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Isolation of Arboviruses from Insects Collected at Beatrice Hill, Northern Territory of Australia, 1974–1976

H. A. Standfast[A], *A. L. Dyce*[B], *T. D. St George*[A], *M. J. Muller*[A], *R. L. Doherty*[C], *J. G. Carley*[C] *and Cheryl Filippich*[C]

[A] Division of Tropical Animal Science, CSIRO, Long Pocket Laboratories, Private Bag No. 3, P.O., Indooroopilly, Qld 4068.
[B] Division of Tropical Animal Science, CSIRO, McMaster Laboratory, Private Bag No. 1, P.O., Glebe, N.S.W. 2037.
[C] Queensland Institute of Medical Research, Bramston Terrace, Herston, Qld 4006.

Abstract

Between October 1974 and May 1976, 57 596 mosquitoes, 169 957 *Culicoides*, 5923 *Lasiohelea* and 1043 phlebotomines were collected for virus isolation at Beatrice Hill (lat. 12°39′S.,long. 131°20′E.) in the Northern Territory of Australia. A total of 94 viruses belonging to 22 different serological groupings was isolated. The following species of insect yielded viruses which were identified and those viruses marked with an asterisk represent a new record of insect host:

Culex annulirostris:	Ross River, Kokobera, Barmah Forest, Corriparta, Eubenangee*, Wongorr;
Anopheles amictus:	Mapputta*;
An bancroftii:	bovine ephemeral fever*;
An farauti:	Eubenangee*;
An annulipes:	Mapputta;
Culicoides marksi:	Barmah Forest*, Belmont, Eubenangee*, Wallal, Warrego, Leanyer*, Parker's Farm*, Humpty Doo*;
C. peregrinus:	Beatrice Hill*;
C. oxystoma:	Bunyip Creek*, Marrakai*;
C. pallidothorax:	Wongorr*;
C. histrio:	Thimiri*;
Lasiohelea spp.:	Humpty Doo*.

Pools of mixed species of *Culicoides* yielded bluetongue, Belmont, CSIRO Village, Warrego and Facey's Paddock viruses. Filter-passing agents not yet identified, were isolated from *Cx annulirostris* and *An bancroftii*.

As well as providing new locality records for all but one of the 22 viruses isolated, the study yielded five new viruses (bluetongue serotype 20, CSIRO Village, Marrakai, Beatrice Hill and Humpty Doo viruses) and a new record for Thimiri virus which had not been recorded previously in Australia nor had it been isolated from an arthropod. Nine of the viruses isolated occur in more than one family of Diptera.

Introduction

In Australia, bovine ephemeral fever has been reported since 1936. Epidemics of the disease occurred in 1936–37, 1955–56, 1967–68, 1970–71 and 1972–74 (St George *et al.* 1977). The indirect evidence accumulated in Australia indicated that bovine ephemeral fever was spread by an insect vector (Seddon 1938; Mackerras *et al.* 1940; Murray 1970; Newton and Wheatley 1970). However, direct evidence of insect involvement was lacking in spite of efforts applied to isolating viruses from potential vectors collected during the 1967–68, 1970–71 and 1972–74 outbreaks (Doherty *et al.* 1972; H. A. Standfast and A. L. Dyce, unpublished data). Consequently the procedure of carrying out insect collections only where and when an epidemic occurred was revised to that of collecting continuously

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in an area where there was a high probability that infection of cattle with bovine ephemeral fever would occur. Beatrice Hill in the Northern Territory was selected because sentinel herd serology (St George *et al.* 1977) indicated that bovine ephemeral fever would be encountered if collections were made to cover two wet seasons. Laboratory facilities were available on site, and importantly, access by road was possible throughout the year.

Materials and Methods

Field Studies Site

The site was on the subcoastal plain, 80 km south-east of Darwin at Beatrice Hill (lat. 12°39′S.,long. 131°20′E.). The period of observation and collection extended for 81 weeks from October 1974 to May 1976. This period included two wet seasons and the intervening dry season. The area contained a rich avian fauna including both resident and migratory waders and a large population of marsupials with the agile wallaby (*Macropus agilis agilis*) the dominant species. Other vertebrates included several species of rodents, bats, small marsupials, and a large population of both feral and domesticated water buffalo (*Bubalus bubalis*) and cattle and feral pigs. A description of the area including sections on climate, geology, geomorphology, soils and vegetation is given by Story (1969).

Insect Collections

Truck traps, light traps and animal (buffalo) bait collections were employed using techniques described by Dyce *et al.* (1972).

Virus Isolation

The insects were sorted at the field site while still alive. In most cases they were identified to species. When the numbers of insects in a collection were too large to process in the time available at least 1000 insects were identified and the remainder were bulked according to genus. Insects containing blood were excluded from pools for virus isolation and submitted for blood meal identification (Muller *et al.* 1981). The species pools of insects were stored and transported in liquid nitrogen to the Long Pocket Laboratories for virus isolation.

