Transmission of Japanese Encephalitis Virus from the Black Flying Fox, *Pteropus alecto*, to *Culex annulirostris* Mosquitoes, Despite the Absence of Detectable Viremia

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Abstract. To determine the potential role of flying foxes in transmission cycles of Japanese encephalitis virus (JEV) in Australia, we exposed *Pteropus alecto* (Megachiroptera: Pteropididae) to JEV via infected *Culex annulirostris* mosquitoes or inoculation. No flying foxes developed symptoms consistent with JEV infection. Anti-JEV IgG antibodies developed in 6/10 flying foxes exposed to infected *C. annulirostris* and in 5/5 inoculated flying foxes. Low-level viremia was detected by real-time reverse transcriptase polymerase chain reaction in 1/5 inoculated flying foxes and this animal was able to infect recipient mosquitoes. Although viremia was not detected in any of the 10 flying foxes that were exposed to JEV by mosquito bite, two animals infected recipient mosquitoes. Likewise, an inoculated flying fox without detectable viremia infected recipient mosquitoes. Although infection rates in recipient mosquitoes were low, the high population densities in roosting camps, coupled with migratory behavior indicate that flying foxes could play a role in the dispersal of JEV.

INTRODUCTION

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus of Southeast Asia responsible for over 40,000 clinical cases annually, with a 25% fatality rate. Japanese encephalitis virus is an emerging virus in the Australasian region with potentially serious public and animal health implications. Widespread JEV activity was first detected in Australia in 1995 on Badu Island in Torres Strait, when it caused an outbreak consisting of three human cases and two deaths. Subsequently, except for 1999, JEV activity was recognized every year in the Torres Strait between 1995 and 2005, when the sentinel pig program was discontinued. In 1998, human and pig infections were identified for the first time on the Australian mainland at the mouth of the Mitchell River on Cape York Peninsula. Entomologic studies implicated *Culex annulirostris* as the primary mosquito vector of JEV in northern Australia.

Pigs and ardeid wading birds, such as herons and egrets are the primary amplifying hosts of JEV, as they produce viremia sufficient to infect mosquitoes. Although humans and horses can develop fatal encephalitis, they are considered to be dead-end hosts of the virus, developing only low-level viremia.

Should JEV become established on the Australian mainland, the potential importance of Australian fauna species acting as vertebrate amplifying hosts is largely unknown. Experimental studies at the Commonwealth Scientific and Industrial Research Organization Australian Animal Health Laboratory previously examined the possible role of a number of Australian native species (such as wallabies and possums) as reservoir or amplifying hosts. However, flying foxes (Megachiroptera: Pteropidae) were not included in these studies, and mosquitoes were not used as the route of virus exposure or to show transmission of JEV. Flying foxes are of particular interest as a potential reservoir or amplifying host of JEV as a result of their nomadic behavior, and because of their increasing abundance in major urban areas in eastern Australia. Certainly, the behavioral ecology of flying foxes deems them potential dispersal hosts of mammalian viruses, and several recently emerged zoonotic viruses, including Hendra virus (HeV) and Australian bat lyssavirus, have been described in flying foxes in Australia. Furthermore, both experimental and natural infection studies have suggested a possible role for bats in the transmission cycles of a number of arboviruses, including JEV (reviewed by Sulkin and Allen). However, the majority of these experiments have examined insectivorous bats of the order Microchiroptera. The only experimental infection of Megachirotteran bats with JEV has been undertaken in India. In these earlier studies, viremia developed in both *Rousettus leschenaultii* and *Cynopterus sphinx*, with the latter species able to infect recipient mosquitoes.

We undertook laboratory-based infection and transmission experiments to determine whether flying foxes can act as amplifying hosts for JEV. Specifically, we examined 1) the ability of flying foxes to develop clinical illness, viremia, and specific antibodies after being exposed to JEV via infected mosquitoes or by inoculation; and 2) the likelihood of recipient mosquitoes becoming infected after feeding on flying foxes that had been previously exposed to JEV. The Black flying fox, *Pteropus alecto*, was chosen because of its widespread geographic distribution throughout northern Australia, including areas where JEV has previously occurred.

