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Isolation of Arboviruses from Mosquitoes (Diptera: Culicidae) Collected from the Gulf Plains Region of Northwest Queensland, Australia

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ABSTRACT As part of investigations into Japanese encephalitis (JE) virus and related flaviviruses in northern Australia, 153,529 mosquitoes were collected and processed for virus isolation from the Gulf Plains region of northwest Queensland. Collections from within 30 km of each of the townships of Croydon, Normanton and Karumba yielded 3,087 (2.0%), 66,009 (43.0%), and 84,433 (55.0%) mosquitoes, respectively, from which 16 viruses were isolated. Four isolates of Murray Valley encephalitis (MVE), two of Kunjin (KUN), three of Ross River (RR), and one of Sindbis (SIN) viruses were obtained from *Culex sitiens* subgroup mosquitoes. Molecular identification of the mosquito species composition of these virus positive pools revealed that most isolates were from pools containing mainly *Culex annulirostris* Skuse and low numbers of *Culex palpalis* (Taylor). Only three pools, one each of MVE, KUN, and RR, were from mosquitoes identified exclusively as *Cx. annulirostris*. Other viruses isolated include one Edge Hill virus from *Ochlerotatus normanensis* (Taylor), an isolate of SIN from *Anopheles meraukensis* Venhuis, two isolates of RR from *Anopheles amictus* Edwards, and single isolates of RR from *Anopheles bancroftii* Giles and *Aedes lineatopennis* (Ludlow). The isolate of RR from *Ae. lineatopennis* was the first reported from this species. The public health implications of these isolations in the Gulf Plains region are discussed briefly.

KEY WORDS mosquitoes, virus isolation, flaviviruses, alphaviruses, Queensland, Australia

MORE THAN 75 ARBOVIRUSES have been recorded in Australia, with many being the etiological agents of human disease. Enzootic mosquito-borne viruses implicated in human disease in Australia include the alphaviruses, Ross River (RR) and Barmah Forest (BF), and the flaviviruses, Murray Valley encephalitis (MVE) and Kunjin (KUN); dengue (DEN) viruses are introduced intermittently into north Queensland by overseas travelers (reviewed in Mackenzie et al. 1994, Russell 1995, Russell and Dwyer 2000).

Japanese encephalitis (JE) has emerged as a disease threat for northern Queensland following an outbreak in the Torres Strait in 1995 (Hanna et al. 1996b). Public health responses to the outbreak included vaccination of the residents of the Torres Strait and the implementation of a sentinel pig program to detect future incursions of the virus (Hanna et al. 1996a; Shield et

al. 1996). Virus activity subsequently has been detected in all years except 1999, resulting in five human cases, including two deaths (Hanna et al. 1996b, Shield et al. 1996, Hanna et al. 1999, Pyke et al. 2001). The 1998 outbreak was the most geographically widespread, with activity recorded on most islands of the Torres Strait, as well as on Cape York Peninsula on mainland Australia (Hanna et al. 1999). Virus isolation studies in northern Australia and Papua New Guinea (PNG) demonstrated that members of the *Culex sitiens* subgroup, predominantly *Culex annulirostris* Skuse, were the primary vectors of JE in the Australasian region (Ritchie et al. 1997b, Johansen et al. 2000, Johansen et al. 2001). Single isolates of JE from *Ochlerotatus vigilax* (Skuse) and *Culex gelidus* Theobald, indicated that these species may play a secondary role in transmission (Johansen et al. 2001, van den Hurk et al. 2001c). Indeed, *Cx. gelidus* is a recognized vector of JE in Southeast Asia (Vaughn and Hoke 1992).