On a day that virus isolation was to be attempted sufficient groups of insect collections were thawed on a refrigerated table and amalgamated according to species, date and method of collection to form groups of up to 50 for mosquitoes and 200 for biting midges. Less common species were processed in smaller groupings. To each pool of insects was added 1 ml of chilled rabbit saline [10% (w/v) sterile rabbit serum in 0·05 M phosphate-buffered saline of pH 7·2 which contained 1·6 mg of streptomycin sulfate and 1000 units of penicillin G per millilitre] and ground in a 5-ml tissue grinder (Catalogue 7927/966, Corning Jobling, Melbourne) in an ice-bath. The ground insect material was centrifuged at 2000 *g* for 30 min at 4°C.

A 0·02 ml aliquot of supernatant from each insect pool was inoculated intracerebrally into each of a litter of six mice 1–3 days old. The brains of mice which died or became sick 48 h or more after inoculation were passaged intracerebrally to further litters of mice until a regular death or paralysis pattern was established. The brains of survivors were harvested at 14 days and passaged in suckling mice before being discarded as negative.

The technique of inoculating part of the supernatant to cell cultures (baby hamster kidney BHK21) was introduced in April 1975, 5 months after the first isolation attempts in suckling mice. Pools were then processed by passaging three times in BHK21 cells in parallel with mice. Pools which had been processed prior to April 1975 in mice, were recultured in cell cultures, although these insect pools had been frozen and thawed once or twice. The tissue culture monolayers were prepared as described by St George *et al.* (1978*a*).

The viruses were screened in a neutralization test using suckling mice and an antiserum prepared against bovine ephemeral fever virus. Those viruses which were negative in this test were tested at The Queensland Institute of Medical Research (QIMR) by standard methods (Doherty *et al.* 1979) against antisera to all known Australian arboviruses (Doherty 1972, 1977). Viruses which did not react with these antisera were submitted to the WHO International Reference Centre, Yale Arbovirus Research Unit.

Results

Thirty isolates of 10 viruses were recovered from the 57 596 mosquitoes processed. Details of the species recorded, number of individual species processed and number of isolations of viruses from each species are summarized in Table 1. *Culex annulirostris* was the most productive of the mosquitoes with 24 isolations of eight viruses from 34 240 insects. Sixty-four isolates of 16 viruses were made from 175 880 biting midges (Table 2). *Culicoides marksi* was the most productive of the *Culicoides* with 55 isolates of seven viruses coming from this species. The viruses and the arthropod species from which they were isolated are listed in Table 3, the isolates are listed in Table 4.

Table 1. Species of Culicidae collected at Beatrice Hill which were submitted for virus isolation[A]

Species	No. of insects	No. of pools	No. of isolations
Aedeomyia catasticta Knab	307	46	0
Aedes (*Neomelaniconion*) *lineatopennis* (Ludlow)	1052	35	0
Ae (*Ochlerotatus*) *normanensis* (Taylor)	111	24	0
Ae (*Och*) *vigilax* (Skuse)	112	17	0
Ae (*Verrallina*) *funereus* (Theobald)	650	12	0
Ae (*Ver*) *reesi* King & Hoogstraal	393	22	0
Anopheles (*Anopheles*) *bancroftii* Giles	5747	113	2
An (*Cellia*) *amictus* Edwards	1810	63	2
An (*Cel*) *annulipes* Walker	2740	83	1
An (*Cel*) *farauti* Laveran	304	13	1
An (*Cel*) *meraukensis* Venhuis	270	27	0
Coquillettidia (*Coquillettidia*) *crassipes* (Van der Wulp)	182	8	0
Cq (*Coq*) *xanthogaster* (Edwards)	1565	66	0
Culex (*Culex*) *annulirostris* Skuse	34 240	493	24
Cx (*Cux*) *bitaeniorhynchus* Giles	799	44	0
Cx (*Cux*) *quinquefasciatus* Say	239	27	0
Cx (*Cux*) *sitiens* Wiedemann	756	49	0
Cx (*Culiciomyia*) *pullus* Theobald	897	37	0
Cx (*Lophoceraomyia*) spp.	301	37	0
Mansonia (*Mansonioides*) *uniformis* (Theobald)	2191	81	0
Mimomyia (*Etorleptiomyia*) *elegans* (Taylor)	687	59	0
Mi (*Mimomyia*) *chamberlaini metallica* (Leicester)	358	48	0
Uranotaenia (*Uranotaenia*) *albescens* Taylor	793	35	0
Ur (*Ura*) *nivipes* (Theobald)	218	37	0
Culicidae processed as pools of more than one species	1074	27	0
Total	57 596	1503	30

[A] Small collections (less than 100) of the following species were processed: *Aedes* (*Finlaya*) *kochi* (Doenitz); *Ae* (*Fin*) *notoscriptus* (Skuse); *Ae* (*Macleaya*) sp. No. 76 Marks; *Ae* (*Mucidus*) *alternans* (Westwood); *Anopheles* (*Anopheles*) *powelli* Lee; *An* (*Cellia*) *novaguinensis* Venhuis; *Culex* (*Culex*) *australicus* Dobrotworsky & Drummond; *Cx* (*Cux*) *squamosus* (Taylor); *Cx* (*Cux*) *starckeae* Stone & Knight; *Cx* (*Cux*) *vicinus* (Taylor) and *Cx* (*Lutzia*) *halifaxii* Theobald.

