

EXPERIMENTAL INFECTION OF AUSTRALIAN BRUSHTAIL POSSUMS, *TRICHOSURUS VULPECULA* (PHALANGERIDAE: MARSUPIALIA), WITH ROSS RIVER AND BARMAH FOREST VIRUSES BY USE OF A NATURAL MOSQUITO VECTOR SYSTEM

ANN MARIE BOYD, ROY A. HALL, ROBERT T. GEMMELL, AND BRIAN H. KAY

Queensland Institute of Medical Research and University of Queensland, Tropical Health Program; University of Queensland,
Department of Microbiology and Parasitology and Department of Physiology and Pharmacology, St. Lucia, Queensland, Australia

Abstract. Brushtail possums, *Trichosurus vulpecula* Kerr, were experimentally infected with Ross River (RR) or Barmah Forest (BF) virus by *Aedes vigilax* (Skuse) mosquitoes. Eight of 10 animals exposed to RR virus developed neutralizing antibody, and 3 possums developed high viremia for < 48 hr after infection, sufficient to infect recipient mosquitoes. Two of 10 animals exposed to BF virus developed neutralizing antibody. Both infected possums maintained detectable neutralizing antibody to BF for at least 45 days after infection (log neutralization index > 2.0 at 45 days). Eight possums did not develop neutralizing antibody to BF despite exposure to infected mosquitoes. These results suggest that *T. vulpecula* may potentially act as a reservoir species for RR in urban areas. However, *T. vulpecula* infected with BF do not develop viremia sufficient to infect mosquitoes and are unlikely to be important hosts for BF.

INTRODUCTION

The vertebrate hosts of Ross River (RR) virus are primarily mammalian, with macropods (kangaroos and wallabies) acting as major reservoirs. However, the vertebrate hosts of Barmah Forest (BF) virus are presently unknown. In Australia, serum neutralizing antibodies to RR are widespread in human and mammal populations. Serological studies consistently indicate that marsupials are commonly infected with RR.¹ Macropods are believed to be the major hosts of RR, with seropositivity rates of > 80% recorded in north Queensland.² Experimental inoculations of animals with RR indicate that marsupials are the most competent of the potential vertebrate hosts, ahead of placental mammals.³ There is seroepidemiological evidence suggesting that mammals, particularly marsupials, also act as vertebrate hosts of BF.^{1,4} However, the molecular epidemiology of the virus indicates that more mobile hosts, such as birds, are likely to play a major role.⁵

Outbreaks of RR in urban areas of Perth, Western Australia, during 1991–1992⁶ and more recently in Brisbane, Queensland, are likely to have been maintained by hosts common in urban environments. The roles of animals common in urban areas, such as brushtail possums, in the transmission cycles of RR and BF have not been investigated until now. In urban areas where the common macropod vertebrate hosts are absent or rare, it is logical that other vertebrates may be responsible for maintaining RR and BF epidemics. Animals common in urban Australia, such as dogs, cats, and possums, may be important hosts. In fact, dogs have commonly been found with RR antibody.^{2,7} However, nothing is known about the role that these species have in the persistence of the virus in urban environments. Given that both BF and RR antibodies have been found in the same host species and have been isolated from the same mosquito species, it is possible that the transmission cycles of both viruses involve these urban animals as reservoir hosts.

Common brushtail possums, *Trichosurus vulpecula*, are distributed from Tasmania through tropical Australia, includ-

ing central and Western Australia. They are highly adaptable, successfully colonizing both natural and urban environments. In urban Australia, brushtail possums commonly inhabit buildings, especially roof cavities, and tree hollows. Peak RR notifications in Queensland occur most frequently in February through April each year, at a time when young possums are relatively common in urban areas of southeast Queensland. Of the 40 possums trapped and tested for this study, 11 (27.5%) had naturally occurring antibodies to RR virus. This indicates that along with other marsupials such as kangaroos,⁸ *T. vulpecula* are commonly exposed to RR virus in the wild.

