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Short Communication

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Survey of Parasitic Bacteria in Bat Bugs, Colorado

Clifton D. McKee,[1,2,4] Lynn M. Osikowicz,[2] Teresa R. Schwedhelm,[2] Ying Bai,[2] Kevin T. Castle,[3] and Michael Y. Kosoy[2]

[1]Department of Biology, Colorado State University, 1878 Campus Delivery, Fort Collins, CO 80523, [2]Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, 3156 Rampart Road, Fort Collins, CO 80521, [3]Wildlife Veterinary Consulting LLC, 2751 Bianco Drive, Fort Collins, CO 80521, and [4]Corresponding author, e-mail: xmm9@cdc.gov

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Abstract

Bat bugs (*Cimex adjunctus* Barber) (Hemiptera: Cimicidae) collected from big brown bats (*Eptesicus fuscus* Palisot de Beauvoir) in Colorado, United States were assessed for the presence of *Bartonella*, *Brucella*, and *Yersinia* spp. using molecular techniques. No evidence of *Brucella* or *Yersinia* infection was found in the 55 specimens collected; however, 4/55 (7.3%) of the specimens were positive for *Bartonella* DNA. Multi-locus characterization of *Bartonella* DNA shows that sequences in bat bugs are phylogenetically related to other *Bartonella* isolates and sequences from European bats.

Key words: *Bartonella*, bat bugs, *Cimex*, Hemiptera

There is increasing evidence that bats host a wide variety of viruses and bacteria, including some that cause important zoonotic infections (Calisher et al. 2006, Mühldorfer 2013). Recent reports of *Bartonella* infections in humans have been linked to *Bartonella* species found in bats (Lin et al. 2010, Podsiadly et al. 2010, Veikkolainen et al. 2014, Urushadze et al. 2017). *Bartonella* infections in bats are prevalent, diverse, and globally distributed (McKee et al. 2016). Previous studies have detected *Bartonella* DNA in the ectoparasites of bats from the United States, including from bat bugs (Loftis et al. 2005; Reeves et al. 2005, 2006, 2007; Morse et al. 2012; Lilley et al. 2017).

Bat bugs (Hemiptera: Cimicidae) are common ectoparasites of bats (Frank et al. 2015) and are known to occasionally bite humans (Whyte et al. 2001). The detection of *Bartonella quintana* (Schmincke 1917) Krieg 1961 (Rhizobiales: Bartonellaceae) in bed bugs (*Cimex hemipterus*) from an African prison (Angelakis et al. 2013) and a subsequent study (Leulmi et al. 2015) demonstrating the acquisition of *B. quintana* by *quintana by Cimex lectularius* L. (Hemiptera: Cimicidae) and excretion of viable bartonellae in bug feces highlight cimicids as potential vectors of *Bartonella* spp. in humans and other animals. Experimental studies have shown that *Brucella* and *Yersinia* bacteria are capable of surviving in the gut of bed bugs (*Cimex hemipterus*) for many weeks (Tovar 1947, Burton 1963). However, there have been no reports of actual transmission of these bacteria by bed bugs. Recently, Bai et al. (2017) detected *Brucella* DNA in insectivorous bats from the Republic of Georgia. The only other evidence of *Brucella* infection in bats comes from an anecdotal report in vampire bats from Brazil based on serology (Ricciardi et al. 1976). *Yersinia enterocolitica* and *Y. pseudotuberculosis* have been isolated from bats from Europe (Mühldorfer et al. 2010). To clarify the role of bat bugs as potential vectors of zoonotic bacteria in bats, we used molecular methods to detect *Bartonella*, *Brucella*, and *Yersinia* DNA in bat bugs (*Cimex adjunctus* Barber) (Hemiptera: Cimicidae) from Colorado.

