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Short communication

# Molecular epidemiology of pathogenic *Leptospira* spp. in the straw-colored fruit bat (*Eidolon helvum*) migrating to Zambia from the Democratic Republic of Congo

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ABSTRACT

The role played by bats as a potential source of transmission of *Leptospira* spp. to humans is poorly understood, despite various pathogenic *Leptospira* spp. being identified in these mammals. Here, we investigated the prevalence and diversity of pathogenic *Leptospira* spp. that infect the straw-colored fruit bat (*Eidolon helvum*). We captured this bat species, which is widely distributed in Africa, in Zambia during 2008–2013. We detected the flagellin B gene (*flaB*) from pathogenic *Leptospira* spp. in kidney samples from 79 of 529 *E. helvum* (14.9%) bats. Phylogenetic analysis of 70 *flaB* fragments amplified from *E. helvum* samples and previously reported sequences, revealed that 12 of the fragments grouped with *Leptospira borgpetersenii* and *Leptospira kirschneri*; however, the remaining 58 *flaB* fragments appeared not to be associated with any reported species. Additionally, the 16S ribosomal RNA gene (*rrs*) amplified from 27 randomly chosen *flaB*-positive samples was compared with previously reported sequences, including bat-derived *Leptospira* spp. All 27 *rrs* fragments clustered into a pathogenic group. Eight fragments were located in unique branches, the other 19 fragments were closely related to *Leptospira* spp. detected in bats. These results show that *rrs* sequences in bats are genetically related to each other without regional variation, suggesting that *Leptospira* are evolutionarily well-adapted to bats and have uniquely evolved in the bat population. Our study indicates that pathogenic *Leptospira* spp. in *E. helvum* in Zambia have unique genotypes.



## 1. Introduction

Leptospirosis is an important reemerging zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira*. The disease is found worldwide, especially in tropical regions. Human leptospirosis presents with a variety of signs and symptoms, including general febrile disease an influenza-like illness, and results in liver or kidney failure. As a result, this disease is often confused with other diseases, such as dengue fever, hemorrhagic fever and malaria, all of which are common in tropical and subtropical regions of the world (World Health Organization, 2003). Pathogenic *Leptospira* spp. can infect the renal tubules of most animals and are excreted in their urine, resulting in contaminated

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environments (e.g., soil and water) (Adler and de la Peña Moctezuma, 2010). Humans become infected mainly through *Leptospira*-contaminated water or soil, or from contact with urine from animals infected with this bacterium (Adler and de la Peña Moctezuma, 2010). Rodents are the most important reservoir of *Leptospira* among a variety of wildlife reservoirs.

Over the past decade, there have been many reports of bats being an important reservoir and vector of emerging infectious diseases, such as Ebola and Marburg viral diseases, severe acute respiratory syndrome (known as SARS), Nipah and Hendra viral infections, and rabies (Calisher et al., 2006). Bats (order Chiroptera) are the second largest order in mammals after rodents (order Rodentia) and are geographically widespread. Loss of habitat for bats, caused by recent anthropogenic activities, may increase contact between bats and humans, resulting in transmission of various pathogens from peridomestic bats to humans (de Jong et al., 2011). Transmission of viral pathogens from bats to humans has been the main focus of studies in this area; however, there have not been many studies on pathogenic bacteria in bats (Mühldorfer, 2013).

A variety of pathogenic *Leptospira* spp. have been identified in bats worldwide (Bessa et al., 2010; Bunnell et al., 2000; Cox et al., 2005; Fennestad and Borg-Petersen, 1972; Harkin et al., 2014; Lagadec et al., 2012; Matthias et al., 2005; Tulsiani et al., 2011); however, little is known about the role of bats in the transmission of leptospirosis.

In this study, we performed a molecular epidemiological investigation of *Leptospira* spp. in straw-colored fruit bats (*Eidolon helvum*) captured from 2008 to 2013, which were migrating from the Democratic Republic of Congo to Zambia (Richter and Cumming, 2008).

## 2. Materials and methods

A total of 529 kidney samples were collected from captured *E. helvum* that were roosting in trees (Muleya et al., 2014; Ogawa et al., 2015) in Kasanka National Park in Central Province and in Ndola in Copperbelt Province of Zambia (Table 1). This research was performed under the research project "Molecular epidemiology of bacterial zoonoses in Zambia" approved by the Zambia Wildlife Authority, in the Republic of Zambia.

The kidney samples collected from *E. helvum* were placed directly in Korthof or Ellinghausen–McCullough–Johnson–Harris (EMJH) media (World Health Organization, 2003) and homogenized for DNA extraction and *Leptospira* isolation by crushing with beads. DNA was extracted from 10% (w/v) kidney homogenates using a DNA Isolation Kit for Mammalian Blood (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions with minor modifications. A nested PCR based on the flagellin B gene (*flaB*) sequence was used to amplify the extracted DNA samples ($n = 529$) to detect the *flaB* gene of pathogenic *Leptospira* spp. (Koizumi et al., 2008). Some of the *flaB*-nested PCR-positive samples ($n = 27$) were examined further. To identify *Leptospira* species, we also performed a nested PCR based on the 16S ribosomal RNA gene (*rrs*) and the preprotein translocase gene (*secY*) using the primer sets shown in Supplementary Tables 1 and 2.