Virus Isolations

Togaviridae

Alphavirus. Three isolations of Ross River virus (CSIRO 6, 17 and 41) were made in both the mouse and tissue-culture systems with cytopathic effect (CPE) appearing at 3 days in the latter; all isolations came from *Cx annulirostris.** One isolate of Barmah Forest virus, (CSIRO 30) was made from *Cx annulirostris* processed in suckling mice and three isolates (CSIRO 83, 104 and 108) came from *C. marksi.** One strain (CSIRO 83) was

*Mosquito generic and subgeneric names are abbreviated according to Reinert (1975). However, *C.* = *Culicoides.*

isolated in mice and not in tissue culture while the other two (CSIRO 104 and 108) were isolated in tissue culture and not in mice.

Flavivirus. Three isolates of Kokobera virus (CSIRO 7, 31 and 37) were made in suckling mice but not in the parallel tissue culture system; all were from *Cx annulirostris.*

Table 2. Species of *Culicoides*, *Lasiohelea* and *Phlebotomus* collected at Beatrice Hill which were submitted for virus isolation

Species	No. of insects	No. of pools	No. of isolations
Culicoides austropalpalis Lee & Reye[A]	7480	74	0
C. (*Avaritia*) *brevitarsis* Kieffer	5490	76	0
C. bundyensis Lee & Reye[A]	3391	41	0
C. dycei Lee & Reye[A]	58	5	0
C. (*Haemophoructus*) sp.	102	11	0
C. (*Meijerehelea*) *histrio* Johannsen	1005	41	1
C. marksi Lee & Reye[A]	72 268	20	55
C. pallidothorax Lee & Reye[A]	66	20	1
C. (*Culicoides*) *peregrinus* Kieffer	15 549	116	1
C. oxystoma Kieffer[A]	44 998	271	2
Culicoides processed as pools of more than one species	19 550	140	4
Total	169 957	1213	64
Lasiohelea spp. undetermined	5923	71	1
Phlebotomines spp. undetermined[B]	1043	40	0

[A] These species have not been allocated to a subgenus.
[B] Largely *Sergentomyia* (*Parrotomyia*) *queenslandi* (Hill).

Bunyaviridae

Bunyavirus. In the Simbu antigenic group Thimiri virus (CSIRO 1) was isolated in mice from *C. histrio.* No attempt was made to isolate the virus in tissue culture. In the same group Facey's Paddock virus (CSIRO 10) was isolated in mice from a mixed pool of *Culicoides.*

Bunyavirus-like. Three isolates of Mapputta virus were isolated. One, CSIRO 78 was isolated from *An amictus* in both mice and tissue culture while the others, CSIRO 84 from *An annulipes* and CSIRO 86 from *An amictus* were isolated only in mice. There was a single isolate of Belmont virus (CSIRO 38) in mice, from a pool of *C. marksi.*

Rhabdoviridae

One isolate of bovine ephemeral fever virus (CSIRO 42) was made from *An bancroftii.* The virus was detected in the second mouse brain passage. It was not isolated in tissue culture.

Reoviridae

Orbivirus: Bluetongue antigenic group. One isolate of bluetongue (CSIRO 19) was made from a pool of 214 *Culicoides* of mixed species processed in tissue culture. No isolation was made in mice.

Orbivirus: Eubenangee antigenic group. Six strains were isolated in this group. CSIRO 20 was isolated in tissue culture but not in mice and came from *C. marksi.* CSIRO 23 from *An farauti* was isolated in both mice and tissue culture. The remaining isolates (CSIRO

Table 3. Species of insects collected at Beatrice Hill from which arboviruses were isolated

Species	Ross River	Kokobera	Barmah Forest	Thimiri	Facey's Paddock	Mapputta	Belmont	Bovine ephemeral fever	Bluetongue	Corriparta	Eubenangee	Bunyip Creek	Marrakai	CSIRO Village	Wallal	Warrego	Leanyer	Parker's Farm	Wongoor	CSIRO 25	CSIRO 79, 80	Unidentified	Total
Biting Midges																							
Culicoides marksi	—	—	3	—	—	—	1	—	—	—	1	—	—	—	27	18	2	2	—	—	1	—	55
C. peregrinus	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	1
C. oxystoma	—	—	—	—	—	—	—	—	—	—	—	1	1	—	—	—	—	—	—	—	—	—	2
C. pallidothorax	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1
C. histrio	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Culicoides spp.	—	—	—	—	1	—	—	—	1	—	—	—	—	1	—	1	—	—	—	—	—	—	4
Lasiohelea spp.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	1
Mosquitoes																							
Culex annulirostris	3	3	1	—	—	—	—	—	—	3	4	—	—	—	—	1	—	—	7	—	—	1	23
Anopheles amictus	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2
An bancroftii	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2
An farauti	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	1
An annulipes	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Total	3	3	4	1	1	3	1	1	1	3	6	1	1	1	27	20	2	2	8	1	2	2	94

32, 33, 34 and 36) were from *Cx annulirostris* and were all isolated in mice but not in tissue culture.

Orbivirus: Palyam antigenic group. Bunyip Creek virus (CSIRO 87) was isolated from *C. oxystoma*,* Marrakai virus (CSIRO 82) from a pool containing 148 *C. oxystoma* and 24 *C. peregrinus* while CSIRO Village virus (CSIRO 11) was isolated from a pool of *Culicoides* spp. All three viruses were isolated in both tissue culture and mice.