Materials and Methods

All sampling occurred at a bat maternity roost co-occupied by big brown bats (*Eptesicus fuscus* Palisot de Beauvoir) and little brown bats (*Myotis lucifugus* Le Conte) in a non-residential building in Fort Collins, Colorado (40.5948°N, 105.0839°W). This colony was one of several described in prior studies (Ellison et al. 2007, O'Shea et al. 2011) and bat bugs were opportunistically collected from bats captured as part of a separate study focused on tracking bat movements (Castle et al. 2015). Bats were captured in May–September 2015 and June–July 2016 using mist nets and harp traps while emerging from the roost. State collection permits were obtained prior to sampling (Colorado Parks and Wildlife Permits 15TR2010 and 16TR2010A1 issued to P. Cryan, USGS) and techniques followed the guidelines of the American Society of Mammologists (Sikes and Gannon 2011). Protocols were approved by the Institutional Animal Care and Use Committee of the U.S. Geological Survey, Fort Collins Science Center (Protocol 2014-08 and Standard Operating Procedure 01-01 for Capture, Handling, Marking, Tagging, Biopsy Sampling, and Collection of Bats). Bat bugs were opportunistically collected from individual bats using forceps to remove visible bugs from the fur and wing membranes with the bat under manual restraint. Collected bugs were stored in 70% ethanol.

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Bat bugs were surface sterilized after collection following published procedures (Billeter et al. 2012). Bugs were homogenized in 1.5 ml microcentrifuge tubes containing Brain Heart Infusion (BHI) broth (CDC, Atlanta, GA). Samples from 2015 were homogenized for 3 min at 12,000 rpm in 200 µl BHI along with 4–6 glass beads (1.5 mm diameter) using a Bullet Blender Gold homogenizer (Next Advance, Averill Park, NY). The homogenate was centrifuged for 3 min at 16,000 rpm and transferred to a clean 1.5 ml tube. Samples from 2016 were homogenized in 500 µl BHI using a sterile pestle. This change was made because manual homogenization was found to be more complete compared to machine homogenization for the small bug specimens. A 200 µl aliquot of homogenate was retained and frozen, 200 µl was used for DNA extraction, and 100 µl was used to attempt culturing of bartonellae following standard techniques (Billeter et al. 2012). DNA extraction was performed using either heat extraction (95°C for 10 min) or using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Bat bug species identification was performed by amplification and sequencing of arthropod mitochondrial DNA. Two loci were targeted, mitochondrial 16S ribosomal RNA and cytochrome oxidase I (COI), using primers and protocols previously published (Szalanski et al. 2004, Quetglas et al. 2012).

Initial detection of *Bartonella*, *Brucella*, and *Yersinia* DNA was implemented using a multiplex real-time PCR reaction targeting the *Bartonella* transfer mRNA gene (*ssrA*), *Brucella* insertion sequence (IS711), and *Yersinia* peptidoglycan-associated lipoprotein (*pal*) (Halling et al. 1993, Diaz et al. 2012). Primers and probes for the *pal* gene were previously developed from whole-genome sequences of *Yersinia* spp. (M. Diaz, unpublished data). Positive (*Bartonella doshiae*, *Brucella melitensis*, and *Yersinia pseudotuberculosis*) and negative (water only) controls were used to evaluate the presence of bacterial DNA and to detect potential contamination, respectively.

Conventional PCR reactions were also performed for the detection and sequencing of *Bartonella* DNA in bat bugs. These tests targeted the *Bartonella* 16S–23S intergenic spacer region (ITS) (Diniz et al. 2007), citrate synthase gene (*gltA*), and cell division protein (*ftsZ*). Nested PCR protocols were run for *gltA* and *ftsZ* using combinations of previously published primers (Norman et al. 1995, Birtles and Raoult 1996, Zeaiter et al. 2002, Colborn et al. 2010, Bai et al. 2016). PCR reactions were followed by gel electrophoresis using 1.5% agar and GelGreen stain (Biotium, Hayward, CA). In all conventional PCR reactions, positive (*Bartonella doshiae*) and negative (water only) controls were included to determine appropriately sized amplicons and to detect potential contamination, respectively. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced in both directions using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were assembled using the SeqMan Pro program in Lasergene v12 (DNASTAR, Madison, WI). Exact 95% binomial confidence intervals (CI) for prevalence estimates based on obtained sequences were calculated using the Clopper–Pearson method (Clopper and Pearson 1934). Sequences are stored in GenBank with the accession numbers KY888207–KY888209 (*ftsZ*), KY888210–KY888211 (*gltA*), and KY888212–KY888215 (ITS).

