Pathogenic Leptospira Species in Insectivorous Bats, China, 2015

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PCR amplification of the rrs2 gene indicated that 50% (62/124) of insectivorous bats from eastern China were infected with Leptospira borgpetersenii, L. kirschneri, and several potentially new Leptospira species. Multilocus sequence typing defined 3 novel sequence types in L. kirschneri, suggesting that bats are major carriers of Leptospira.

Leptospirosis is a zoonotic disease caused by the pathogenic spirochetes of the bacterial genus Leptospira (1). Although leptospirosis is mainly prevalent in tropical and subtropical countries (2), it is considered an emerging or reemerging zoonosis of global public health concern (3). In China, leptospirosis is listed as a category B notifiable disease (3). Globally, rodents are recognized as important reservoir hosts (4); however, a growing number of studies highlight the potential role of bats in the epidemiology of Leptospirosis (4). So far, knowledge on Leptospira in bats in China is lacking. Therefore, we screened archived bat kidney samples for Leptospira species to evaluate the potential role of bats in the ecology of Leptospira in China.

The Study

During July–October 2015, we captured 124 insectivorous bats with nets in Mengyin County, Shandong Province, China; the bats were initially intended for viral metagenomic analysis. Details regarding anesthetization of bats and tissue sampling were described previously (5). We collected the kidneys, storing them at −80°C until analysis. We identified bat species by using PCR amplification and DNA sequencing of the cytochrome B (cytB) gene as described previously (6). The 124 bats were classified into 4 species of the Vespertilionidae family (26 Eptesicus serotinus bats from 2 farmers’ houses, 30 Myotis fimбриatus bats and 10 M. ricketti bats from a city sewer, and 58 Myotis pecinius bats from a karst cave).

We designated bat kidney samples by using the abbreviation of Shandong plus the sample identification number (e.g., SD-49). We extracted DNA from bat kidney samples by using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. To identify the species of Leptospira in bats, we amplified the 16S rRNA gene (rrs2) by using nested PCR with primers Lepto 16S-1st-F, Lepto 16S-1st-R, Lepto 16S-2nd-F, and Lepto 16S-2nd-R (7). We cloned the rrs2 PCR products (642 bp) into the pMD 19-T vectors (TaKaRa, Shiga, Japan) and randomly picked 1 colony for Sanger sequencing using M13 universal primers on both DNA strands.

On the basis of rrs2 amplification results, 62 (50.0%) of 124 bats tested were positive for Leptospira. All the E. serotinus bats (0/26, 0.0%) were negative for Leptospira, whereas the M. fimбриatus bats (19/30 [63.3%]), M. ricketti bats (6/10 [60.0%]), and M. pecinius bats (37/58 [63.7%]) showed high rates of infection.

Using ClustalW with MEGA 7.0 (http://www.megasoftware.net), we aligned the rrs2 sequences from this study with reference Leptospira species downloaded from GenBank. We constructed a neighbor-joining phylogenetic tree (also by using MEGA 7.0) (8). On the basis of the rrs2 phylogeny, all Leptospira detected in bats in Mengyin County clustered into the pathogenic group and could be divided into ≥14 clades (clades A–N). Although clade H was most likely associated with L. borgpetersenii, the Leptospira detected in bats in Mengyin County were divergent from any known Leptospira species (Figure 1) and any previously published Leptospira sequences from bats (Figure 2); therefore, these organisms might represent potentially new Leptospira species. We deposited the 62 rrs2 sequences of Leptospira in the Mengyin County bats into GenBank (accession nos. MF498596–657).

To characterize the Leptospira species detected in bats from Mengyin County, we attempted multilocus sequence typing (MLST) on 7 housekeeping genes (glmU, pntA, sucA, tpiA, pfkB, mreA, and citA) as previously described (9). We assigned alleles for all 7 loci by using a publicly available Leptospira MLST website (https://pubmlst.org/leptospira) and defined sequence types (STs) by using the allelic profiles (glmU-pntA-sucA-tpiA-pfkB-mreA-citA).

Because we conducted MLST using kidney DNA rather than DNA from isolates, the results were arduous to
Figure 1. Neighbor-joining phylogenetic tree based on rrs2 gene of *Leptospira* isolates from bats, Mengyin County, Shandong Province, China, and reference *Leptospira* sequences from GenBank (boldface). We constructed the tree with the rrs2 sequences (642 bp) from this study by using the Kimura 2-parameter model with MEGA 7.0 (http://www.megasoftware.net); we calculated bootstrap values with 1,000 replicates. Sequences of *Leptospira* detected in bats in this study are shown with the GenBank accession number, the Latin name of the bat species in which *Leptospira* was detected, and the corresponding bat number. Only bootstrap values >75% are shown. Scale bar indicates nucleotide substitutions per site.
obtain. Only 35 of the 62 rrs2-positive bats were successfully amplified for ≥1 gene. For three samples, SD-49, SD-88, and SD-112, all 7 loci were obtained. We uploaded the allele data to the *Leptospira* MLST database and assigned a novel allele number for each gene (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/6/17-1585-Techapp1.pdf). According to the allelic profiles, we classified the organisms as *L. kirschneri*, assigning all 3 with novel STs (ST244 for SD-49, ST246 for SD-88, and ST245 for SD112).