Orbivirus: Corriparta antigenic group. Three isolates of Corriparta virus (CSIRO 76, 109 and 134) were made from *Cx annulirostris*. The isolations were made in tissue culture. No isolations were made in mice.

Table 4. Strains of virus isolated from insects collected at Beatrice Hill

Family	Genus	Antigenic group	Virus	CSIRO isolate No.
Togaviridae	*Alphavirus*	A	Ross River	6, 17, 41
	Alphavirus	A	Barmah Forest	30, 83, 104, 108
	Flavivirus	B	Kokobera	7, 31, 37
Bunyaviridae	*Bunyavirus*	Simbu	Thimiri	1
	Bunyavirus	Simbu	Facey's Paddock	10
	Bunyavirus-like	Mapputta	Mapputta	78, 84, 86
	Bunyavirus-like	—	Belmont	38
Rhabdoviridae	—	—	Bovine ephemeral fever	42
Reoviridae	*Orbivirus*	Bluetongue	Bluetongue	19
		Eubenangee	Eubenangee	20, 23, 32, 33, 34, 36
		Palyam	Bunyip Creek	87
		Palyam	Marrakai	82
		Palyam	CSIRO Village	11
		Corriparta	Corriparta	76, 109, 134
		Wallal	Wallal	21, 22, 24, 44, 47, 56, 66, 67, 68, 70, 71, 72, 74, 85, 88, 93, 94, 95, 96, 97, 98, 99, 102, 103, 105, 106, 107
Reoviridae	*Orbivirus*	Warrego	Warrego	8, 9, 12, 13, 18, 40, 46, 48, 52, 60, 61, 63, 64, 65, 69, 73, 77, 81, 100, 260
Ungrouped	Ungrouped	Ungrouped	Leanyer	2, 62
Ungrouped	Ungrouped	Ungrouped	Parker's Farm	57, 59
Ungrouped	Ungrouped	Ungrouped	Wongorr	26, 27, 29, 35, 39, 43, 45, 131
Ungrouped	Ungrouped	Ungrouped	New	25
Ungrouped	Ungrouped	Ungrouped	New	79, 80
Unidentified	Unidentified	Unidentified	Unidentified	51, 75

Orbivirus: Wallal antigenic group. All 27 isolates came from *C. marksi*. Sixteen isolates (CSIRO 21, 22, 24, 47, 56, 66, 67, 68, 70, 71, 72, 74, 85, 88, 93, and 105) were made in both mice and tissue culture while the remaining isolates (CSIRO 44, 94, 95, 96, 97, 98, 99, 102, 103, 106 and 107) were not detected in the mouse system.

Orbivirus: Warrego antigenic group. Of the 20 isolates of Warrego virus, 19 were from *C. marksi* and one (CSIRO 18) from *Cx annulirostris*. Fourteen strains (CSIRO 12, 18, 46, 48, 52, 60, 61, 63, 64, 65, 69, 73, 77 and 81) were isolated in both mice and tissue

*A recent revision of the group indicates that the species previously referred to as *C. schultzei* is *C. oxystoma* Kieffer (W. W. Wirth, personal communication).

culture while three strains (CSIRO 40, 100 and 260) were isolated only in tissue culture, with no virus detected in the mouse system. For three others (CSIRO 8, 9 and 13) no isolation attempt was made in tissue culture.

Table 5. Characterization of isolates CSIRO 25 and CSIRO 79 prototype strains of Beatrice Hill and Humpty Doo viruses

Source and details of collection	CSIRO 25 (Beatrice Hill)[A]	CSIRO 79 (Humpty Doo)[B]
Re-isolation	+	−
Titres[C] of virus pools:		
In mice:		
Intracerebral infant	7·0	6·2, 7·7
Intraperitoneal infant	<6·0	<4·5, <3·5
Intracerebral weaned	<4·5	<4·5, <3·5
In cell culture:		
Plaque assay on PS-EK cells	<2·0	n.t.
Sucrose–acetone extract of infected mouse brain:		
Haemagglutination of gander cells[D]	—	—
Fixation of complement[E]	128/16	128/16
Titre[F] after filtration through Millipore membranes of average pore diameter:		
650 nm	6·0	5·6
300 nm	6·0	5·5
220 nm	4·3	5·0
100 nm	Trace at 2·5	<2·0
50 nm	<2·5	<2·0
Sensitivity to sodium deoxycholate[G]:		
1/500	3·0/5·5	<2·0/5·0
1/1000	2·0/5·5	<2·0/5·0
Sensitivity to ether[G]:		
1 h at 4°C	n.t.	<2·0/5·5
8 h at 4°C	<2·0/4·1	<2·0/5·6
Sensitivity to 5-iododeoxyuridine	5·6/5·7	n.t.
Growth in inoculated *Aedes aegypti*:		
7 days incubation[H]	1·3/3·3	n.t.