Assembled *ftsZ*, *gltA*, and ITS sequences were aligned to other known *Bartonella* species and *Bartonella* sequences from bats (see supp Table S1 for GenBank accession numbers [online only]) using the local, accurate L-INS-I algorithm in MAFFT v7.187 (Katoh and Standley 2013). Alignments were trimmed to a common length of 696 bp (*ftsZ*) and 356 bp (*gltA*). Sequences from *ftsZ* and *gltA* were concatenated and a Bayesian phylogeny was inferred by Markov chain Monte-Carlo (MCMC) sampling in BEAST v1.8.4 (Drummond and Rambaut 2007, Drummond et al. 2012) using the generalized time reversible molecular evolution model with a proportion of invariant sites (GTR+Γ+I) with four gamma categories (Nei and Kumar 2000), a fixed molecular clock rate, and a birth–death tree prior with incomplete sampling (Stadler 2009). Three MCMC chains were run for 2×10^7 iterations, discarding the first 10% as burn-in. Chains were visually assessed for convergence and mixing of parameters in Tracer v1.6.0 (Drummond et al. 2012). The maximum clade credibility tree was found using all trees from the combined chains. The ITS alignment was trimmed to a common length of 1,920 bp, including gaps, and a separate Bayesian tree was inferred using the same procedure as above.

Results and Discussion

In total, 226 *Eptesicus fuscus* and 10 *Myotis lucifugus* were captured over 10 nights of sampling. All bat bugs ($n = 55$) collected came from *Eptesicus fuscus* and were identified as *C. adjunctus* according to both mitochondrial 16S and COI sequences. Using a combination of markers, a total of 4/55 (7.3%, 95% CI: 2–17.6%) *C. adjunctus* were positive for *Bartonella* DNA. However, the different markers showed varying sensitivity: 4/55 samples were positive by ITS, 2/55 (3.6%, 95% CI: 0.4–12.5%) were positive using nested *gltA*, and 3/55 were positive using nested *ftsZ* (5.4%, 95% CI: 1.1–15.1%). All attempts at culturing bartonellae from bat bugs were unsuccessful, primarily due to overgrowth by other contaminating bacteria (a common issue with *Bartonella* spp.). Other bug specimens may have had insufficient numbers of bacteria to successfully culture. The multiplex real-time PCR assay for *Bartonella*, *Brucella*, and *Yersinia* spp. was negative for the three bacteria.