Introduced from Australia in 1837 to establish a fur industry, *T. vulpecula* numbers in New Zealand are estimated to be in excess of 70 million⁹ and are established in > 91% of New Zealand. In 1998, the southern salt-marsh mosquito *Aedes camptorhynchus* (Thompson) was imported from Australia into the Hawkes Bay region of New Zealand.¹⁰ This mosquito is an efficient vector of RR¹¹ and has returned isolates of BF.¹² Accordingly, the status of brushtail possums as potential vertebrate hosts of these viruses in New Zealand has assumed new significance, particularly because large numbers of people, some infected with RR,¹³ travel there from Australia.

The purpose of this work is to determine whether brushtail possums can be infected with RR or BF via mosquito inoculation and whether mosquitoes can subsequently become infected by feeding on infected possums.

MATERIALS AND METHODS

Trichosurus vulpecula were trapped with wire box traps in urban areas of Brisbane Department of Environment (DOE) permit E5/000015/97/SAA, University of Queensland Animal Ethics Experimentation approval (QIMR/ANAT/338/97/PHD) and held at the Native Animal Research Unit at the University of Queensland Pinjarra Hills Veterinary Farm. Ten adult possums were used for each experimental infection. Possums were held for 7 days before being in-

fectured to ensure that they were maintaining weight and eating well before being included in experiments.

All animals were tested on arrival for antibodies to RR and BF viruses by the varying virus/constant sera microneutralization method.¹⁴ Neutralization tests were considered positive if cell and sera control cells were normal and the log neutralization index (LNI)¹⁵ was > 2.0 . Individuals without neutralizing antibodies (LNI < 2) were tagged, weighed, and held in mosquito-proofed facilities.

Virus pools. An isolate of RR (B94/20 SMB passage 1, C6/36 [*Aedes albopictus* cells] passage 1), from *Culex annulirostris* Skuse collected in Brisbane, Australia, in 1994 was used. Stock virus pools had a titer of $10^{8.7}$ of 50% Vero cell culture infective dose (CCID₅₀). The BF isolate (BF2078/120) isolated from *Ae. vigilax* collected in 1996 at Mount Coolum, ~ 100 km north of Brisbane, Australia, was used. Stock BF virus (C6/36 passage 2) had a titer of 10^7 Vero CCID₅₀ per 100 μ L. Each virus sample was formed into aliquots of 300 μ L lots and stored at -70°C until required.

Mosquitoes. *Aedes vigilax* mosquitoes were selected as infecting vectors on the basis of their efficacy of transmission for both BF¹⁶ and RR.¹⁷ Laboratory transmission rates (which were assessed by use of suckling Quackenbush mice) for RR and BF by *Ae. vigilax* range approaches 80 and 60%, respectively, after incubation at 27°C for 6 days.¹⁶ Adult female *Ae. vigilax* were from a laboratory colony at the Queensland Institute of Medical Research (QIMR). The colony was established in 1987 from eggs collected in Townsville, North Queensland, by Mr. S. Doggett, Westmead Hospital, Sydney, New South Wales. Newly emerged mosquitoes were maintained at 28°C and 70% relative humidity in a 12:12 (light:dark) hr photoperiod regimen, with a 10% sucrose solution. Before blood feeding for infection studies under the above conditions, 2–3-day-old females were starved for 24 hr.

The mosquitoes were fed overnight on stock virus in heparinized rabbit blood (25 U/mL) containing 1% sucrose. To determine the per os virus dose, blood-virus mixture was titrated on Vero (African green monkey) cells. The median virus dose per mosquito was calculated on the basis of an average *Ae. vigilax* bloodmeal of 3 μ L.¹⁸ Virus doses were estimated as $10^{2.5}$ per os for BF and $10^{3.5}$ for RR (CCID₅₀ per mosquito). After feeding, engorged mosquitoes were sorted on a chill table and held for 7 days before being used to infect the possums.