[A] Isolated from *Culicoides peregrinus* 173, light trap, 9 April 1975.
[B] Isolated from *Lasiohelea* spp. 141, truck trap, 15 May 1975.
[C] Titres as $\log_{10}LD_{50}$/g (inoculation of mice) on fifth passage in infant mice. Cell culture titre as $\log_{10}ID_{50}$/ml. n.t., not tested.
[D] Studies at Yale Arbovirus Research Unit at pH 5·75–7·2 and at 1·5 and 0·4 M.
[E] Reciprocal serum titre/reciprocal antigen titre for homologous system.
[F] Titres as $\log_{10}LD_{50}$/0·015 ml (i.c. in infant mice).
[G] Titres as $\log_{10}LD_{50}$/0·015 ml (i.c. in infant mice). Test titre/control titre.
[H] Titres as $\log_{10}LD_{50}$/0·015 ml (i.c. in infant mice). 0 time/7 day.

Ungrouped

Two isolates of Leanyer virus (CSIRO 2 and 62) were made from *C. marksi* in both the mouse and tissue culture systems.

Parker's Farm virus was isolated twice (CSIRO 57 and 59) from *C. marksi*, each in both the mouse and tissue culture systems.

Eight isolates of Wongorr virus were made. One (CSIRO 29) originated from *C. pallidothorax* while the other seven (CSIRO 26, 27, 35, 39, 43, 45 and 131) came from *Cx annulirostris*. Four isolates (CSIRO 26, 27, 29 and 31) were made only in mice, no

virus was detected in tissue culture. Three isolates (CSIRO 39, 43 and 45) were made only in tissue culture, no virus was detected in mice. One isolate (CSIRO 35) was detected in both systems from a pool of *C. marksi*.

Three isolates, CSIRO 25 from *C. peregrinus*, CSIRO 79 from *Lasiohelea* spp. and CSIRO 80 from *C. marksi* were made only in mice. CSIRO 79 was indistinguishable from CSIRO 80 and all three did not react with antisera held at QIMR. Prototype strains of CSIRO 25 and CSIRO 79 were freeze-dried and submitted to the WHO International Reference Centre, Yale Arbovirus Research Unit, for comparison with the world range of arboviruses. At Yale, CSIRO 25 and CSIRO 79 antigens reacted positively with their homologous ascitic fluids and were negative at 1 : 4 with 39 different polyvalent or grouping fluids. A detailed list has been lodged as accessory publication with the Subcommittee on Evaluation of Arthropod-borne Status of the American Committee on Arthropod-borne Viruses, P.O. Box 3333, New Haven, Connecticut, 06510, U.S.A.

Thin-section electron microscopy of CSIRO 79 virus at Yale revealed particles with Rhabdovirus-like morphology and morphogenesis.

CSIRO 25 and CSIRO 79 are considered to be new viruses and the names 'Beatrice Hill' (CSIRO 25) and 'Humpty Doo' (CSIRO 79) are suggested. The properties of the two new viruses are summarized in Table 5.

Table 6. Isolations of virus from insect material processed in parallel in suckling mice and BHK21 tissue culture

Virus	Mice	BHK21 tissue culture	Insect species	Virus	Mice	BHK21 tissue culture	Insect species
Ross River	3	3	*Culex annulirostris*	CSIRO Village	1	1	*Culicoides* mixed spp.
Barmah Forest	1	0	*Cx annulirostris*				
Barmah Forest	1	0	*Culicoides marksi*	Marrakai	1	1	*C. oxystoma* + *C. peregrinus*
Barmah Forest	0	2	*C. marksi*				
Kokobera	3	0	*Cx annulirostris*	Bunyip Creek	1	1	*C. oxystoma*
Mapputta	1	1	*Anopheles amictus*	Wallal	16	16	*C. marksi*
Mapputta	2	0	*An amictus*, *An annulipes*	Wallal	0	11	*C. marksi*
				Warrego	13	13	*C. marksi*
Belmont	1	0	*C. marksi*	Warrego	0	3	*C. marksi*
Borine ephemeral fever	1	0	*An bancroftii*	Warrego	1	1	*Cx annulirostris*
				Leanyer	2	2	*C. marksi*
Bluetongue	0	1	*C.* mixed spp.	Parker's Farm	2	0	*C. marksi*
Corriparta	0	3	*Cx annulirostris*	Wongorr	3	0	*Cx annulirostris*
Eubenangee	0	1	*C. marksi*	Wongorr	1	1	*Cx annulirostris*
Eubenangee	1	1	*An farauti*	Wongorr	0	3	*Cx annulirostris*
Eubenangee	2	0	*Cx annulirostris*	Wongorr	1	0	*C. pallidothorax*

Unidentified Viruses

Two isolates remain unidentified. One of these, CSIRO 51, was isolated from *An bancroftii* in tissue culture, the other, CSIRO 75, from *Cx annulirostris* in mice. Both have passed a 220 nm filter but neither have been grown sufficiently well to establish an homologous complement fixation or neutralizing system.

Virus Isolation Systems

Details of the isolations made in parallel in the mouse and tissue culture systems are summarized in Table 6.

In the suckling mouse system 85% of the isolations were made in the first passage in mouse brain and 15% in the second passage, while in the BHK tissue culture system 48% of isolations were made in the first passage in cells, 44% in the second passage and 8% in the third.

Discussion

Alphavirus

The isolation of Ross River virus from *Cx annulirostris* collected at Beatrice Hill extends the known range of the virus to the Northern Territory. It has been recovered previously from a range of mosquito species: *An amictus*, *Aedes normanensis*, *Ae theobaldi*, *Ae vigilax*, *Coquillettidia linealis*, *Cx annulirostris* and *Mansonia uniformis* (Kay *et al.* 1982). The vertebrate hosts from which the virus has been isolated, man (Doherty *et al.* 1971*a*), horse (Pascoe *et al.* 1978) and agile wallaby (Doherty *et al.* 1971*b*), were well represented in the project area.

