

Use of PCR to Identify *Leptospira* in Kidneys of Big Brown Bats (*Eptesicus fuscus*) in Kansas and Nebraska, USA

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ABSTRACT: Bats have been implicated as potential carriers of *Leptospira* as a result of surveys, mostly in Australia and South America. We measured the prevalence of pathogenic leptospires in kidneys of bats from Kansas and Nebraska. From 7 August 2012 to 21 August 2012, we extracted DNA from kidneys of 98 big brown bats (*Eptesicus fuscus*) submitted and found negative for rabies. The DNA was processed in a two-step, seminested PCR assay with a dual-labeled Taqman probe specific for pathogenic leptospires. As a negative control, we used a saprophytic leptospire (*Leptospira biflexa* Patoc) and, as a pathogenic control, *Leptospira interrogans* Canicola. All bat kidneys were negative for pathogenic leptospires, suggesting that it is unlikely that the big brown bat, one of the most prevalent bat species in North America, is a reservoir for transmission of leptospires to dogs or humans.

Key words: Big brown bat, *Eptesicus fuscus*, leptospirosis, PCR.

Leptospirosis is a worldwide zoonosis transmitted through direct or indirect contact with urine (Bharti et al. 2003). The principal domestic animal sources implicated in human leptospirosis are cattle (*Bos primigenius*), pigs (*Sus scrofa*), and dogs (*Canis lupus familiaris*). Wildlife sources commonly implicated in both human and canine leptospirosis include rodents (Rodentia) and raccoons (*Procyon lotor*; Levett 2001). Evidence of leptospiuria and incidents of leptospirosis acquired from other wildlife sources, including flying squirrels (*Graecomys volans*), skunks (*Mephitis mephitis*), foxes (Canidae), elephant seals (*Mirounga angustirostris*), and sea lions (*Zalophus californianus*) have been documented, but the low frequency of direct or indirect contact of these species with humans and dogs makes them of less concern (Galton et al. 1959; Roth et al.

1963; Colagross-Schouten et al. 2002; Colegrove et al. 2005; Masuzawa et al. 2006).

Studies of the risk factors for leptospirosis in dogs have suggested that there is greater risk to dogs residing in urban as compared with rural areas (Alton et al. 2009; Raghavan et al. 2011). The peri-urban wildlife of greatest concern are rats (*Rattus* spp.), mice (*Mus* spp.), and raccoons (*Procyon lotor*). Bats (Chiroptera) are prevalent in urban areas, often roosting in accessible parts of houses, and guano and bat urine contaminate the area below the roosting site. There is abundant evidence that bats can be naturally infected with and excrete pathogenic leptospires, yet no study has conclusively demonstrated that the bats are reservoir hosts for infection in humans or dogs (Bunnell et al. 2000; Cox et al. 2005). In 2009, a reported case of human leptospirosis in Chicago, Illinois, USA, was attributed to contact with a bat in a swimming pool, but the species of bat was not identified, nor was the bat tested for *Leptospira* (Vashi et al. 2009).

One of the most prevalent species of bat in the US is *Eptesicus fuscus*, the big brown bat, and it is common in urban centers (Bat Conservation International 2013). We used PCR to investigate the prevalence of pathogenic *Leptospira* infection in kidneys of big brown bats.

All samples were taken from bats submitted to the Rabies Laboratory at Kansas State University, Manhattan, Kansas, USA, after they were confirmed negative for rabies. All bats were captured alive and euthanized (methods of euthanasia were not reported) by the submitting

agency prior to overnight shipment with a frozen gel pack (bats were not frozen). Pending the results of rabies testing, bats were refrigerated up to 8 hr prior to collection of both kidneys, which were then homogenized and extracted, or frozen until extraction could be performed. Information obtained about the bats included genus and species, type of building where the bat was caught or found, date of submission, and city.

A 0.5-cm² section of bat kidney was homogenized (FastPrep-24 High Speed Homogenizer, MP Biochemicals, Santa Ana, California, USA) in 1.5 mL of Dulbecco's Modified Eagle medium (Thermo Fisher Scientific, Hanover Park, Illinois, USA) at 5.0 M/sec (60 sec) and centrifuged (600 × G for 5 min) to pellet cell debris. Lysed tissue (50 µL) was removed for nucleic acid extraction using a viral isolation kit (MagMax Viral-96 RNA Extraction Kit, Life Technologies, Grand Island, New York, USA) on a magnetic particle processor (KingFisher 96 Flex Magnetic Particle Processor, Thermo Fisher Scientific), both according to manufacturer's protocols. Total nucleic acid from each sample was eluted in a final volume of 75 µL and stored at -20 C until PCR screening.

For the initial PCR, 5.0 µL of each DNA template was added to 22.5 µL of master mix (1 × PCR Buffer B, 4.0 mM MgCl₂, 800 nM dNTP mix, 2.5 U of Taq Polymerase (Fisher Bioreagents, Thermo Fisher Scientific); 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA); and 400 nM each of L737 forward primer (5'-GAC CCG AAG CCT GTC GAG-3') and L1218 reverse primer (5' GCC ATG CTT AGT CCC GAT TAC-3'; Woo et al. 1997; Harkin et al. 2003). The reactions were performed on a standard thermal cycler for 30 cycles at 95 C for 30 sec, 60 C for 1 min, and 72 C for 1 min. For each run, a no-template control of 10 mM Tris pH 8.0 and 1 mM ethylenediaminetetraacetic acid buffer, DNA extracted from a saprophytic control, *Leptospira biflexa* Patoc, and a

pathogenic control, *Leptospira interrogans* Canicola, each diluted 1:5,000, were used as DNA controls for the assay.