We obtained 32 incomplete allelic profiles in all. After searching the *Leptospira* MLST database for each gene, we found that the loci of this study could not match with any known alleles and that they represented novel

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**Figure 2.** Neighbor-joining phylogenetic tree based on rrs2 gene of pathogenic *Leptospira* isolates from bats, Mengyin County, Shandong Province, China, and reference *Leptospira* sequences from GenBank that had been previously isolated from bats (boldface). We constructed the tree with bat *Leptospira* rrs2 sequences (446 bp) from this study and previous studies by using Kimura 2-parameter model with MEGA 7.0 (http://www.megasoftware.net); we calculated bootstrap values with 1,000 replicates. *Leptospira* detected in this study are shown with compressed clades, and *Leptospira* sequences from previous studies are shown with the GenBank accession number and the Latin name of the bat species in which *Leptospira* was detected. Only bootstrap values >75% are shown. Scale bar indicates nucleotide substitutions per site.
alleles; however, novel alleles could not be assigned for incomplete allelic profiles. Phylogenies based on each of the 7 genes showed inconsistent topologies for individual bats, indicating co-infection with different *Leptospira* species in the Mengyin County bats (online Technical Appendix Table, online Technical Appendix Figures 1–7). For SD-103, for example, we amplified 5 of the 7 genes. On the basis of *glmU, tpiA, pfkB*, and *mreA*, SD-103 clustered with *L. borgpetersenii*; however, on the basis of *sucA*, SD-103 fell into the group of the potentially new *Leptospira* sp. 2.

Because we did not conduct culture isolation with the archived bat kidney samples, further isolation with fresh bat kidney or urine samples will be needed to enable a thorough genotyping analysis of *Leptospira* species in the Mengyin County bats. Altogether, our study suggested that bats in Mengyin County carried a wide diversity of *Leptospira*, and the actual genetic diversity is likely even higher.

In our study, all the *E. serotinus* bats were negative for *Leptospira*, whereas *M. fimbriatus*, *M. ricketti*, and *M. pequinus* bats showed a high rate of infection. This finding might be explained by a widely accepted belief that *Leptospira* mainly circulate in humid environments (10). *E. serotinus* bats were captured from the eaves of 2 farmers’ houses, where the habitats were dry, whereas the *M. fimbriatus*, *M. ricketti*, and *M. pequinus* bats were sampled from the humid city sewer and karst cave.

So far, *Leptospira* has been detected in ≈50 bat species belonging to 8 bat families from tropical and subtropical regions as well as part of Europe (4). Although the role of bats as carriers of *Leptospira* associated with human leptospirosis remains uncertain, intrusion into bat habitats and increasing urbanization that results in the cohabitation of bats and humans is likely to increase the opportunity for batborne *Leptospira* spillover (11). Moreover, bats might play an important role in the epidemiology of *Leptospira* through transmission between bats and rodents, with rodents being a major source of human infection (12).