Three isolates of Barmah Forest virus were made from *C. marksi* and one from *Cx annulirostris*. Previously the virus has been isolated from *Cx annulirostris* collected in the Murray Valley (Marshall *et al.* 1982) and from *Cx annulirostris* and *Ae normanensis* (Doherty *et al.* 1979) collected in south-western Queensland, so the isolations from the Northern Territory represent a considerable extension in the known range of this virus. The virus has not previously been isolated from a *Culicoides*.

Flavivirus

The isolation of Kokobera virus from *Cx annulirostris* was not unexpected. This virus had previously been isolated from *Cx annulirostris* collected in similar environments in north Queensland (Doherty *et al.* 1963, 1968, 1971*b*) and in Western Australia (Liehne *et al.* 1981).

Bunyavirus

Simbu serogroup

Thimiri virus was isolated from *C. histrio*. Standfast and Dyce (1982) described the isolation and concluded that the virus was circulating in a *Culicoides*-bird cycle. The only previous isolations of Thimiri virus were from birds in India and Egypt (Carey *et al.* 1971) but the vertebrate host in Australia is unknown. A serological survey of 582 cattle, buffalo, deer, goats, horses and sheep showed no neutralizing antibodies (St George *et al.* 1979), but neutralizing antibodies were detected in the little whimbrel (*Numerius minutus*) collected in 1976 at Beatrice Hill (D. H. Cybinski, personal communication). As these birds are migrants which do not breed in Australia it is not possible to say if the virus was transmitted locally.

A second Simbu group virus, Facey's Paddock, was isolated from a pool of *Culicoides* which contained several species. The four isolates of this virus previously reported by Doherty *et al.* (1979) were all from mosquitoes collected near Charleville in south-western Queensland. Thus the isolation from Beatrice Hill considerably broadens the geographical and potential vector diversity.

Rhabdoviridae

Bovine ephemeral fever virus (CSIRO 42), was isolated from *An bancroftii* (Standfast *et al.* 1976). Previously the virus had been isolated from a mixed pool of 14 mosquitoes [*Culex* (*Lophoceraomyia*) spp. 4, *Uranotaenia* (*Uranotaenia*) *nivipes* 8, *Ur* (*Ura*) *albescens* 1, and *Ae* (*Verrallina*) *carmenti* 1] collected near Rockhampton, Qld (Standfast *et al.* 1976) and from a mixed pool of 4000 *Culicoides* collected at Lake Nakuru, Kenya (Davies and Walker 1974). In the laboratory the virus had been shown to grow in *Cx annulirostris* fed

on a blood virus mixture (Kay *et al.* 1975; H. A. Standfast, unpublished data) and in *C. brevitarsis* and *C. marksi* fed on sugar virus mixtures (H. A. Standfast, unpublished data).

The isolation of bovine ephemeral fever virus from *An bancroftii* contributes to the understanding of the natural history of the virus. It confirms that in Australia the virus exists in more than one species of mosquito. *An bancroftii* is abundant at the end of the monsoon season in areas of northern Australia with rainfall in excess of 750 mm per annum (H. A. Standfast and A. L. Dyce, unpublished data), but this species cannot play a significant role in the spread of the virus in south-eastern Australia.

Reoviridae

Orbivirus

The greatest number of isolates (60) were of the genus *Orbivirus*. Six serological groups were represented: bluetongue, Eubenangee, Palyam, Corriparta, Wallal and Warrego. Of the 60 isolates 51 came from *Culicoides* and of these, 46 came from *C. marksi*.

Bluetongue Serogroup

One isolation (CSIRO 19) was identified as bluetongue virus at the Yale Arbovirus Research Unit (St George *et al.* 1978*b*). It was later typed at the World Reference Laboratory, Onderstepoort, South Africa, as serotype 20, a new serotype of bluetongue virus (Snowdon 1979). This was the first indication that viruses of the bluetongue serogroup were present in Australia and resulted in numerous studies to map the distribution of the virus and identify its vectors (Standfast *et al.* 1979; Dyce and Standfast 1979; St George *et al.* 1979; St George 1982). Despite extensive sampling of both insects and cattle in northern and eastern Australia no further isolates of serotype 20 have been made although numerous isolations of four other serotypes were made (St George *et al.* 1980; T. D. St George, unpublished data).

Eubenangee Serogroup

The prototype Eubenangee isolate (IN 1074) came from a pool of 31 mosquitoes which contained 11 species including *An farauti*, but the pool did not contain any *Cx annulirostris* (Doherty *et al.* 1968). The isolation was made in mice. At Beatrice Hill one isolate came from *An farauti* and was made in both tissue culture and mice. Four isolates came from *Cx annulirostris* and were made only in mice while the sixth isolate came from *C. marksi* and was made only in tissue culture. These isolates which behaved differently in the isolation systems used and which came from different genera of Culicidae and different families of Diptera warrant further study and detailed comparison with each other and the prototype strain.