Experimental infections. During all mosquito work and subsequent blood testing, the possums were anesthetized with a halothane-oxygen mix via inhalation. Two to 3 virus-fed mosquitoes were used as the infection source. Because virus titers are scaled logarithmically, multiple donor mosquitoes do little to affect dosage. Donor mosquitoes, in 30×40 mm plastic containers with mesh covering the opening, were held onto each animal to allow blood feeding. Small areas, ~ 2.5 cm², were shaved on the abdomen to facilitate exposure to the skin. Mosquitoes that fed on the animals were processed for infection and virus content, and the number of infected mosquitoes that had fed on each animal was recorded.

Clinical signs were expected to be limited to those shown by other species, such as the muscular and joint stiffness and

ataxia,¹⁹ pyrexia, lethargy, and mild colic observed in horses.^{20,21} Other species (birds, cattle, kangaroos) inoculated with RR have remained asymptomatic.²⁰

Possums were checked daily for these potential clinical signs and were weighed daily for the first 7 days, then weekly.

Mosquito assays. Individual blood-fed mosquitoes were homogenized separately in sterile 1.5-mL Microfuge tubes in 500 μ L tissue culture (TCM) diluent (RPMI 1640, 10% fetal calf serum, 2 mM L-glutamine [ICN Biomedicals, Costa Mesa, CA], 200 μ g/mL benzylpenicillin, 200 μ g/mL streptomycin, and 2 μ g/mL Fungizone [Apothecan, Princeton, NJ]). Specimens were centrifuged at $8,000 \times g$ for 5 min. The supernatant was transferred into clean, sterile Microfuge tubes and respun. One hundred microliters of the resultant supernatant were transferred to duplicate wells of a 96-well microtiter plate and titrated as 10-fold dilutions. Each well was then seeded with 1.5×10^5 /mL Vero cells. Unfed mosquitoes were processed with each batch of plates as negative controls. Plates were incubated at 37°C and were examined daily for cytopathic effect (CPE) due to virus, which was confirmed by staining with 0.5% crystal violet in 10% formalin after 7 days.

Testing for viremia and antibody. To determine infection rate and to demonstrate passage of virus from host to vector, batches of up to 30 uninfected, colonized *Ae. vigilax* (recipients) were induced to feed on individual animals, as described above. Recipient mosquitoes were fed on individual animals daily for 5 days, and then at weekly intervals from 7 days for up to 28 days after infection.

To assay for virus content, engorged recipient mosquitoes were held 5–7 days after feeding under insectary conditions before processing. In the case of animals from which we isolated no virus in blood or individual mosquitoes, but which subsequently produced neutralizing antibodies, pools of supernatant from ground recipient mosquitoes were passaged through C6/36 cells for 48 hr and then titrated on Vero cells. This was done to amplify any small quantities of virus that may have been present but not detected.

In addition, sera from each bleed was diluted 1:10 with TCM and titrated onto Vero cells within 4 hr of collection for virus detection, and the balance was held for antibody analysis by microneutralization. Supernatant from titrated sera that resulted in CPE, indicating the presence of virus, was tested for *Alphavirus* infection by C6/36 cell culture immunoassay in 24-well tissue culture plates.²² Fixed cells were examined for *Alphavirus*, and RR and BF antigens by use of specific monoclonal antibodies (for cross-reactive *Alphavirus* detection B10, for RR virus specific identification D7 and A10A2, and for BF virus 3B3 monoclonals) at the QIMR.

For antibody analysis, a blood sample was taken from each animal and tested by the varying virus–constant sera microneutralization (NT) method.¹⁴ Sera were heat-inactivated at 56°C for 30 min to destroy any nonspecific cellular or viral inhibitors. In brief, the protocol followed for NT was as follows. Ten-fold serial dilutions of virus stock in 50 μ L were added to the wells of a 96-well tissue culture plate. Fifty microliters of heat-inactivated (56°C for 30 min) sera diluted 1:20 with TCM was added to duplicate wells down the plate, except for the control columns. Plates were incu-

