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FLYING FOXES AS CARRIERS OF PATHOGENIC *LEPTOSPIRA* SPECIES

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ABSTRACT: Recent serologic studies have identified flying foxes (*Pteropus* spp.) as carriers of leptospirosis; however, little is known about the role of flying foxes as carriers of pathogenic *Leptospira* spp. To determine if Australian *Pteropus* spp. are carriers of pathogenic *Leptospira* spp., TaqMan real-time polymerase chain reaction (PCR) was used to detect leptospiral DNA in kidney and urine specimens from four species of flying fox, including the spectacled flying fox (*Pteropus conspicillatus),* black flying fox *(Pteropus alecto),* grey-headed flying fox *(Pteropus poliocephalus),* and little red flying fox (*Pteropus scapulatus).* Of the 173 kidney samples tested, 19 (11%) were positive for leptospiral DNA. Positive individuals were detected in all four species; significant differences in prevalence were not detected between species, between species within the same geographic area, or between geographically separated samples from the same species. Of the 46 urine samples tested, 18 (39%) tested positive by PCR, confirming that flying foxes shed leptospires into the environment. The detection of leptospiral DNA in the kidneys and urine of flying foxes suggests that flying foxes are carriers of pathogenic *Leptospira* spp. No evidence collected in the present study, however, suggests that flying foxes pose a significant risk of leptospirosis to the wider community or that humans who are in regular, close contact with flying foxes are at risk for leptospirosis.

Key words: Flying fox, leptospirosis, polymerase chain reaction, *Pteropus*.

INTRODUCTION

Leptospirosis is a re-emerging bacterial zoonosis that affects both domestic and wild animals as well as humans (Hartskeerl and Terpstra, 1996). This acute, febrile disease occurs in humans and animals worldwide and is potentially lethal with involvement of the hepatic, renal, and central nervous systems (Queensland Health, 2002). Wildlife species often are involved in both the maintenance and the spread of leptospirosis to livestock and humans (Cirone et al., 1978), and they include a variety of wild animals, such as rodents, monkeys, camels, toads, platypus, and sea lions (Faine, 1994).

Initial evidence of infection with *Leptospira* spp. in Australian flying foxes was reported by Smythe et al. (2002a) based on detection of leptospiral antibodies in all four species of flying fox from mainland Australia. The development of a real-time polymerase chain reaction (PCR) for the detection of pathogenic leptospira (Smythe et al., 2002b) has facilitated the abilities to detect leptospiral DNA rapidly, to differentiate between pathogenic and saprophytic strains of leptospirosis, and to provide relative estimates for the number of leptospires present in an individual sample.

In recent years, considerable research has been conducted on the relationship between emerging and zoonotic diseases in Australia and Southeast Asia and pteropid bats (Smythe et al., 2002a). Pteropid bats have been implicated as a natural host of Hendra virus (Rogers et al., 1996; Field et al., 2000) and may be involved in the natural history of Australian bat lyssavirus and Menangle virus (Philbey et al., 1998) as well as Nipah virus in peninsular Malaysia (Field et al., 2001).

Little is known about the role of flying foxes as carriers of pathogenic *Leptospira* spp. Leptospiral serovars were isolated from bats in Indonesia (Alston and Broom, 1958), and leptospiral antibodies have been identified in the spectacled flying fox (*Pteropus conspicillatus*) (Emanual et al., 1964). Leptospiral antibodies also have

been identified in long-tongued fruit bats (*Sycnycteris crassa*), fruit bats (*Dobsonia moluccensis*), and a single flying fox (*Pteropus temmicki*) on the Molucca Islands in Indonesia (Van Peenen et al., 1971). More recently, preliminary findings by Smythe et al. (2002a) suggest a previously unrecognized role of pteropid bats in the natural history of leptospirosis, including the transmission of leptospira to other species residing under flying fox roosts and an endemic leptospiral infection in pteropid species. Results from serologic testing of pteropid bats suggest the presence of seven *Leptospira* serovars, including *L. interrogans* serovar cynopteri, which is considered to be exotic to Australia (Smythe et al., 2002a).

Flying foxes may make no significant contribution to the leptospiral transmission cycle, or they may be an as-yet-unidentified carrier, facilitating the spread and maintenance of leptospiral populations to other species, including humans. They also may provide a pathway for the introduction of exotic serovars (Smythe et al., 2002a) through contact with pteropid colonies on the islands off northern Queensland, Australia.