Phylogenetic analysis of concatenated *Bartonella ftsZ* and *gltA* sequences showed that sequences from *C. adjunctus* were identical to one another and clustered with other *Bartonella* isolates or sequences amplified from bats (Fig. 1). Specifically, the *C. adjunctus* sequences most closely matched a sequence amplified from blood from a *Pipistrellus* sp. bat from the United Kingdom (Concannon et al. 2005) and a *Bartonella* isolate from *Rhinolophus ferrumequinum* from the Republic of Georgia (Urushadze et al. 2017). Additional sequences that were part of the same clade (99.5% posterior support) include sequences amplified from *Rhinolophus pusillus* in China (Han et al. 2017), a *Myotis* sp. individual in Peru (Bai et al. 2012), and a bat fly (*Anatrichobius scorzai*) removed from *Myotis keaysi* in Costa Rica (Judson et al. 2015). Also part of this clade are *Bartonella* isolates from *Eidolon helvum* from Kenya (Bai et al. 2015) and *Myotis blythii* from the Republic of Georgia (Urushadze et al. 2017). Other sequences from bats and ectoparasites in the United States (Morse et al. 2012, Lilley et al. 2017) cluster into three distinct, well-supported (>90% posterior support) clades with other *Bartonella* sequences from Europe, Asia, and Central America (Concannon et al. 2005, Veikkolainen et al. 2014, Lilley et al. 2015, Urushadze et al. 2017). One of these clades includes *Candidatus* Bartonella mayotimonensis, the etiological agent of endocarditis in a patient from Iowa (Lin et al. 2010), and previously linked with insectivorous bats (Veikkolainen et al. 2014). GenBank accession numbers and associated citations for reference *Bartonella* Species and closely matching sequences are listed in Supp Table S1 (online only).

Phylogenetic analysis of ITS sequences showed similar results to *ftsZ* and *gltA* (Fig. 2). All sequences from *C. adjunctus* were identical and clustered within the same clade of other bat *Bartonella* as with *ftsZ* and *gltA* (98.4% posterior support). ITS sequences from *C. adjunctus* most closely matched sequences amplified from the liver of *R. ferrumequinum* (Bai et al. 2017), isolates from *Myotis blythii*

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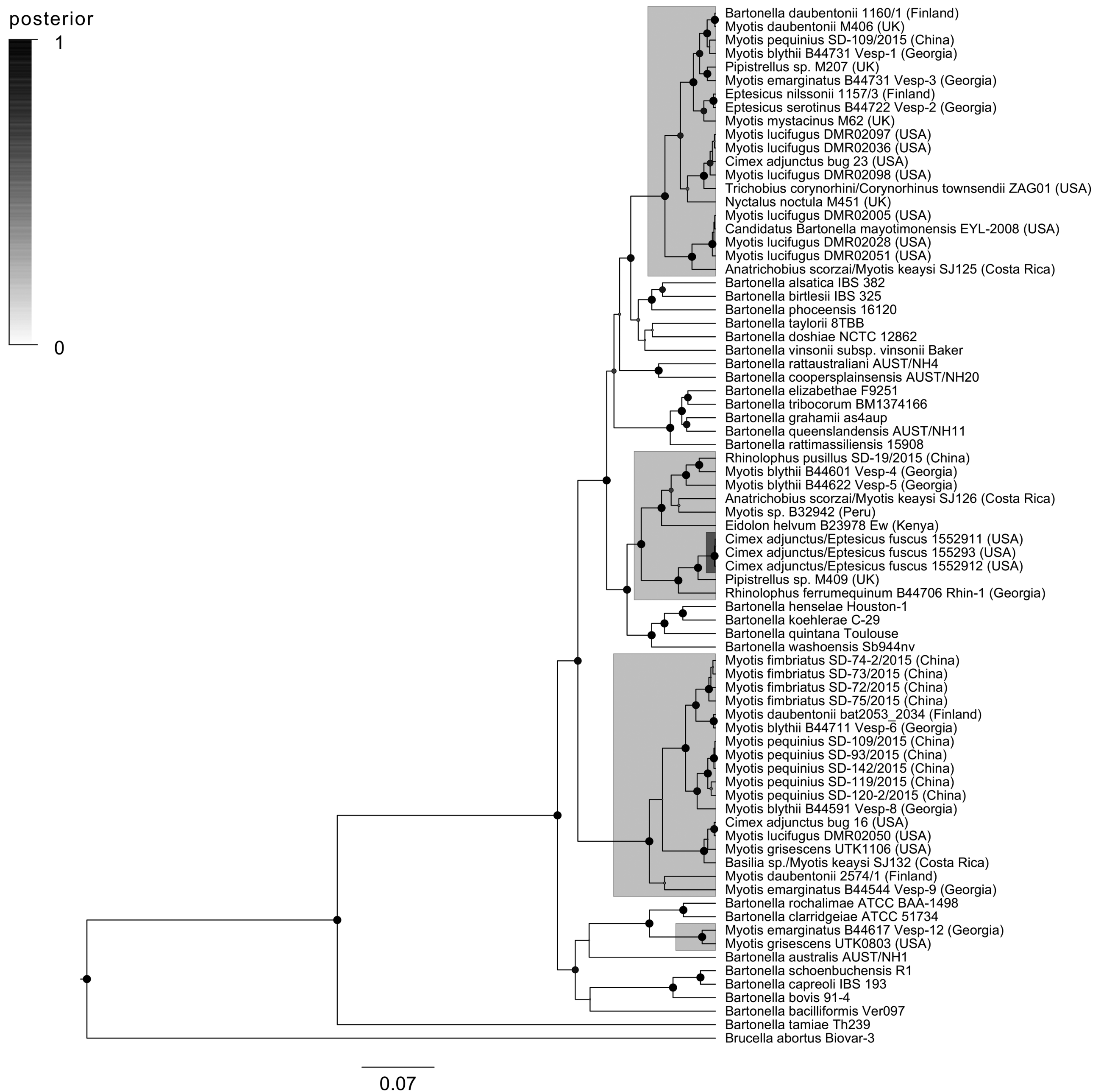