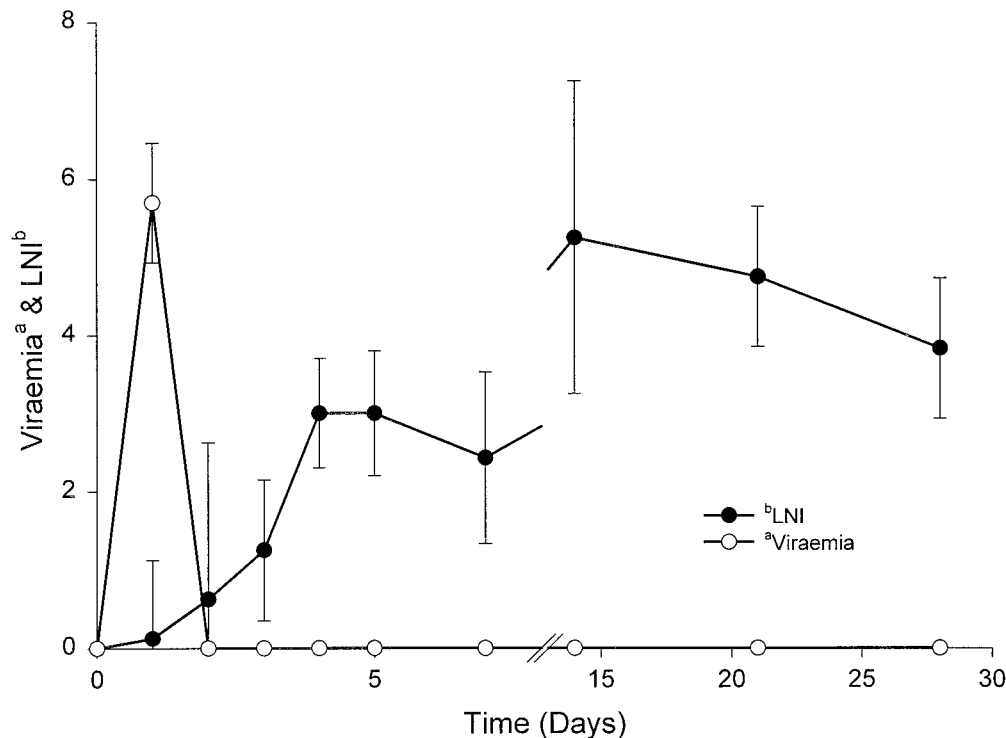


FIGURE 1. Viraemia and neutralizing antibodies in brushtail possums exposed to Ross River virus. ^a Viraemia log₁₀ CCID₅₀ ($n = 3$) \pm 1 standard deviation (SD). ^b Neutralizing antibody log neutralization index (LNI) ($n = 8$) \pm 1 SD.

bated at 37°C for 60 min before each well was seeded with $\sim 1.5 \times 10^5$ of Vero cells in 100 μ L of TCM and returned to 37°C with 5% CO₂ and examined daily for CPE with a phase-contrast microscope. Those wells showing partial CPE were marked and rechecked more often. The plates were stained with crystal violet after 7 days, or when there had been no observed increase in CPE for 24 hr. Neutralization tests were considered positive if serum controls were normal and the LNI¹⁵ was > 2.0 .

RESULTS

Donor mosquitoes. Individual animals were infected via bites from 1–3 infected mosquitoes with viral titers (whole bodies) of $10^{2.7-6.7}$ CCID₅₀/mosquito and $10^{1.7-7.0}$ CCID₅₀/mosquito for RR and BF, respectively.

Viremic response to infection. Three of the 10 possums exposed to RR became viremic (R829, R830, and R831) with a peak blood virus titer of log₁₀ Vero CCID₅₀ 7.5/100 μ L at 24 hr after infection (Figure 1). Of the 34 recipient *Ae. vigilax* that fed on the individuals at 24 hr after infection, 18 (53%) became infected (Table 1). After incubation for 5 days under insectary conditions, assayed infected recipient mosquitoes had virus titers of 1.7–3.7 log₁₀ Vero CCID₅₀. No further recipient mosquitoes became infected, and no virus was recovered by cell culture in any other individuals.