Attempts to isolate JE on mainland Australia have been undertaken as far south as Kowanyama on Cape York Peninsula (van den Hurk et al. 2001a, 2001b). The objective of the current study was to isolate JE and other medically important viruses from mosquitoes collected from the Gulf Plains region of northwest Queensland, ≈300 km south of Kowanyama. This region marks the southern limit of the sentinel pig

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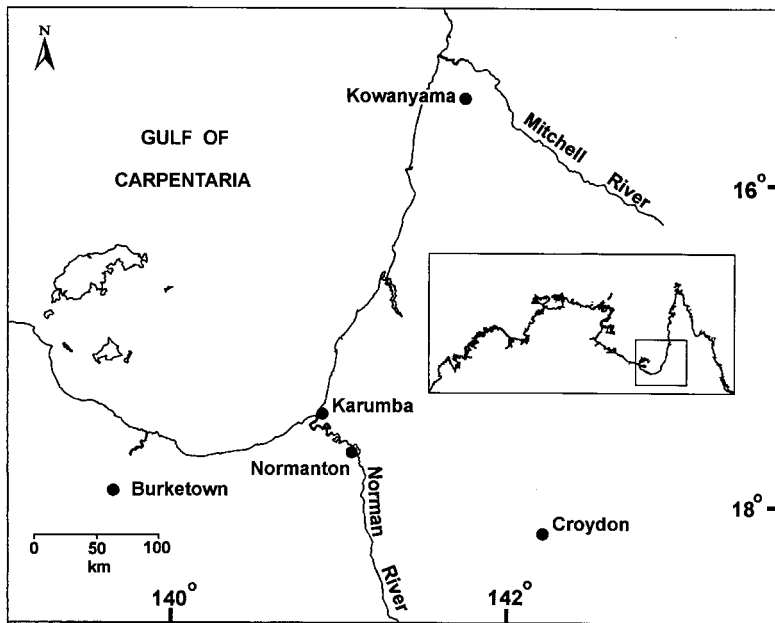


Fig. 1. Study sites within the Gulf Plains region of northwest Queensland, Australia.

program that was established in 1995 and extended into the Cape York Peninsula in 1998 (Shield et al. 1996, Hanna et al. 1999). Previously, Doherty et al. (1963) isolated Getah and Koongal viruses from mosquito collections from Normanton in 1961. Serosurveys detected neutralizing antibodies to MVE in children from Normanton and Burketown in 1957 (Doherty et al. 1959). Due to the remoteness of this location, our studies are the first to be undertaken on arbovirus ecology in the Gulf Plains region since the early 1960s. They form a vital component in assessing risk for the establishment of JE in northern Australia and the role other arboviruses may play in public health.

A PCR-restriction fragment length polymorphism (RFLP) method was used to identify morphologically indistinguishable members of the *Cx. sitiens* subgroup in virus positive pools. This procedure reliably distinguishes *Cx. annulirostris*, *Culex papalis* (Taylor) and *Culex sitiens* Wiedmann, the three polymorphic members of the *Cx. sitiens* subgroup present in northern Australia (Beebe et al. 2002).

Materials and Methods

Study Sites. The Gulf Plains region occupies an area of $\approx 180,000$ km² from the Great Dividing Range in the east to the Gulf of Carpentaria in the west (Fig. 1). Mosquito collections were undertaken within 30 km of each of the townships of Croydon (population, 232), Normanton (population, 1,328) and Karumba (population, 1,043). The Gulf Plains region is the largest tropical savannah in northern Australia, with vegetation characterized by open grasslands, dominated by blue grasses (*Dicanthium* spp.) and Mitchell grass (*As-*

trebla spp.), and woodlands, dominated by eucalypts (*Eucalyptus* spp.) and tea trees (*Melaleuca* spp.) (National Land and Water Resources Audit 2001). Seasonal freshwater floodplains occur over much of the area between Normanton and Karumba, giving way to salt marshes near the coast. Mangroves are restricted to the tidal reaches of rivers and creeks around Karumba.

The climate of this region is monsoonal with $\approx 90\%$ of rain falling in the wet season from December to May. Both Normanton and Karumba have a mean annual rainfall >920 mm, whereas Croydon has a mean annual rainfall of 730 mm. Daily maximum temperatures are generally warm, often exceeding 40°C in the wet season, whereas dry season minimums can fall below 10°C.