MATERIALS AND METHODS

All animal experimentation complied with the Queensland Animal Care and Protection Act 2001 and was approved by the Queensland Department of Primary Industries Animal Ethics Committee (AEC; approval no. 33/08/02), University of Queensland AEC (approval no. MICRO/PARA/463/02), and Queensland Health Forensic and Scientific Services AEC (QHFSS; approval no. VIR 1/03/32). In addition, the collection of wild flying foxes was approved by the Queensland Parks and Wildlife Service (Scientific Purposes permit no. WISP01419503). As JEV is considered an exotic virus in Australia, all infection and transmission experiments...
were undertaken at the Physical Containment Level 3 insectary/animal house at QHFSS, Brisbane.

**Animals.** *Pteropus alecto* were collected from flying fox colonies located in the Brisbane suburbs of East Brisbane (27°28′S, 153°03′E) and Indooroopilly (27°30′S, 152°58′E), which also contained the Grey-headed flying fox, *Pteropus poliocephalus* and the Little red flying fox, *Pteropus scapulatus*. Flying foxes returning from overnight foraging were captured using mist nets for a period of approximately 90 min before sunrise. Flying foxes were anaesthetized using the inhalation anesthetic Isoflurane (Isoflurane Inhalation Anesthetic, Laser Animal Health, Salisbury, Australia) administered by a portable Ohmeda Iso Tec 3 vaporizer (BOC Health Care, West Yorkshire, England) at a concentration of 5% for induction and 1.5% during maintenance with an oxygen flow rate of 1 L/min.17 Each England) at a concentration of 5% for induction and 1.5% Ohmeda Iso Tec 3 vaporizer (BOC Health Care, West Yorkshire, England) at a concentration of 5% for induction and 1.5% during maintenance with an oxygen flow rate of 1 L/min.17 Each bat was assessed for age, weight, forearm length, and female bats for pregnancy status by abdomen palpation. Pregnant females were excluded from the study and released. To aid in future identification, each flying fox was injected with a microchip containing a unique identification number (LifeChip, Destron Fearing, South St. Paul, MN). A 1–2 mL blood sample was obtained from the propatagial vein using a 3 mL syringe (Terumo [Philippines] Corporation, Laguna, Philippines), a 0.50 × 16 mm needle (Terumo [Philippines] Corporation), and lithium heparin (Heparin Injection BP; David Bull Laboratories, Mulgrave North, Australia) as an anticoagulant. The serum fraction was tested for JEV-specific antibodies using a neutralization assay.18 As HeV circulates within wild flying fox populations in Brisbane,19 the serum samples also were tested for antibodies to this virus, using the HeV Serum Neutralization Test developed and performed at the CSIRO Australian Animal Health Laboratory, Geelong.20

Flying foxes were held at the Queensland Department of Primary Industries and Fisheries, Animal Research Institute (ARI), Yeerongpilly, for ≤ 14 d to allow for a second serum sample to be obtained for the completion of baseline serology. Any animals showing evidence of current or previous infection with either JEV or HeV were excluded from the study.

**Virus strain.** All experiments used the JEV TS3306 strain isolated from *Aedes vigilax* collected from Badu Island in February 1998. The virus had been passaged once in C6/36 (*Ae. albopictus* salivary gland) cells and twice in porcine stabil-equine kidney (PS-EK) cells. The titer of virus stock was 10^6 PS-EK TCID₅₀ (tissue culture infectious dose)₅₀/mL.21

**Mosquito strain.** The mosquitoes used for all infection and transmission studies were *Culex annulirostris* from colonies housed at the Australian Army Malaria Institute, Brisbane. The *Cx. annulirostris* colony was established from adults collected from the Boondall Wetlands, Brisbane in 1998. Vector competence experiments had previously shown that this strain of *Cx. annulirostris* had infection and transmission rates of 100% and 83%, respectively, for the same strain of JEV used in the current study.14

**Infection of mosquitoes with JEV.** The 2- to 4-day-old mosquitoes were starved for 24 hr before feeding for 30 min to 1 hr on a virus suspension using the hanging drop method of Goddard and others.21 The virus suspension contained JEV stock diluted in heparinized rabbit blood and 1% sucrose. Two to three milliliter (mL) of this suspension (first heated to 37 ± 1.0°C) was dropped onto the gauze covering the open end of 700 mL plastic containers that housed the mosquitoes. To determine the virus titer of the blood meal at the commencement and cessation of feeding, samples of the blood/virus suspension were diluted 1:20 in growth media (GM; Opti-MEM [Invitrogen, Grand Island, NY] with 3% fetal bovine serum, antibiotics and fungizone) and stored at −70°C for later titration.