Palyam Serogroup

The three Palyam group viruses isolated have been shown by Cybinski and St George (1982) to be three different viruses (Bunyip Creek, Marrakai and CSIRO Village). Two of the isolates came from *C. oxystoma* or *C. peregrinus*, insects very closely associated with the water buffalo and known in Australia only from a very restricted area in the northern part of the Northern Territory and Western Australia, especially where water buffalo are found (A. L. Dyce, H. A. Standfast and M. J. Muller, unpublished data).

Corriparta Serogroup

This virus has previously been isolated from Mitchell River in Cape York Peninsula in north Queensland (Doherty *et al.* 1963) and from the Ord River region in Western Australia (Liehne *et al.* 1976, 1981). Its presence in the Northern Territory in the same species of mosquito (*Cx annulirostris*) is not unexpected.

Wallal Serogroup

The first isolates of Wallal virus came from *C. marksi* and *C. dycei* collected at Charleville in south-western Queensland (Doherty *et al.* 1973). The related Mudjinbarry virus was isolated by Doherty *et al.* (1978) from *C. marksi* collected at Mudginberri Station 170 km east of Beatrice Hill. The Beatrice Hill isolates all came from *C. marksi.* They were all related to Wallal virus by complement fixation. The isolates were studied by Gorman *et al.* (1977) who attempted to characterize them by analysis of the genome segments of the virus and reported extensive heterogeneity within this group of serologically closely related viruses (Gorman *et al.* 1977, 1983). The vertebrate hosts for the Wallal viruses are thought to be marsupials (Doherty *et al.* 1973). At Beatrice Hill the agile wallaby was one of the most abundant large mammals in the project area and is one of the most important hosts of *C. marksi*, the source of the isolates.

Warrego Serogroup

The 20 isolates were indistinguishable from Warrego virus by complement fixation but distinguishable from Mitchell River virus, the other Warrego group virus used in the test. Detailed comparison of the 20 isolates by serum neutralization tests have not been made. Neutralizing antibody to Warrego virus has been recorded in 7 out of 62 cattle and 20 out of 51 macropods by Doherty *et al.* (1973). The high incidence of antibodies in macropods and the large number of isolates from *C. marksi* at Beatrice Hill is compatible with the virus surviving in a *C. marksi*–marsupial cycle as postulated by Doherty (1972).

Ungrouped Viruses

Leanyer virus was isolated from *An meraukensis* collected at Leanyer Swamp near Darwin (Doherty *et al.* 1977) whereas both the isolates reported here were from *C. marksi.*

Parker's Farm (CH 19520) was originally isolated from *Cx annulirostris* collected at Charleville (Doherty *et al.* 1979). The isolates reported here came from *C. marksi* and are another example of a virus isolated from two different families of blood sucking insects from localities 2000 km apart.

Wongorr Virus

Previous isolates were from *Cx annulirostris* and *Ae lineatopennis* from Mitchell River (Doherty *et al.* 1973, 1979). Seven of the eight isolates reported here came from *Cx annulirostris* the other from a *Culicoides* (*C. pallidothorax*). Once more there is a wide geographic range and isolations from two families of insects.

Comparison of Virus Isolation Systems

Isolations made in parallel in the mouse and tissue culture systems clearly indicates the importance of using more than one isolation system when undertaking arbovirus surveys. The mouse system was found to be more efficient for isolating Togaviridae, Bunyaviridae and Rhabdoviridae while the tissue culture system was the more efficient for isolating Reoviridae.

Unfortunately the original mosquito material was destroyed in a freezer accident in 1976 so that re-isolation attempts could not be made. However, prior to their isolation from the Beatrice Hill collections Barmah Forest, Kokobera, Thimiri, Facey's Paddock, Mapputta, Belmont, bluetongue, Eubenangee, Bunyip Creek, Marrakai, CSIRO Village, Corriparta, Warrego, Leanyer, Parker's Farm, Wongorr, Beatrice Hill and Humpty Doo viruses were not held at this laboratory.

Viruses Isolated from more than One Family of Diptera

Several species of insect were found to be sources of named viruses for the first time (*C. oxystoma*, Palyam group viruses; *C. pallidothorax*, Wongorr virus; *C. histrio*, Thimiri

virus). Some viruses were shown to occur in the same locality in both mosquitoes and *Culicoides*. Eubenangee virus was isolated from *Cx annulirostris*, *An farauti* and *C. marksi*; Leanyer virus was isolated twice from *C. marksi*. These instances of the isolation of the same virus from both mosquitoes and *Culicoides* complement the report of the isolation of Warrego virus from widely different insect families (Doherty *et al.* 1979).

The isolation of the same arbovirus from two different families of Diptera is not an unexpected occurrence. There is no more reason to expect a virus to be confined to one insect family than there is to expect it to be restricted to one vertebrate family. Nine of the 22 viruses isolated at Beatrice Hill occur in more than one family of Diptera, while only 35 of the 409 arboviruses listed in the International Catalogue of Arboviruses are recorded as occurring in more than one family of arthropods.