Fig. 1. Bayesian phylogeny of concatenated *Bartonella ftsZ* and *gltA* sequences from *C. adjunctus* in Colorado, Snited States. Posterior support for nodes is indicated by the size and shade of circles at each node. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were 1052 positions in the final dataset (including gaps). Codes next to sequence names indicate strains. For bat-associated sequences, the country of detection is indicated in parentheses. Clades containing sequences from bats or ectoparasites from the United States are highlighted in grey; Colorado *C. adjunctus* sequences are highlighted in dark grey. GenBank accession numbers are listed in Supp Table S1 (online only).

from the Republic of Georgia (Urushadze et al. 2017), an isolate from *Eidolon helvum* from Kenya (Kosoy et al. 2010, Bai et al. 2015), and a sequence from a bat fly (*Trichobius major*) removed from *Myotis austroriparius* (Rhoads, 1897) (Chiroptera: Vespertilionidae) in Florida (Reeves et al. 2005). ITS sequences from *C. adjunctus* in Colorado were not closely related to sequences from *C. adjunctus* in Florida, a bat flea (*Sternopsylla texanus*) from Oklahoma, or a bat tick (*Carios kelleyi*) from Iowa (Loftis et al. 2005; Reeves et al. 2005, 2007).

In this study, we demonstrated that bat bugs (*C. adjunctus*) might harbor *Bartonella* bacteria in the United States. We found that sequences detected in bat bugs collected from *Eptesicus fuscus* cluster with other *Bartonella* isolates and sequences from bats in Europe. This suggests that *Bartonella* may have codiverged with their bat hosts during the colonization of North America, a general pattern that has been inferred for bats globally (Lei and Olival 2014, McKee et al. 2016). Attempts to detect *Brucella* and *Yersinia* in *C. adjunctus* were unsuccessful. It is possible that bats and their ectoparasites in the US harbor these bacteria or our detection approach was not sensitive enough. We suggest that further studies on the bacteria infecting bats and their ectoparasites in the United States are needed. Greater sampling from a diverse selection of bats and their ectoparasites will further clarify the diversity of bacteria in these hosts, increase our understanding of the