Mosquito Collections. Adult mosquitoes were collected using Centers for Disease Control (CDC) light traps (John W. Hock Company, Gainesville, FL) baited with CO₂ (1 kg dry ice) and 1-octen-3-ol (release rate: 4.5 mg/h) operated overnight for 12 h (1800–0600 hours) (Ritchie and Kline 1995). During April 2000, collections were obtained from 48 sites on an east-west transect from Croydon to Karumba. In April 2001, most mosquitoes were used for other experiments (A.F.v.d.H., unpublished data), limiting the number of specimens for virus isolation to four sites within 5 km of Normanton. Mosquitoes were killed on dry ice before being stored in liquid N₂ for transport to the laboratory for storage at -70°C. Mosquitoes were identified morphologically to species on a refrigerated cold table and sorted into pools of ≤ 100 for virus isolation attempts. Due to very large collections, aliquots of ≤ 500 *Anopheles bancroftii* Giles and *Anoph-*

Table 1. Mosquitoes processed for virus isolation from three study sites located in the Gulf Plains region of north Queensland

Mosquito Species ^a	Location			
	Croydon	Normanton		Karumba
	2000	2000	2001	2000
<i>Aedes imprimens</i> (Walker)	1			
<i>Ae. lineatopennis</i> (Ludlow)	8	1,176	2	3,458
<i>Aedeomyia catacticta</i> Knab				4
<i>Anopheles amictus</i> Edwards	54	3,145	65	604
<i>An. amictus</i> (male)	1			
<i>An. annulipes</i> Walker <i>sensu lato</i>	61	428	65	84
<i>An. bancroftii</i> Giles	123	7,627	224	10,369
<i>An. bancroftii</i> (male)		11		
<i>An. hilli</i> Woodhill and Lee		98		60
<i>An. meraukensis</i> Venhuis		622	75	2,335
<i>Coquillettidia crassipes</i> (van der Wulp)		2		1
<i>Culex sitiens</i> subgroup mosquitoes ^b	1,429	21,009	278	56,198
<i>Cx. sitiens</i> subgroup mosquitoes (male)	4			1
<i>Cx. bitaeniorhynchus</i> Giles	140	1,042	36	7,872
<i>Cx. gelidus</i> Theobald		30		6
<i>Cx. pullus</i> Theobald		3		1
<i>Cx. quinquefasciatus</i> Say	1	1		
<i>Cx. squamosus</i> (Taylor)	1			9
<i>Cx. whitmorei</i> (Giles)		110		
<i>Mansonia uniformis</i> (Theobald)		131		72
<i>Ochlerotatus alboannulatus</i> (Macquart)		1		1
<i>Oc. alternans</i> (Westwood)	23	38		5
<i>Oc. normanensis</i> (Taylor)	1,231	29,503	160	1,983
<i>Oc. normanensis</i> (male)	2			
<i>Oc. purpureus</i> (Theobald)	1	42		
<i>Oc. rupestris</i> Dobrotworsky		38		
<i>Oc. tremulus</i> (Theobald)	6	44		
<i>Oc. tremulus</i> (male)		1		4
<i>Oc. vigilax</i> (Skuse)		1		1,362
<i>Oc. vittiger</i> (Skuse)	1			
<i>Uranotaenia albescens</i> Taylor		1		4
Total	3,087	65,104	905	84,433

^a Mosquitoes processed for virus isolation were female unless specified.

^b Consists of *Cx. annulirostris*, *Cx. sitiens* and *Cx. palpalis*.

eles meraukensis Venhuis and $\leq 2,000$ *Ochlerotatus normanensis* (Taylor) per trap were tested.