The PCR products from the *flaB*-nested PCR (732 bp including the 41 bp primer sequence), the *rrs*-nested PCR (~642 bp including the 48 bp primer sequence) and the *secY*-nested PCR (~329 bp including the primer sequence) were purified and subjected to direct sequencing using a BigDye Terminator v3.1 Cycle Sequencing a Kit (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions, and a 3130xl Genetic Analyzer (Life Technologies). The sequence data were aligned using the Clustal W software, and a maximum-likelihood phylogenetic tree was generated with 1,000 bootstrap replications using MEGA 5.2.2 software (Tamura et al., 2011).

The DDBJ accession numbers for the *flaB* and *rrs* sequences from the uncultured *Leptospira* spp. detected in *E. helvum* comprised LC005103 to LC005172 and LC005173 to LC005199, respectively (Supplementary Table 3).

## 3. Results and discussion

A 732 bp fragment of the *Leptospira flaB* gene was detected in 79 out of 529 *E. helvum* kidney samples (14.9%, Table 1). Among the 79 *flaB*-nested PCR-positive samples, 70 were used for direct sequencing and nine samples were not able to be sequenced because of insufficient DNA. Phylogenetic analysis (Fig. 1) revealed that the *flaB* sequences fell into seven clusters (FC1–FC7). Six *flaB* fragments (ZFB08-62, ZFB09-25, ZFB09-32, ZFB12-05, ZFB12-107 and ZFB12-110) in the FC5 cluster were related to the corresponding gene sequences, all of which were identical to *Leptospira borgpetersenii* strains including Jules, De 10, Arborea, Poi, and Veldrat Batavia 46. The six fragments shared sequence identities ranging from 96.2% to 96.4% with the *L. borgpetersenii* strains described above. The nucleotide identity of the *flaB* fragment for ZFB12-96 in the FC6 cluster with the *Leptospira kirschneri* strains Moskva V and 3522C was 95.5% and 95.4%, respectively. The nucleotide sequence identities of five *flaB* fragments (ZFB08-92, ZFB11-56, ZFB12-98, ZFB12-105 and ZFB13-104) in the FC7 cluster with Moskva V and 3522C *L. kirschneri* strains were 95.3% and 95.1%, respectively. The nucleotide sequence identities of the remaining 58 *flaB* fragments belonging to FC1 to FC4 with that of the closest species, *L. borgpetersenii*, were from 91.2% to 94.5%. In a previous report, *L.*

**Table 1**
Summary of the kidney samples analyzed from fruit bats and *Leptospira flaB* prevalence.

| Year | Sample ID | Location | No. of samples | | | No. of positives | | | Positive rate (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total | M | F | Total | M | F | Total | M | F |
| 2008 | ZFB08-01 – ZFB08-104 | Kasanka National Park | 104 | 38 | 66 | 28 | 10 | 18 | 26.9 | 26.3 | 27.3 |
| 2009 | ZFB09-01 – ZFB09-60 | Kasanka National Park | 60 | 15 | 45 | 7 | 2 | 5 | 11.7 | 13.3 | 11.1 |
| 2010 | ZFB10-01 – ZFB10-47 | Kasanka National Park | 47 | 13 | 34 | 4 | 1 | 3 | 8.5 | 7.7 | 8.8 |
| | ZFB10-48 – ZFB10-52 | Ndola | 4 | 3 | 1 | 1 | 1 | 0 | 25.0 | 33.3 | 0 |
| 2011 | ZFB11-01 – ZFB11-38 | Ndola | 38 | 18 | 20 | 3 | 0 | 3 | 7.9 | 0 | 15.0 |
| | ZFB11-39 – ZFB11-95 | Kasanka National Park | 57 | 24 | 33 | 7 | 4 | 3 | 12.3 | 12.5 | 9.1 |
| 2012 | ZFB12-01 – ZFB12-60 | Ndola | 60 | 22 | 38 | 4 | 2 | 2 | 6.7 | 9.1 | 5.3 |
| | ZFB12-61 – ZFB12-110[a] | Kasanka National Park | 49 | 15 | 34 | 18 | 7 | 11 | 36.7 | 46.7 | 32.4 |
| 2013 | ZFB13-01 – ZFB13-76 | Ndola | 76 | 23 | 53 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ZFB13-77 – ZFB13-111[b] | Kasanka National Park | 34 | 9 | 25 | 7 | 2 | 5 | 20.6 | 22.2 | 20.0 |
| Total | | | 529 | 180 | 349 | 79 | 28 | 50 | 14.9 | 15.6 | 14.3 |

[a] Kidney sample from ZFB12-97 was not available for PCR screening.
[b] Kidney sample from ZFB13-93 was not available for PCR screening.