From the experimental infections with BF, no virus was recovered from the 10 possums exposed. However, at 3 days after infection, one of the 30 recipient *Ae. vigilax* from possum B828 was infected (Table 2). After incubation for 5 days, the assayed infected recipient mosquito had a virus

titer of 2.7 log₁₀ Vero CCID₅₀. A further 51 recipient mosquitoes feeding on possum B828 were negative.

Clinical signs. The 3 possums that tested positive for RR infection developed clinical signs by 4 days after infection. Clinical signs included wobbly gait, lethargy, inappetence, and relatively slow recovery from gaseous anesthesia. Possum R830 was killed at 7 days after infection, and possum R831 died during the night at 8 days after infection. Possum R829 recovered and remained healthy until killed at 270 days after infection. The 7 other animals, which did not develop viraemia, remained healthy and active, despite being subjected to the same protocols. Possum R829 developed protective antibody and displayed no more clinical signs by 7 days after infection.

Autopsies were carried out on possums R830 and R831 at the University of Queensland's Veterinary Pathology Department (reference 98/0636-637). Both animals were found to be in good general condition with no external injuries. Postmortem examination found that there was clotted blood in pericardial and thoracic regions, presumably initiated by the cardiac puncture procedure. However, in both cases, the cardiac puncture wounds were unremarkable—in one animal, it was “inconspicuous,” and in the other, it was “sealed by a fibrinous plug” at the time of death. In both R830 and R831, the brains were soft, with wet hemorrhagic and edematous meninges. Petechial hemorrhages were also present on the left cerebral occipital lobe of R831. In R830, the liver was mildly congested with blood, and in R831, the pulmonary alveolar spaces were filled with blood. That the brains of possums R830 and R831 were soft and the meninges edematous and hemorrhagic was unambiguous and raises

TABLE 1

Results for donor and recipient *Aedes vigilax* mosquitoes over time (T) in days for 7 days during the infection studies for Ross River virus

Possum no.	Sex	Weight (g) at T0	Weight (g) at T7	Mean titer donor mosquitoes*	Donor mosquitoes infected (fed)	Recipient mosquitoes infected (no. fed)					
						T1	T2	T3	T4	T5	T7
R829	F	1,292	1,253	3.7	1 (3)	6 (12)	0 (4)	0 (4)	0 (23)	0 (14)	0 (12)
R830	M	1,850	1,772	6.7	1 (3)	6 (10)	0 (2)	0 (10)	0 (12)	0 (13)	0 (12)
R831	F	2,095	1,811	6.7	3 (3)	6 (12)	0 (2)	0 (9)	0 (13)	0 (8)	0 (13)
R833	M	1,419	1,502	4.7	3 (3)	0 (11)	0 (12)	0 (11)	0 (21)	0 (20)	0 (20)
R835	M	2,283	2,288	4.7	2 (3)	0 (18)	0 (14)	0 (25)	0 (34)	0 (9)	0 (12)
R842	M	2,307	2,316	3.7	1 (3)	0 (12)	0 (23)	0 (12)	0 (17)	0 (21)	0 (10)
R846	M	2,680	2,674	4.7	1 (2)	0 (0)	0 (11)	0 (18)	0 (30)	0 (12)	0 (27)
R850	M	2,013	2,115	4.7	1 (3)	0 (6)	0 (12)	0 (18)	0 (13)	0 (7)	0 (19)
R851	M	1,823	1,866	5.0	1 (2)	0 (10)	0 (12)	0 (10)	0 (18)	0 (14)	0 (29)
R852	M	2,320	2,300	4.7	1 (2)	0 (12)	0 (16)	0 (4)	0 (18)	0 (9)	0 (25)

* Mean viral titer for donor mosquitoes: mean Ross River virus titers (whole bodies) of tested mosquitoes 50% Vero cell culture infective dose per mosquito.

some questions concerning meningitis and hemorrhagic symptoms possibly associated with RR infection. None of the BF-exposed possums developed clinical signs.