Virus Isolation. At the Department of Microbiology and Parasitology at the University of Queensland, Brisbane, mosquito pools were processed for virus isolation using methods described previously (Ritchie et al. 1997b, Johansen et al. 2000). Briefly, mosquitoes were homogenized in 5 ml of diluent (M199 tissue culture media containing 2% fetal bovine serum, L-glutamine and antibiotics) using a Spex 8000 mixer/mill (Spex Industries, Edison, NJ), before being centrifuged at $1,560 \times g$ for 25 min. The filtered supernatant then was inoculated onto confluent C6/36 cell monolayers and then incubated at 28°C for 6 d. Cell monolayers were fixed in PBS/acetone before being screened for the presence of viral growth using a panel of monoclonal antibodies in a tissue culture enzyme immunoassay (Broom et al. 1998). Virus isolates were confirmed by reisolation from a different aliquot of the original suspensions. Minimum infection rates (MIRs) for pools of infected mosquitoes were calculated using the formula of Chiang and Reeves (1962).

Identification of cryptic species in virus positive pools. Members of the *Cx. sitiens* subgroup were not identified morphologically to species. Identification of species present in virus positive pools containing spe-

cies of the *Cx. sitiens* subgroup was undertaken using a species diagnostic PCR-restriction fragment length polymorphism (PCR-RFLP) procedure (Beebe et al. 2002). A 10 μ l aliquot of the original unfiltered mosquito homogenate was added to 100 μ l of lysis buffer [1.0 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl (pH 9.0), 0.5 M EDTA and 0.5% sodium dodecyl sulfate (SDS)] before being frozen at -20°C. At the University of Technology, Sydney, mosquito DNA was extracted using the Bresa-clean (Bresatec, Adelaide SA) DNA purification kit following the manufacturer's recommendations for large molecular weight DNA. The purified DNA was reconstituted in 50 μ l of TE containing 5 μ g/ml RNase. The species composition was assessed using 1.0 μ l of the purified DNA and the PCR-RFLP method described in Beebe et al. (2002). These results were confirmed using an allele-specific PCR developed from species-specific sequences in the rDNA ITS1 region (N.W.B., unpublished data).

Results

From 2000, a total of 152,660 mosquitoes were processed for virus isolation, with 3,087 (2.0%), 65,140 (42.7%) and 84,433 (55.3%) collected from Croydon, Normanton and Karumba, respectively (Table 1). Of

Table 2. Virus isolates and minimum infection rates (MIR) of mosquitoes collected from the Gulf Plains region of northern Queensland

Location	Species (morphological ID)	Viruses isolated	PCR-RFLP identification of species ^a	MIR/1000
Normanton	<i>Culex sitiens</i> subgroup	3 MVE	2 <i>Cx. annulirostris</i> / <i>Cx. palpalis</i> , 1 <i>Cx. annulirostris</i>	0.14
	<i>Culex sitiens</i> subgroup	1 KUN	1 <i>Cx. annulirostris</i>	0.05
	<i>Ochlerotatus normanensis</i>	1 EH		0.03
	<i>Anopheles amictus</i>	2 RR		0.65
Karumba	<i>Culex sitiens</i> subgroup	1 MVE	1 <i>Cx. annulirostris</i> / <i>Cx. palpalis</i>	0.02
	<i>Culex sitiens</i> subgroup	1 KUN	1 <i>Cx. annulirostris</i> / <i>Cx. palpalis</i>	0.02
	<i>Culex sitiens</i> subgroup	3 RR	2 <i>Cx. annulirostris</i> / <i>Cx. palpalis</i> , 1 <i>Cx. annulirostris</i>	0.05
	<i>Aedes lineatopennis</i>	1 RR		0.29
	<i>An. bancroftii</i>	1 RR		0.10
	<i>Culex sitiens</i> subgroup	1 SIN	1 <i>Cx. annulirostris</i> / <i>Cx. palpalis</i>	0.02
	<i>An. merakensis</i>	1 SIN		0.43

MVE, Murray Valley encephalitis virus; KUN, Kunjin virus; EH, Edge Hill virus; RR, Ross River virus; SIN, Sindbis virus.

^a Number of virus positive pools of *Cx. sitiens* subgroup mosquitoes identified as either one or more species by PCR-RFLP analysis.

these mosquitoes, *Cx. sitiens* subgroup mosquitoes accounted for 51.5% of mosquitoes processed, followed by *Oc. normanensis* (21.4%), *An. bancroftii* (11.9%), and *Culex bitaeniorhynchus* Giles (5.9%). Only 905 mosquitoes were processed for virus isolation in 2001, of which the abundant species processed were *Cx. sitiens* subgroup mosquitoes (30.7%), *An. bancroftii* (24.8%) and 17.7% *Oc. normanensis*.