After 18 hr, mosquitoes were anaesthetized with CO₂, and blood engorged mosquitoes were placed into 1 L containers within an environmental growth cabinet (Sanyo Electric, Gunma, Japan) maintained at 28°C, 70–75% RH, and 12:12 (L:D), and offered 10% sucrose as a nutrient source. Mosquitoes were held under such conditions for an extrinsic incubation period of 13 d. To determine if the mosquitoes intended for the infection of the flying foxes were indeed infected, a sample of ≤ 6 individual mosquitoes were removed on day 12 post virus exposure and tested for the presence of virus.

**Flying fox husbandry.** On the afternoon before the infection experiments, flying foxes were moved from ARI to QHFSS and placed in individual cages in a room within the PC3 animal house. The dimensions of the cages were 900 × 900 × 900 mm, and were of wire mesh construction (25 × 25 × 25 mm aperture), elevated 1 m above the ground, with 10–15 cm between cages. Seasonal fruits (including rockmelon, bananas, mangoes, apples, etc.), supplemented by full cream milk powder, were fed to the flying foxes between 4 and 6 pm daily; water was provided ad libitum via drip bottles. Artificial light was provided for the flying foxes each day between the hours of usual sunrise and sunset.

**Exposure of flying foxes to JEV.** Individual flying foxes were restrained, placed in calico bags, and transferred from the animal room to the insectary, located within the QHFSS PC3 insectary/animal house. Before infection and transmission procedures, flying foxes were anesthetized as described previously. Ten flying foxes were exposed to JEV via mosquito bite, during two separate trials, each involving five flying foxes. Fifteen donor mosquitoes (previously exposed to JEV), in 30 × 40 mm containers with gauze covering the open end, were allowed to feed on the upper leg for ≤ 15 min. At the same time, 0.5 mL blood samples were obtained from the propatagial vein and 50 μL diluted 1/10 in GM for virus detection. The serum fraction of the remaining 450 μL was retained for serology. Animals were also weighed and rectal temperatures obtained during each flying fox/mosquito interaction. After recovering from the anesthesia (usually within 5 min), flying foxes were returned to their cages. To determine the virus titer of donor mosquitoes at the time of exposure, mosquitoes were killed with CO₂ and blood engorged mosquitoes stored at −70°C to await analysis. Five additional animals were exposed to JEV by being inoculated subcutaneously in the upper thigh area with 0.1 mL of 10^4.8 PS-EK TCID₅₀/mL of virus.

Flying foxes were observed at least twice daily for clinical symptoms of encephalitis (including fever, reduced appetite, ruffled fur, decreased grooming, increased vocalization, ataxia, eye closure, muscular rigidity, etc.) caused by infection with JEV.

To determine if infected bats could produce a viremia capable of infecting mosquitoes, batches of recipient mosquitoes were allowed to feed on the upper leg, interfemoral membrane, or the plagiopatagium of each flying fox on days 1–5, 7, 9, 14, 20/21, 23/28 post exposure for the mosquito-exposed animals, and days 3–5 for the inoculated animals. Because of unforeseen external circumstances independent of this study, the first series of infections were concluded on day 23 instead
mosquitoes were pooled in batches of dilutions on confluent PS-EK cell monolayers. Recipient and filtered using a 0.2 μm filter. Mosquitoes were homogenized in 1 mL of tissue culture medium to determine viral titers of donor mosquitoes, whole individual unit (PFU) of virus, and was deemed to be not detected. To replicate when inoculated onto PS-EK cells.