After centrifugation of the initial PCR reactions, 1.0 µL of each reaction was used as template for a second seminested PCR in 25-µL reaction volume. The master mix for this step was identical as for the initial PCR except that L1218 reverse primer was replaced with Lep2R reverse primer (5'-TTA TCC CCC GTA GTC TGA CTG C-3') and a dual-labeled Taqman probe (LEP883F-FAM Probe [5'-56-FAM/CTC CGA AAT AGG TTT AGG CCT AGC GTC AG/BHQ-1-3']) was added. The PCR was performed on a real-time PCR system (SmartCycler II, Cepheid, Sunnyvale, California, USA) using the following protocol: 94 C for 1 min with the optical sensor off, then 45 cycles at 94 C for 10 sec, 60 C for 20 sec, and 72 C for 30 sec, with the optical sensor on during the 72 C extension step. The initial and final fluorescence were recorded with the background off. With the background off, the initial and final raw fluorescence value for each sample was recorded, with the difference in these values calculated as the change in fluorescence. A positive sample was defined as a positive change in fluorescence greater than 50 fluorescent units. The PCR assay as reported here has a sensitivity of 93% and specificity of 100% (M.H. unpubl. data).

From 7 August 2012 to 21 August 2012, 98 big brown bats were found negative for rabies and were processed for testing for leptospirosis. Twenty-three bats were submitted from Kansas (Salina [nine bats], Manhattan [six], Kansas City metropolitan area [four], and one each from Wichita, Belleville, Emporia, and Hutchinson); 75 were from Nebraska (Lincoln [39], Omaha [21], Columbus [four], Osceola [two], and one each from Norfolk, Wayne, Seward, Hastings, McCook, Albion, Pender, Hartington, and O'Neill). With the exception of two bats found in university buildings, all bats were found in homes. All 98 bat

kidney samples were negative for pathogenic leptospires. Controls gave the anticipated results.

Although this was a convenience sampling of bats submitted for testing, the results of this study suggest that the big brown bat is not a carrier of pathogenic leptospires and is unlikely to be involved in their transmission to humans or dogs. We are unaware of any other studies investigating *Leptospira* in bats of North America.

Various detection methods used in studies outside of North America have documented that some bats are carriers of leptospirosis. On the island of Mayotte, five of 49 flying foxes (*Pteropus seychellensis*; 10%) were antibody positive, as were 11% of black rats (*Rattus rattus*), but unvaccinated dogs had a much higher prevalence (85.7%; Desvars et al. 2012). In a serologic survey of 271 flying foxes (*Pteropus poliocephalus*, *Pteropus scapulatus*, *Pteropus alecto*, and *Pteropus conspicillatus*) from Australia, Smythe et al. (2002) found that 75 of 271 (27.7%) were antibody positive. In another study from Australia investigating the same four species of flying foxes using a PCR assay, Cox et al. (2005) found 11% (19 of 173) positive when testing kidneys from flying foxes on the mainland, but 39% (18 of 46) positive when testing urine from flying foxes on Indooroopilly Island. In the Peruvian Amazon basin, Bunnell et al. (2000) found that kidneys from seven of 20 bats (35%) were PCR positive for pathogenic leptospires. Matthias et al. (2005), tested 589 bats from the same area and found only 20 positive using PCR on kidneys and three positive by culturing urine samples (overall 3.4%).

We speculate that feeding habitats may play a role in the likelihood of exposure of bats to leptospires. The big brown bat, being insectivorous, would have a lower risk of exposure than frugivorous bats, which might share their food with rodents. Bessa et al. (2010), working in São Paulo, Brazil, failed to identify any insectivorous

bats that were positive by serology or PCR (182 examined) but found six of 161 frugivorous or nectarivorous bats positive by PCR of kidney samples. In Denmark, Fennestad et al. (1972) used dark-field microscopy to examine urine or kidney suspensions of 166 insectivorous bats from four genera (*Myotis*, *Pipistrellus*, *Nyctalus*, and *Eptesicus*). Thirty-one bats (19%) were positive, and three of those had spirochetes identified in renal tubules using silver-stained histologic kidney sections. Whether those findings represent pathogenic leptospires is not known because the authors failed to culture leptospires from any sample.

The bats in this study were submitted from urban areas that have been shown to have cases of leptospirosis in dogs (Raghavan et al. 2011), yet we found no PCR-positive bats. Additionally, the bats were all submitted in August, just prior to the peak season (September–November) for leptospirosis in dogs identified by Raghavan et al. (2011). If big brown bats were a reservoir of *Leptospira*, one would expect to see the highest renal colonization rates immediately prior and during the peak season. Although bats may play a role in the transmission of leptospires in other parts of the world, we found no evidence that big brown bats from Kansas and Nebraska are carriers of *Leptospira*.

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