A total of 16 isolates of five different viruses were obtained from mosquitoes collected in 2000 (Table 2). Collections from Normanton yielded isolates of MVE, KUN, EH and RR viruses, whereas collections from Karumba yielded isolates of MVE, KUN, RR, and SIN viruses. Identification of the species composition in *Cx. sitiens* subgroup virus positive pools by PCR-RFLP documented that three MVE, one KUN, two RR and one SIN positive pools contained both *Cx. annulirostris* and *Cx. palpalis*. However, the low amplification intensity of the *Cx. palpalis* specific banding profiles indicated that the pools contained mainly *Cx. annulirostris*, with only low numbers of whole (or parts) of *Cx. palpalis*. One positive pool each of MVE, KUN and RR contained exclusively *Cx. annulirostris*. *Cx. sitiens* was not detected in any of the pools. Virus isolates were not obtained from mosquitoes collected from Croydon in 2000 or from Normanton in 2001.

Discussion

Because JE emerged in the Australasian region, numerous entomological studies have been undertaken to incriminate vector species, identify the presence of antigenically related flaviviruses, and assess the potential for establishment of this virus on the Australian mainland. The geographical regions that have been investigated include the Western Province of PNG (Johansen et al. 2000), the Torres Strait (Ritchie et al. 1997b, Johansen et al. 2001, van den Hurk et al. 2001c) and northern and western Cape York Peninsula (van den Hurk et al. 2001b). Our study covers the southern geographical limit of these investigations and despite documented JE activity on Cape York Peninsula in 1998 (Hanna et al. 1999), there was no evidence that JE has become established on the Australian mainland. Additionally, there have been no seroconversions de-

tected in sentinel pigs on mainland Australia since 1998. The failure of JE to become established on mainland Australia has been linked to the presence of alternative blood meal hosts, such as wallabies and cattle, cocirculating flaviviruses, and differences in pig husbandry compared with the Torres Strait (Hanna et al. 1999; van den Hurk et al. 2001a, 2001b).

Despite no isolations of JE from mosquitoes, several other medically important arboviruses were detected in the Gulf Plains region of northern Queensland. Indeed, the sentinel pigs located at Normanton have demonstrated either specific KUN virus or cross-reactive flavivirus seroconversions by early March from 1999–2001 (A. Pyke, Queensland Health Scientific Services, personal communication). Both MVE and KUN viruses are members of the JE virus serological complex that can cause acute encephalitis in humans. Like JE, the vertebrate hosts of both MVE and KUN viruses include ardeid wading birds, of which large populations are present in the swamps and floodplains surrounding Normanton and Karumba. The primary mosquito vector of both these viruses is *Cx. annulirostris*, so the present isolates from this species are consistent with previous surveys in north Queensland (Doherty et al. 1963, van den Hurk et al. 2001a). A single isolate of MVE was obtained from *Cx. palpalis* collected from Western Australia (A. K. Broom et al. unpublished data cited by Mackenzie et al. 1994).

Interestingly, the timing of our 2000 collections coincided with a widespread outbreak of acute encephalitis caused by MVE that occurred in Western Australia and central Australia, resulting in 14 human cases, two of whom died (Broom et al. 2001). The isolates of MVE obtained from mosquitoes collected from the Gulf Plains region in 2000 indicated that enzootic transmission extended into north Queensland, and it is possible that future human cases could occur, as was evidenced at Burketown, 150 km west of Normanton in 1994 (Hanna et al. 1994). As part of the response to a case of MVE in Mount Isa in 2001 (Hills 2001), sentinel chicken flocks were deployed at Mt Isa and Normanton for the 2002 wet season.