Virus assay. The blood/virus mixture used for mosquito infection was titrated as 10-fold dilutions in a 96-well microtiter plate containing confluent PS-EK cell monolayers. Plates were incubated at 37°C with 5% CO2 and checked daily for any cytopathic effect (CPE) for ≤7 d. The amount of virus ingested by individual mosquitoes was estimated based on an average blood meal volume of approximately 3 μL. The heads and bodies of the representative sample of donor mosquitoes were homogenized separately in 1 mL of GM using a SPEX 8000 mixer/mill (Speks Industries, Edison, NJ). Viral RNA was extracted using the QIAamp Viral RNA kit (Qiagen, Clifton Hill, Australia) before being tested for JEV using a real time TaqMan reverse transcriptase-polymerase chain reaction procedure (RT-PCR). In this assay, a cycle threshold (Ct) value of 40 corresponded to < 0.001 of one plaque-forming unit (PFU) of virus, and was deemed to be not detected. To determine viral titers of donor mosquitoes, whole individual mosquitoes were homogenized in 1 mL of tissue culture medium and filtered using a 0.2 μm filter, before being titrated as 10-fold dilutions on confluent PS-EK cell monolayers. Recipient mosquitoes were pooled in batches of ≤ 12 according to bat number and day post exposure. Pools of recipient mosquitoes were then homogenized in 1 mL of GM. Viral RNA was extracted from the supernatant using the QIAamp Viral RNA kit before being tested using the TaqMan RT-PCR. Infection rates were calculated for the pools of recipient mosquitoes using the PooledInfRate statistical software package.

To test for viremia, all blood samples collected from the first five flying foxes exposed to JEV by mosquito bite, were initially titrated as serial 10-fold dilutions on PS-EK cell monolayers as described previously. When virus was not detected using the cell culture system, the blood samples from this trial and from the remaining flying foxes exposed to JEV by mosquito bite and by inoculation were screened using the TaqMan RT-PCR. Any positive blood samples in the RT-PCR were titrated as 10-fold dilutions on PS-EK cell monolayers as described above.

Serology. To establish if seroconversion to JEV had occurred in flying foxes after JEV exposure, an enzyme-linked immunosorbent assay (ELISA) was developed using peroxidase conjugated Protein A/G to monitor IgG response. Porcine sera, which had previously been shown to contain JEV-specific antibodies, were used for assay optimization and as positive controls. Negative controls consisted of porcine sera, which had previously tested negative for flavivirus antibodies. The ELISA was performed using Maxisorp strips (NUNC A/S, Roskilde, Denmark), which were coated with inactivated JEV antigen in carbonate buffer (pH 9.6). After overnight incubation at 4°C, the strips were washed five times in phosphate buffered saline (PBS)-TWEEN. Sera collected from the 15 flying foxes during the experiments were added at a 1/100 dilution to the wells and incubated for 1 hr at 37°C before a second wash. The presence of JEV-specific antibody was detected by the addition of ImmunoPure Protein A/G-peroxidase conjugate (Pierce, Rockford, IL) at a dilution of 1/160,000. The conjugate was incubated for 1 hr at 37°C. The strips were again washed before the addition of KBlue substrate (Neogen, Lexington, KY) for 10 min. The reaction was stopped by the addition of 1N H2SO4. Absorbances were measured at 450 nm with a reference wavelength of 650 nm.

RESULTS

Virus titer of donor mosquitoes. After 13 d extrinsic incubation, 1–7 infected donor mosquitoes fed on each of the flying foxes. The virus titer of donor mosquitoes ranged between 10^1.0 and 10^1.1 TCID50 per mosquito (Table 1).

Clinical symptoms of JEV infection in the flying foxes. None of the flying foxes displayed any clinical symptoms of encephalitis consistent with infection by JEV.

Viremia. None of the flying foxes exposed to JEV by mosquito bite developed a detectable viremia. Only one inoculated flying fox (microchip no. 3003) developed a viremia on the fourth day post inoculation. However, this viremia was of a low level, as evidenced by the high cycle threshold score of 36 cycles in the TaqMan RT-PCR and a lack of detectable viral replication when inoculated onto PS-EK cells.

