry Road, West End, Queensland, 4101, Australia. Alyssa T. Pyke, World Health Organization Collaborating Centre for Arbovirus Reference and Research, Public Health Virology, Queensland Health Scientific Services, 39 Kessels Rd, Coopers Plains, Queensland, 4108, Australia.

Reprint requests: Andrew F. van den Hurk, Department of Microbiology and Parasitology, The University of Queensland, C/- Public Health Virology, Queensland Health Scientific Services, 39 Kessels Rd, Coopers Plains, Queensland, 4108, Australia. E-mail: andrew.hurk@health.qld.gov.au.

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