Conclusions

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About the Author

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Technical Appendix

| Bat species | Bat no. | glmU | pntA | sucA | tpiA | pfkB | mreA | caaB | ST  | Co-infection |
|-------------|---------|------|------|------|------|------|------|------|-----|--------------|
| *M. fimbriatus* | SD-88  | Lk (62) | Lk (73) | Lk (68) | Lk (63) | Lk (88) | Lk (85) | Lk (60) | ST246 | 1             |
| *M. fimbriatus* | SD-112 | Lk (63) | Lk (74) | Lk (69) | Lk (64) | Lk (89) | Lk (86) | Lk (60) | ST245 | 1             |
| *M. fimbriatus* | SD-124 | Lb     | N1    | N1    | N1    | N1    | N1    | N1    |      |              |
| *M. fimbriatus* | SD-122 | N1     | N1    | N1    | N1    | N1    | N1    | N1    |      |              |
| *M. fimbriatus* | SD-93  | Lb     | N2    | Lb    | Lb    | Lb    | Lb    | Lb    |      |              |
| *M. fimbriatus* | SD-103 | Lb     | N1    | N1/N2 | N1    | N1/Lb | N1    | N1    |      |              |
| *M. rickettii*   | SD-92  | N1     | N1    | N1/Lb | N1    | N1    | N1    |      |      | 2             |
| *M. pequinius*   | SD-49  | Lk      | Lk     | Lk    | Lk    | Lk    | Lk    | Lk    | ST244 | 1             |
| *M. pequinius*   | SD-10  | Lk      | Lk     | Lk    | Lk    | Lk    | Lk    |      |      | 1             |
| *M. pequinius*   | SD-15  | Lk      | Lk     | Lk    | Lk    | Lk    | Lk    |      |      |              |
| *M. pequinius*   | SD-36  | Lk      | Lk     | Lk    | Lk    |      |      |      |      | 1             |
| *M. pequinius*   | SD-21  | Lb     | N2    | Lb    | Lb    | Lb    | Lb    | Lb    |      | 1             |
| *M. pequinius*   | SD-23  | Lb     | N2    | Lb    | Lb    | Lb    | Lb    |      |      | 1             |
| *M. pequinius*   | SD-38  | Lb     | N2    | Lb    | Lb    | Lb    | Lb    |      |      |              |
| *M. pequinius*   | SD-30  | Lb     | N2    | Lb    |      |      |      |      |      | 1             |
| *M. pequinius*   | SD-62  | Lb     | N2    | Lb    |      |      |      |      |      | 1             |
| *M. pequinius*   | SD-41  | Lb     | N2    | Lb    |      |      |      |      |      |              |
| *M. pequinius*   | SD-39  | Lb     | N2    | Lb    |      |      |      |      |      |              |
| *M. pequinius*   | SD-25  | Lk     | Lk     | Lk    | Lk    | Lk    | Lk    | Lk    |      | 2             |
| *M. pequinius*   | SD-9   | Lk     | Lk     | Lk    | Lk    | Lk    | Lk    |      |      | 2             |
| *M. pequinius*   | SD-27  | Lk     | Lk     | Lk    | Lk    | Lk    | Lk    |      |      | 2             |
| *M. pequinius*   | SD-3   | Lb     | N2    | Lb    | N2    | Lb    | N2    |      |      | 2             |
| *M. pequinius*   | SD-40  | Lb     | N2    | Lb    | N2    | Lb    |      |      |      | 2             |
| *M. pequinius*   | SD-5   | Lb     | N2    | Lb    | N2    | Lb    |      |      |      | 2             |
| *M. pequinius*   | SD-69  | Lb     | N2    | Lb    | N2    | Lb    |      |      |      | 2             |
| *M. pequinius*   | SD-33  | Lb     | N2    | Lb    | N2    | Lb    |      |      |      | 2             |
| *M. pequinius*   | SD-37  | Lb     | N2    | Lb    | N2    | Lb    |      |      |      | 2             |
| *M. pequinius*   | SD-45  | Lb     | N2    | Lb    | N2    | Lb    |      |      |      | 2             |
| *M. pequinius*   | SD-26  | Lk     | N1    | N1    | N1    | N1    | N1    | Lk    |      | 2             |
| *M. pequinius*   | SD-31  | Lk     | N1    | N1    | N1    | N1    | N1    | Lk    |      | 2             |
| *M. pequinius*   | SD-16  | N1     | Lb    | N2    | Lb    | N2    | Lb    |      |      | 3             |
| *M. pequinius*   | SD-11  | Lb     | N2    | Lb    |      |      |      |      |      | 3             |
| *M. pequinius*   | SD-17  | N1     | N3    | N1    | N1    | N1    | Lk    |      |      | 3             |

For SD-49, SD-88, and SD-112, novel alleles assigned are shown in the parentheses. Lb, *L. borgpeterseni*; Lk, *L. kirschneri*; N1, potential new *Leptospira* sp. 1; N2, potential new *Leptospira* sp. 2; N3, potential new *Leptospira* sp. 3; N4, potential new *Leptospira* sp.
Technical Appendix Figure 1. Neighbor-joining phylogenetic tree for glmU gene of Leptospira in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura’s 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. Leptospira species are shown on the right, and potential new Leptospira species detected in this study are shown in red.
Technical Appendix Figure 2. Neighbor-joining phylogenetic tree for pntA gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura's 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.
**Technical Appendix Figure 3.** Neighbor-joining phylogenetic tree for *sucA* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura’s 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.
Technical Appendix Figure 4. Neighbor-joining phylogenetic tree for *tpiA* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura’s 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.
Technical Appendix Figure 5. Neighbor-joining phylogenetic tree for pfkB gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura’s 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.
Technical Appendix Figure 6. Neighbor-joining phylogenetic tree for mreA gene of Leptospira in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura’s 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. Leptospira species are shown on the right, and potential new Leptospira species detected in this study are shown in red.
Technical Appendix Figure 7. Neighbor-joining phylogenetic tree for *caiB* gene of *Leptospira* in bats, Mengyin County, Shandong Province, China. The tree was constructed with Kimura’s 2-parameter model using MEGA 7.0, and bootstrap values were calculated with 1,000 replicates. Alleles of each locus detected in this study are shown with the sample number highlighted with a triangle, and novel allele numbers assigned in this study are indicated in red. *Leptospira* species are shown on the right, and potential new *Leptospira* species detected in this study are shown in red.