Antibody response to infection. Of the 10 possums subjected to RR infection, 8 developed detectable neutralizing antibody, measured by microneutralization assay from 5 days after infection to least 28 days after infection (Figure 1). Possum R829 had neutralizing antibody to RR that remained detectable at 250 days after infection, after which time, the animal was killed.

Of the 10 possums exposed to BF, only 2 (B802 and B828) developed neutralizing antibodies to BF, detectable at 7–14 days after infection (Figure 2). Both possums maintained detectable neutralizing antibody to BF at similar levels for at least 45 days after infection (LNI > 2.0 at 45 days). Eight possums did not develop any neutralizing antibody to BF despite being exposed to infected mosquitoes.

DISCUSSION

This study demonstrates that brushtail possums are potential hosts of RR in urban Australia, capable of infecting an average of 53% of susceptible recipient vector mosquitoes while viremic. Because the known vertebrate hosts of RR are primarily mammalian, with macropods known to be major reservoirs, it was not surprising that the most common urban marsupial in Australia should prove to be a capable host. This study also raises questions about possible clinical

symptoms and suggests a new model for the study of RR pathology. This did not apply to BF. This study shows that ~ 30% of brushtail possums exposed to RR may develop high titer viremia after a bite by an infected mosquito. Even though the possums have viremia lasting for < 2 days, at 1 day, an average of 53% of recipient *Ae. vigilax* became infected. This could be considered an efficient system. The high infection rate in recipient *Ae. vigilax* mosquitoes suggests that *T. vulpecula* is a competent host for RR virus, which may potentially act as a reservoir in urban areas.

In contrast, all possums infected with BF remained aviremic, or had undetectable titers. Of the recipient mosquitoes that fed on the viremic possum, only a single recipient mosquito (equivalent to 3.3% of mosquitoes exposed on Day 3) was infected with detectable BF. These results indicate that viremias were very low and of 3–4 days' duration in the infected animals. The resulting low infection rate in recipient mosquitoes suggests that *T. vulpecula* is not a competent host for the strain of BF tested.

These data are consistent with the understanding that RR is associated with marsupials and suggest the upgrading of mosquito control, especially for peridomestic species, particularly if the level of mosquitoes feeding on brushtail possums proves high. For New Zealand, with its high numbers of feral brushtail possums, the recent introduction of the RR vector *Ae. camptorhynchus* from southern Australia is cause for concern.

TABLE 2

Results for donor and recipient *Aedes vigilax* mosquitoes over time (T) for 7 days during the infection studies for Barmah Forest virus

Possum no.	Sex	Weight (g) at T0	Weight (g) at T1	Mean titer donor mosquitoes	Donor mosquitoes infected (fed)	Recipient mosquitoes infected (no. fed)					
						T1	T2	T3	T4	T5	T7
B802	F	1,790	1,745	6	2 (3)	0 (19)	0 (23)	0 (20)	0 (15)	0 (8)	0 (13)
B828	F	1,444	1,410	4	1 (3)	0 (12)	0 (27)	1 (30)	0 (10)	0 (11)	0 (6)
B832	M	1,853	1,852	5.7	1 (1)	0 (42)	0 (22)	0 (16)	–	0 (43)	0 (25)
B835	M	2,068	2,077	4.7	1 (3)	0 (20)	0 (20)	0 (17)	–	0 (38)	0 (23)
B842	M	2,310	2,146	5.7	1 (1)	0 (29)	0 (14)	0 (26)	–	0 (11)	0 (18)
B843	F	1,897	1,893	4.7	3 (3)	0 (10)	0 (10)	0 (18)	0 (9)	0 (11)	0 (27)
B844	M	1,772	1,773	7	3 (3)	0 (19)	0 (12)	0 (26)	–	0 (26)	0 (13)
B846	F	1,991	2,014	6	2 (3)	0 (27)	0 (19)	0 (11)	0 (12)	0 (24)	0 (32)
B847	M	1,623	1,673	2.7	1 (3)	0 (27)	0 (20)	0 (28)	0 (17)	0 (15)	0 (12)
B848	M	2,156	2,182	1.7	1 (3)	0 (32)	0 (13)	0 (12)	–	0 (10)	0 (8)

* Mean viral titer for donor mosquitoes: mean Barmah Forest virus titers (whole bodies) of tested mosquitoes 50% Vero cell culture infective dose per mosquito.