Ross River virus has been isolated from over 42 mosquito species in Australia, with the main vectors being *Oc. vigilax* and *Ochlerotatus camptorhynchus*

(Thomson) in coastal areas and *Cx. annulirostris* in inland areas (reviewed in Russell 1995; Harley et al. 2001; Russell 2002). The isolation of RR from *Cx. annulirostris* is consistent with its role as a major vector of this virus in Queensland (Doherty et al. 1979; Ritchie et al. 1997a; Harley et al. 2000). Collections from Western Australia have yielded isolates of RR from *Cx. palpalis* (Lindsay et al. 1992). The isolates from *Anopheles amictus* Edwards and *An. bancroftii* also are consistent with the results of previous studies (Doherty et al. 1979; M. D. Lindsay, personal communication cited by Russell 2002). However, the isolate of RR from *Aedes lineatopennis* (Ludlow) reported herein is the first reported for this species, although its role in transmission has not been determined.

Ross River virus, the causative agent of epidemic polyarthritis, is clinically the most widespread of the Australian arboviruses, with >47,000 cases of RR virus infection recorded for the period 1991–2000 (Russell 2002). Infection causes a polyarticular disease, which can be accompanied by fever and rash (reviewed in Harley et al. 2001, Russell 2002). The vertebrate hosts of RR are thought to be macropods, such as wallabies and kangaroos (Russell 1995, Harley et al. 2001, Russell 2002), although flying foxes recently have been implicated in transmission (Ryan et al. 1997, Harley et al. 2000). In the Gulf Plains region, large populations of marsupials and flying foxes, coupled with high mosquito numbers and preliminary evidence that *Cx. annulirostris* preferentially feeds on marsupials (A.F. van den Hurk and A. Griffith, unpublished data) could contribute to epizootic transmission of RR to humans. Indeed, for the period 1996–2001, 21 cases of epidemic polyarthritis were reported from the Gulf of Carpentaria Statistical Area (Tropical Public Health Unit Network, unpublished data).

Both EH and SIN cause mild or inapparent infection in humans (Doherty et al. 1969, Boughton et al. 1984; Hawkes et al. 1985, Aaskov et al. 1993). Marsupials are believed to be the main vertebrate hosts of EH (Doherty et al. 1964), whereas birds are the primary hosts of SIN (Doherty 1972; Mackenzie et al. 1994). EH was first isolated from *Oc. vigilax* and *Cx. annulirostris* from north Queensland (Doherty et al. 1963) and since has been isolated from several other species including *Oc. normanensis* (Doherty et al. 1979). SIN is considered to be the most common virus isolated from mosquitoes in Australia (Mackenzie et al. 1994) and previously has been isolated from *An. meraukensis* in Western Australia (A. Broom, personal communication cited by Russell 1995).

The *Cx. sitiens* subgroup consists of eight morphological species (Lee et al. 1989), of which *Cx. annulirostris* is considered to be the most important vector of arboviruses in Australia (Kay and Standfast 1987). However, *Cx. annulirostris*, *Cx. sitiens* and *Cx. palpalis* share overlapping morphology, which can make accurate identification difficult. The Gulf Plains region contains sympatric populations of all three species, increasing the problems associated with accurate identification. This can be compounded even further

when diagnostic characters have been damaged or are missing due to rubbing or other damage. We used a diagnostic PCR-RFLP technique to confirm the species composition in virus positive pools of *Cx. sitiens* subgroup mosquitoes. Similar molecular techniques have identified the species present in West Nile virus positive pools of *Culex* collected from New York and New Jersey (Nasci et al. 2001). Further development of this procedure may be required to quantify the amount of DNA of each species present in a pool of mixed mosquitoes, as recently has been demonstrated with La Crosse virus positive pools of *Aedes albopictus* (Skuse) and *Ochlerotatus triseriatus* (Say) collected from the southeastern United States (Gerhardt et al. 2001). Future virus isolation studies in areas where members of the *Cx. sitiens* subgroup occur sympatrically could employ this PCR-RFLP diagnostic procedure to confirm the morphological identification of species in virus positive pools.

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