Table 7. Arboviruses recorded at Beatrice Hill, Northern Territory, which have been isolated from more than one family of Diptera

Virus	*Culicoides* spp.	Locality	Ref.[A]	Mosquito spp.	Locality	Ref.[A]
Barmah Forest	*C. marksi*	Beatrice Hill		*Cx annulirostris*	Charleville	(3)
				Ae normanensis	Charleville	(3)
Belmont	*C. marksi*	Beatrice Hill		*Cx annulirostris*	Rockhampton	(2)
Facey's Paddock	Mixed	Beatrice Hill		*Cx annulirostris*	Charleville	(3)
Bovine ephemeral fever	Mixed	Kenya	(1)	*An bancroftii*	Beatrice Hill	(4)
				Mixed	Rockhampton	(4)
Eubenangee	*C. marksi*	Beatrice Hill		*Cx annulirostris*	Beatrice Hill	
				Mixed	Innisfail	(5)
Warrego	*C. marksi*	Beatrice Hill		*An meraukensis*	Kowanyama	(3)
	C. marksi	Charleville	(2)	*Cx annulirostris*	Charleville	(2)
	C. dycei	Charleville	(2)			
Leanyer	*C. marksi*	Beatrice Hill		*An meraukensis*	Darwin	(6)
Parker's Farm	*C. marksi*	Beatrice Hill		*Cx annulirostris*	Charleville	(3)
Wongorr	*C. pallidothorax*	Beatrice Hill		*Cx annulirostris*	Beatrice Hill	
				Ae lineatopennis	Kowanyama	(2)
				Cx annulirostris	Kowanyama	(3)

[A](1) Davies and Walker (1974); (2) Doherty *et al.* (1973); (3) Doherty *et al.* (1979); (4) Standfast *et al.* (1976); (5) Doherty *et al.* (1968); (6) Doherty *et al.* (1977).

The nine viruses listed in Table 7 have (with the exception of Barmah Forest and Facey's Paddock) been shown by serological studies (Doherty 1972, 1977) to infect cattle or marsupials or both, while the arthropods listed are known to feed readily on a range of hosts including marsupials and cattle (Muller *et al.* 1981). Where insects were being collected for virus isolation, emphasis was placed on collecting those groups associated with cattle and water buffalo. At Beatrice Hill the agile wallaby was abundant and grazed in company with the cattle and buffalo being sampled, so that collection of insects included species attracted to both groups. Thus the conditions prevailing at Beatrice Hill and the procedures followed in the project were more likely to reveal broad host ranges of viruses than projects limited in the period for which collections were made and which concentrated on one group of biting flies.

Disease

Three of the virus species listed in Table 4 have been associated with disease in man or domestic animals, namely Ross River virus, bovine ephemeral fever virus and bluetongue virus. Each has been isolated in Australia from domestic animals (Pascoe *et al.* 1978; Doherty *et al.* 1969; St George *et al.* 1980), but only Ross River virus has been associated with human disease (Doherty 1972, 1977).

Bovine ephemeral fever is the cause of considerable economic loss to the dairy and pastoral industries in northern and eastern Australia. Mortalities in infected herds are low but major losses are due to interruption of lactation in dairy herds, loss of fertility in stud bulls and the general disruption of management procedures in the beef cattle industry (St George 1981).

Bluetongue virus (serotype 20) has not been recorded as causing clinical illness in the field although antibodies have been demonstrated to be widespread in cattle in northern Australia (St George, unpublished data). In the laboratory it is recorded as producing mild disease in sheep (Uren and Squire 1982).

Subclinical infection of cattle with two of the Palyam group viruses [CSIRO 11 and CSIRO 87 (Bunyip Creek)] has been established by virus isolation (Cybinski and St George 1982), and of buffalo by CSIRO 82 by seroconversion. Each of the *Culicoides* species from which Palyam group viruses were isolated is known to bite cattle and buffalo (Muller *et al.* 1981).

Entomology

The mosquito *Cx annulirostris* described as the most important vector of arboviruses in Australia (Doherty *et al.* 1979) was the most productive mosquito in the collections. The biting midge, *C. marksi*, was the most productive *Culicoides*, a reflection of this insect's abundance and its close association with marsupials (Muller *et al.* 1981). It was interesting that no isolates were made from the 5490 *C. brevitarsis* processed. This insect has yielded in excess of 217 isolates of 11 viruses from insects collected in eastern Australia (St George *et al.* 1983). However, only two of the Beatrice Hill viruses (CSIRO Village and Bunyip Creek) have been isolated from *C. brevitarsis*. A number of the other viruses from Beatrice Hill appear to have marsupials as their main vertebrate host, a group of animals seldom attacked by this midge which is closely associated with cattle (Muller *et al.* 1981).

It should be noted that the isolation of a virus from an insect does not necessarily mean that the species is a vector of the virus.

Conclusion

The isolation of viruses of many serological groups was evidence that Beatrice Hill was a focus of arbovirus activity. The number of different viruses isolated remains to be determined but will certainly exceed 22. The study resulted in the collection of five new viruses (bluetongue serotype 20, CSIRO Village, Marrakai, Beatrice Hill and Humpty Doo) and revealed the presence of Thimiri virus in Australia. In addition it has extended the geographic range of most of the viruses to the north of the Northern Territory and recorded a number from more than one family of biting Diptera.

While the isolation of viruses from both previously recorded and new invertebrate hosts at a site some 1200–2000 km from previous study sites will aid the study of the natural history of these viruses, more importantly this study has shown that this part of Australia which is in close proximity to the Oriental region would be highly receptive to exotic arboviruses.

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