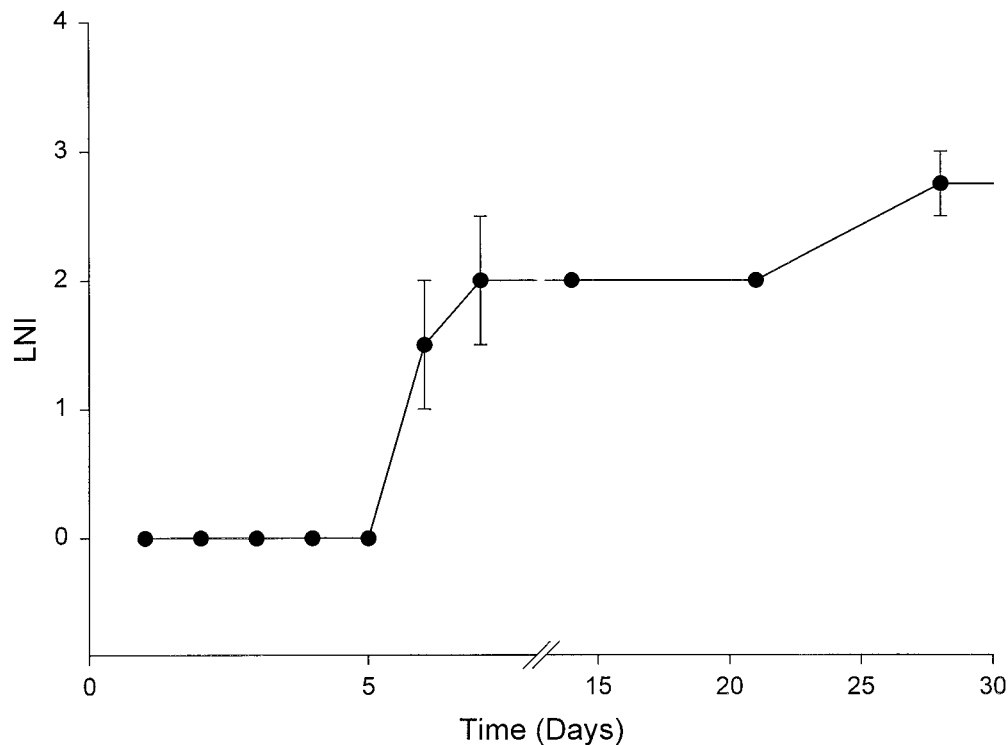


FIGURE 2. Neutralizing antibodies in brushtail possums exposed to Barmah Forest virus. Neutralizing antibody log neutralization index (LNI) ($n = 2$) \pm 1 standard deviation (SD).

Authors' note: *Aedes vigilax* and *Ae. camptorhynchus* are now revised to *Ochlerotatus vigilax* and *Oc. camptorhynchus*.

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Authors' addresses: Ann Marie Boyd and Brian H. Kay, Queensland Institute of Medical Research and University of Queensland, Tropical Health Program, Post Office Royal Brisbane, Hospital Queensland 4029, Australia. Roy A. Hall, University of Queensland, Department of Microbiology and Parasitology, St. Lucia, Queensland 4072, Australia. Robert T. Gemmell, University of Queensland, Department of Physiology and Pharmacology, St. Lucia, Queensland 4072, Australia.

Reprint requests: Ann Marie Boyd, Queensland Institute of Medical Research, P.O. Royal Brisbane Hospital 4029, Australia. Telephone: 61-7-3362-0351, Fax: 61-7-3362-0106 (e-mail: annB@qimr.edu.au).

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