In the present study, samples of kidney and urine from four *Pteropus* species were tested by PCR to determine if flying foxes are carriers of pathogenic *Leptospira* spp. and if they shed *Leptosira* spp. into the environment. Potential effects of species and geographic location on prevalence of infection also were evaluated.

MATERIALS AND METHODS

Kidney samples from the four *Pteropus* species found on mainland Australia were analyzed by TaqMan PCR (Smythe et al., 2002b); urine samples from flying foxes on Indooroopilly Island, Brisbane, Queensland, Australia, also were tested. All flying foxes were collected by the Queensland Department of Primary Industries (QDPI) Animal Research Institute, Yerongpilly, Queensland, Australia, as part of other studies primarily related to research on Lyssavirus and Hendra virus. Samples used in the present study were from northern and eastern Australia.

Whole kidneys were removed from individual animals, and a longitudinal cross-section was taken and placed in a collection jar. From this cross-section, a 25-mg tissue sample (~2 mm^3) was taken from the renal tubule area, where leptospires are known to colonize. Tissue samples were lysed overnight at room temperature. The DNA was extracted from kidney samples using the QIAamp DNA Mini Kit (catalog no. 51404, lot no. 11238137) and QIAamp DNA Blood Mini Kit (catalog no. 51306, lot no. 11243081; Qiagen, Doncaster, Victoria, Australia). The DNA from urine samples was provided by the Virology Section, Queensland Health Scientific Services (QHSS), Brisbane, Queensland, Australia. Urine samples were collected by the QDPI from flying foxes on Indooroopilly Island (27°59′23″S, 149°18′48″E). Each sample represented a pool of eight individuals. Duplicates of each DNA sample were tested and analyzed by TaqMan PCR using an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer, Applied Biosytems, Foster City, California, USA) as described previously (Smythe et al., 2002b). Leptospiral tissue levels were estimated based on cycle threshold (C_t) values and were categorized as heavy (C_t<27), moderate (C_t=27–35), or light (C_t>35). For positive control, C_t values ranged from 19 to 21.

Prevalence data were analyzed by Fisher's exact test using the SAS FREQ procedure (Version 8.2; SAS Institute Inc., Cary, North Carolina, USA). The Cramer's *V* test (crv) (Kendall and Stuart, 1979) also was used to analyze prevalence data related to both species and location. For this test, a value of +1 or −1 indicates a perfect association between samples and, therefore, a strong correlation between samples. A value of zero indicates no association between samples.

RESULTS

Of the 173 kidney samples tested, 19 (11%) were positive for leptospiral DNA. Positive results were detected from all four flying fox species, and prevalence varied between populations (Table 1). Based on C_t values, the number of leptospires in kidney samples was classified as high in 42% of the positive samples, moderate in one sample from *Pteropus alecto* (5%), and low in the remaining 53% of positive samples. The percentage of individuals testing positive for leptospiral DNA varied considerably within each species: *P. alecto*, 16%; *P. conspicillatus*, 4%; *Pteropus polioce-*

TABLE 1. Sample distribution and the number of flying foxes testing positive by polymerase chain reaction for leptospiral DNA.

Species	Location[a]	Sample size	% Positive
Pteropus alecto	Southeast Queensland	40	11
	Northern Territory	49	20
Pteropus conspicillatus	North Queensland	24	4
Pteropus poliocephalus	Southeast Queensland	35	6
	New South Wales	7	0
Pteropus scapulatus	Southeast Queensland	18	29
Total		173	

[a] All locations are in Australia.

phalus, 5%; and *Pteropus scapulatus,* 29%. Using both Fisher's exact test and Cramer's *V* test, significant differences in prevalence were not detected between geographically separated populations of the same species ($P=0.5358$, crv=−0.0921), between species in the same geographic area ($P=0.1870$, crv=0.1917), or between all four species of flying fox found on mainland Australia ($P=0.1326$, crv=0.1815).

The PCR results for the *P. alecto* samples from the Northern Territory were compared to previously reported serologic results as determined using the Microscopic Agglutination Test (MAT) (Smythe et al., 2002a). No clear association was found between serologic status and infection as detected by PCR, with only 47% of the seropositive samples also testing positive by PCR (Table 2). Of the 46 urine samples from the flying fox population on Indooroopilly Island, 18 (39%) were positive for leptospiral DNA, with C_t values of 32–40.