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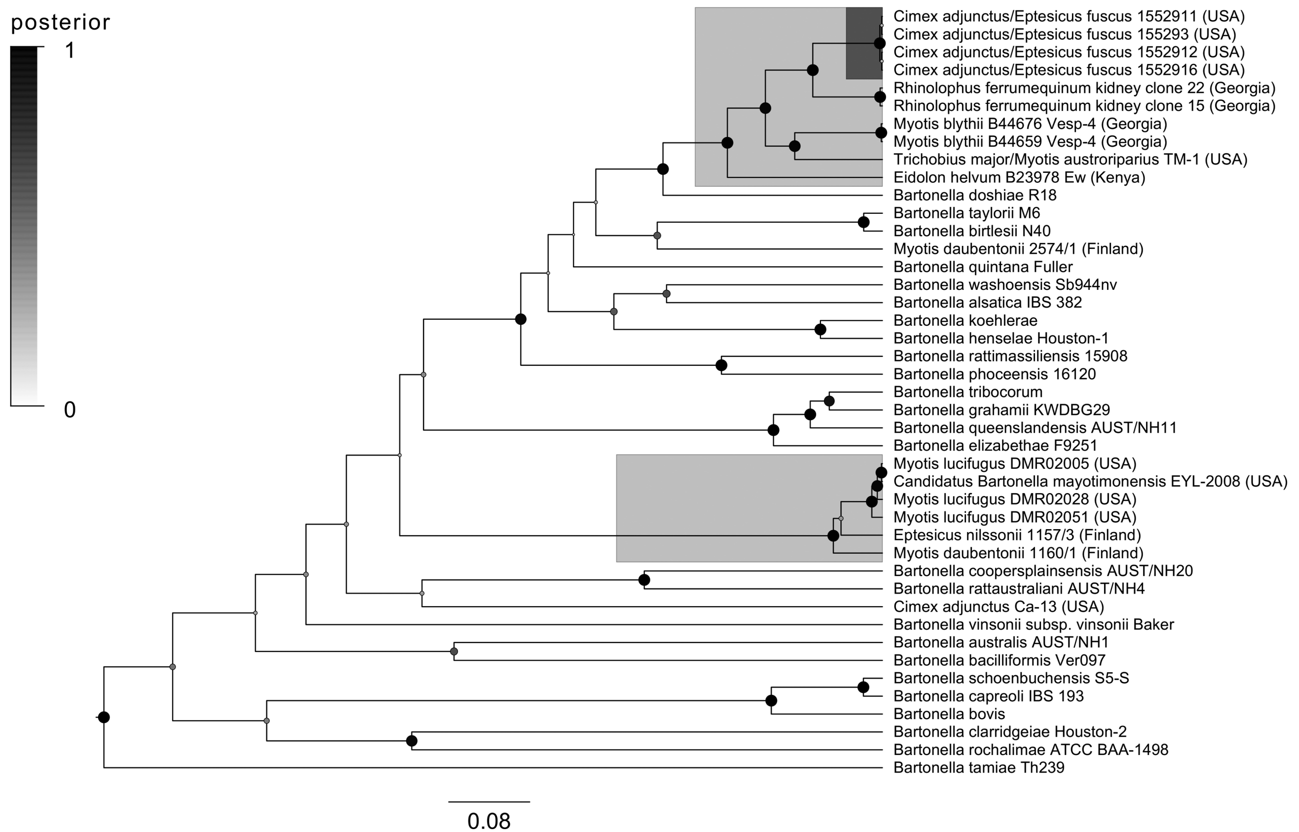

Fig. 2. Bayesian phylogeny of *Bartonella* ITS sequences from *C. adjunctus* in Colorado, United States. Posterior support for nodes is indicated by the size and shade of circles at each node. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were 1920 positions in the final dataset (including gaps). Codes next to sequence names indicate strains. For bat-associated sequences, the country of detection is indicated in parentheses. Clades containing sequences from bats or ectoparasites from the United States are highlighted in grey; *C. adjunctus* sequences are highlighted in dark grey. GenBank accession numbers are listed in Supp Table S1 (online only).

maintenance of bacterial infections in bats, and provide data necessary to assess the risk to humans posed by zoonotic bacteria in bats.

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Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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