Infection of recipient mosquitoes. A total of 2,464 recipient mosquitoes in 305 pools were processed for detection of JEV RNA. Of these mosquitoes, 2,191 had fed on the flying foxes that had been infected by mosquito bite and 273 on the inoculated flying foxes. JEV RNA was detected in 7 pools of recipient mosquitoes that had fed on 4 flying foxes, indicating virus transmission (Table 2). Recipient mosquitoes were infected on either Days 3, 4, or 5 post exposure and infection rates for these pools ranged between 38.5 and 90.9 per 1,000 mosquitoes.

Antibody response to infection. Sixty percent (6/10) of the flying foxes that were exposed to JEV via mosquito had detectable IgG responses in the Protein A/G ELISA, indicating a seroconversion to JEV (Figure 1). In the experiments using inoculation as the source of infection, 100% of the flying foxes seroconverted to JEV (Figure 2).

Table 1

<table>
<thead>
<tr>
<th>No. of infected donor mosquitoes (no. fed)</th>
<th>Mean (range) titer of infected mosquitoes†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flying fox no.*</td>
<td></td>
</tr>
<tr>
<td>3924</td>
<td>1 (4)</td>
</tr>
<tr>
<td>9919</td>
<td>2 (6)</td>
</tr>
<tr>
<td>8309</td>
<td>2 (4)</td>
</tr>
<tr>
<td>2258</td>
<td>2 (5)</td>
</tr>
<tr>
<td>1459</td>
<td>6 (9)</td>
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<tr>
<td>3023</td>
<td>6 (6)</td>
</tr>
<tr>
<td>3466</td>
<td>4 (7)</td>
</tr>
<tr>
<td>6594</td>
<td>3 (4)</td>
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<tr>
<td>3332</td>
<td>5 (7)</td>
</tr>
<tr>
<td>2355</td>
<td>7 (11)</td>
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</tbody>
</table>

*Flying fox number refers to the microchip number.
†Titer expressed as log10 PS-EK TCID50 per mosquito.
‡Threshold of detection was 1.0 log10 PS-EK TCID50 per mosquito.
As shown by serologic studies, numerous animals are naturally infected with JEV,\textsuperscript{27,28} although humans and horses are the only animals that develop severe and fatal disease, characterized by acute encephalitis. In some instances, infection in pigs can result in abortion and stillbirth in sows, and reduced sperm production in boars.\textsuperscript{29,30} In the current study, infection with JEV did not result in overt clinical illness in any of the flying foxes. These results support previous studies of both Microchiropteran and Megachiropteran bats, in which experimental infection with JEV failed to produce any signs of encephalitis, even if inoculated directly into the brain.\textsuperscript{13–15}

Common opinion considers that virus circulating in the blood is essential to facilitate ingestion by a mosquito and that a threshold level of viremia must be reached before infections of vectors can occur.\textsuperscript{31} Therefore, infection of recipient mosquitoes after feeding on flying foxes without detectable viremia was unexpected. However, this is not a novel phenomenon, as earlier experiments have shown that recipient mosquitoes can be infected after feeding on horses and Agile wallabies (\textit{Macropus agilis}) without detectable viremia that had previously been exposed to JEV and Murray Valley encephalitis virus, respectively.\textsuperscript{32,33}

In our study, we used a sensitive real-time RT-PCR, which is able to detect viral RNA from 0.005 PFU of virus.\textsuperscript{23} Therefore, it should have been more likely to detect viral RNA than earlier methods of virus assay used in vertebrate studies. Importantly, the results of this study emphasize the need to use highly susceptible recipient arthropods to show transmission when assessing the relative ability of vertebrate species to serve as amplifying hosts of arboviruses.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{IgG antibody response of flying foxes exposed to Japanese encephalitis virus (JEV) by infected mosquitoes, as detected by enzyme-linked immunosorbent assay (ELISA).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{IgG antibody response of flying foxes exposed to Japanese encephalitis virus (JEV) by inoculation, as detected by enzyme-linked immunosorbent assay (ELISA).}
\end{figure}