DISCUSSION

The detection of pathogenic leptospiral DNA in 11% of flying fox kidney samples indicates that flying foxes are carriers of pathogenic *Leptospira* spp. Because lep-

TABLE 2. Positive polymerase chain reaction results from 49 *Pteropus alecto* from the Northern Territory, Australia, and their corresponding serologic status.

Sample	Cycle threshold	Serology	MAT results[a]
1	22.12	-ve[b]	<50
2	27.57	Australis/Panama	100/200
3	28.67	-ve	<50
4	31.60	Australis	50
5	34.64	Australis	200
6	35.15	Australis/Cynopteri	100/100
7	36.29	Cynopteri	100
8	38.85	-ve	<50
9	40.00	Cynopteri	400
10	40.00	Cynopteri	200
11	40.00	Australis	100
12	40.00	Australis	50
13	40.00	Australis	50
14	40.00	Australis	50
15	40.00	Cynopteri	800
16	40.00	Australis	50
17	40.00	Australis	50

[a] Microagglutination test (MAT) results of less than 50 are considered to be negative. Serologic results were reported previously by Smythe et al. (2002a).
[b] -ve=negative.

tospires do not colonize the kidneys of noncarrier species (Hartskeerl and Terpstra, 1996; Faine et al., 1999), the detection of leptospires in kidney samples is expected to be minimal if the species is not a carrier (~1%, as seen in human hospital admissions) (Yersin, 1995). The identification of one in 10 animals with leptospiral DNA suggests that leptospires are more prevalent in flying fox populations than would be expected for a species that is not a carrier.

The highest prevalence was observed in *P. scapulatus,* with 29% of kidney samples testing positive by PCR. *Pteropus conspicillatus,* which is found in northern Queensland, where more than half of Australia's human leptospirosis cases occur, had the lowest prevalence, with 4% of the sample testing positive by PCR. Leptospires were not detected in the New South Wales population of *P. poliocephalus,* but this was not unexpected because of the small sample size of only seven individuals. Because of these negative results, we did not test for potential differences in prevalence between geographically separated *P. poliocephalus* populations; however, negative results from this population were included for species comparisons.

The limited agreement between previously reported serologic results from the Northern Territory *P. alecto* samples (Smythe et al., 2002b) and PCR results from the present study indicate that negative serologic result do not necessarily rule out carrier status (Table 2). Of the 17 samples tested, only 8 MAT-positive samples (47%) had positive PCR readings. It is not uncommon for animals that are infected with leptospires to have negative serologic results (Merien et al., 1995), and some carriers excrete spirochetes in their urine even after becoming seronegative (Day et al., 1998; Faine et al., 1999). The detection of leptospiral DNA in urine further confirms that the flying foxes are carriers and that flying foxes are shedding the bacteria into the environment.

The identification of flying foxes as carriers of pathogenic *Leptospira* spp. does not imply that flying foxes contribute to human cases of leptospirosis. While leptospiral DNA has been detected in flying fox kidneys, specific serovars have not been identified, and it is unknown if the serovars associated with human leptospirosis are present. Additional work that includes the culture and identification of *Leptospira* spp. from flying foxes and assessment of the leptospiral antibody status of humans who have close contact with flying foxes is warranted.

Our data confirm the suggestion by Smythe et al. (2002a) that leptospiral infection is endemic in Australian flying foxes and that pteropid bats have an unidentified role in the natural history of leptospirosis. As such, these endemic infections are not expected to impact flying fox populations negatively. The present findings, however, are significant, because they suggest a previously unrecognized vector of a re-emerging disease. Without supporting culture data, it is difficult to draw conclusions about the role that flying foxes play in the transmission of leptospires to species that reside beneath flying fox roosts and about the role that flying foxes play in the introduction of exotic serovars to Australia. The introduction of exotic serovars provides a potential for the establishment of leptospira colonization in new carrier species and for the transmission of new serovars to known carrier species (Smythe et al., 2002a). The identification of specific serovars within these populations is required to gain a sound understanding of the role played by flying foxes in the maintenance and transmission of pathogenic *Leptospira* spp.

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