DISCUSSION

This cryptic viremia may be explained by the replication of virus in skin or dendritic cells at the site of inoculation of the flying fox, with or without entering the bloodstream. In our experiments, some recipient mosquitoes fed at the same site (i.e., the upper leg) as the donor mosquitoes and may have subsequently imbibed virus liberated from the cells and tissues damaged by the action of the probing mouthparts. Indeed, West Nile virus (WNV), a member of the JEV serologic group, has been shown to replicate in the skin cells of eastern grey squirrels (\textit{Sciurus carolinensis}) and mice at the site of inoculation, with viral loads in the latter species increasing from an undetectable level on the first day post exposure to a titer of $\leq 10^6$ PFU/g at day 7 post exposure.\textsuperscript{34,35} Initial infection of skin tissue is possibly caused by the deposition of virions extravascularly in tissue surrounding capillaries and not directly into the vascular system while mosquitoes are probing and locating blood vessels.\textsuperscript{9,37}

This is a different concept to purported nonviremic transmission (NVT) of arboviruses, where infection of recipient mosquitoes with WNV occurs when they feed simultaneously with infected donor mosquitoes and, importantly, before viral replication in the host.\textsuperscript{38} When McGee and others\textsuperscript{39} assessed the effects of time and space on NVT, recipient mosquitoes could be infected $\leq 45$ min after the donor mosquitoes had fed. However, in our experiments, transmission to recipient mosquitoes occurred 3 to 5 d after exposure, providing adequate time for the virus to replicate at the site of inoculation. Further experiments are required to elucidate whether recipient mosquitoes can be infected while probing cells and tissues before cannulation of the blood vessel.

Although infection rates in recipient mosquitoes were relatively low in this experiment, the large number of animals in \textit{P. alecto} colonies, in which populations can reach tens of thousands,\textsuperscript{40} may provide sufficient numbers of hosts to infect local mosquitoes. Indeed, large flying fox colonies are present on some Torres Strait islands where JEV activity has been observed, including Badu Island, the island that has yielded the majority of JEV isolates. Furthermore, analysis of mosquito host feeding patterns has revealed that \textit{Cx. annulirostris} does feed on flying foxes on Badu Island (van den Hurk AF, Hall-Mendelin S, and Cheah WY, unpublished data).

Only a small number of infected flying foxes would be required to facilitate spillover of JEV into local bird and/or pig populations via mosquito bite and thus initiate epizootic

\begin{table}[h]
\centering
\caption{Infection rates (IRs) of pools of infected recipient mosquitoes that were allowed to feed on Japanese encephalitis virus (JEV) exposed flying foxes*}
\begin{tabular}{|cccc|c|}
\hline
Bat no. & Source of exposure & Day post exposure & No. mosquitos & No. PCR positive pools (no. pools tested) & IR per 1,000 mosquitoes \\
\hline
1459 & Mosquito & 3 & 24 & 2 (3) & 83.3 \\
3466 & Mosquito & 4 & 22 & 1 (3) & 38.5 \\
3003 & Inoculation & 4 & 26 & 2 (2) & 90.9 \\
0752 & Inoculation & 5 & 16 & 1 (4) & 62.5 \\
\hline
\end{tabular}
\label{table2}
\end{table}

* PCR = polymerase chain reaction.
activity. Importantly, because there is considerable movement of *P. alecto* between Cape York Peninsula, the Torres Strait, and Papua New Guinea, it is conceivable that flying foxes may have introduced JEV into northern Australia from the New Guinea landmass, as suggested by Mackenzie and others. Flying foxes can travel > 50 km in a single night, so that it would take approximately 2 and 4 d for a flying fox to traverse the distance between southern Papua New Guinea and Badu Island, or Cape York Peninsula, respectively. This is well within the 3–5 d time period after JEV exposure that flying foxes were able to infect recipient mosquitoes in our experiments. However, whereas flying foxes undoubtedly undertake foraging flights to different islands, such sporadic activity could not account for the synchronicity of the widespread appearance of JEV on eastern Torres Strait islands. This phenomenon suggests an alternative mechanism of incursion, such as wind-blowen mosquitoes, may be more likely.

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