

Serologic survey of *Eptesicus fuscus* from Georgia, U.S.A. for *Rickettsia* and *Borrelia* and laboratory transmission of a *Rickettsia* by bat ticks

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ABSTRACT: Bats and their ectoparasites are associated with bacterial agents of unknown pathogenicity. We tested sera from 56 *Eptesicus fuscus* from Georgia against *Borrelia hermsii*, *Orientia tsutsugamushi*, *Rickettsia conorii*, and *Rickettsia rickettsii*. We detected antibodies reactive against a relapsing fever *Borrelia* and spotted fever group *Rickettsia* in 3/56 and 1/56 bats, respectively. We attempted to culture *Bartonella* from the blood of these bats but were unsuccessful. In addition, we fed bat ticks, *Carios kelleyi*, infected with *Rickettsia* on a specific pathogen-free guinea pig. The guinea pig had a weak seroconversion to *R. rickettsii* with a peak titer of 1:32 starting on day 14. *Rickettsia* was not detected in any of the tissue samples from the guinea pig by molecular means. Our results indicate that *E. fuscus* is naturally exposed to both a spotted fever group *Rickettsia* and a relapsing fever group *Borrelia*. If these agents are transmitted by bat ticks, then people living in close proximity to bat ticks might be exposed. **Journal of Vector Ecology 31 (2): 386-389. 2006.**

Keyword Index: *Carios kelleyi*, *Rickettsia*, *Borrelia*, relapsing fever spirochetes, zoonosis.

INTRODUCTION

Arthropod-borne spirochetes and rickettsial agents are pathogens of humans and domestic animals. These pathogens cause diseases such as Lyme disease, cat scratch disease, and Rocky Mountain spotted fever. A bat tick, *Carios kelleyi* (Cooley and Kohls), has recently been associated with *Bartonella henselae* and a variety of potential bacterial pathogens such as *Rickettsia* and *Borrelia* spp. (Loftis et al. 2005). In certain circumstances, this tick feeds on humans, and its bite is associated with erythematous rashes, fever, and malaise (Gill et al. 2004, Vargas 1984). These can be symptoms of exposure to pathogens or allergens. *Eptesicus fuscus*, the big brown bat, is among the most common bat species associated with human dwellings throughout most of its North American range (Agosta 2002). *Eptesicus fuscus* was one of the primary hosts of *C. kelleyi* studied by Loftis et al. (2005). There is no experimental evidence that *B. henselae* or the novel *Rickettsia* or *Borrelia* from *C. kelleyi* are transmitted to vertebrate hosts, including bats. Spirochaetes that are morphologically similar to relapsing fever *Borrelia* have been reported in other bats and their associated ticks, but these have not been well characterized (e.g. Heisch 1952). *Carios kelleyi* often infests houses with bats, is known from Georgia, and will feed on *E. fuscus* (Cooley and Kohls 1944, Reeves et al. 2000, Dick et al. 2003). We conducted a serologic survey for antibodies reactive with *Rickettsia* spp. and *Borrelia hermsii* in *E. fuscus* from Georgia. Bats in this study were trapped as part of an ongoing rabies surveillance project. We attempted to culture *Bartonella* from the blood clots associated with the sera using previously described techniques. In addition, we fed larvae of *C. kelleyi* that were naturally infected with a spotted fever group *Rickettsia* on a guinea pig.

Both rickettsial agents and *Borrelia* were identified

in bat ticks from Iowa that were associated with *E. fuscus* (Loftis et al. 2005). The goal of our study was to determine if *E. fuscus* is naturally exposed to either rickettsial agents or *Borrelia*. A second goal was to determine if the spotted fever group *Rickettsia* previously reported by Loftis et al. (2005) could be transmitted by tick feeding.

MATERIALS AND METHODS

Specimen collection

Capture of bats and collection of blood was performed in accordance with the protocols specified by CDC's Institutional Animal Care and Use Committee and Georgia Scientific Collecting Permit #29-WFS-05-14. All specimen collection was performed by rabies-vaccinated personnel with recently verified rabies virus neutralizing antibody titers. One hundred and ninety-one big brown bats were collected from 13 houses in the Atlanta area from 12 July 2005 through 29 September 2005, and again from 17 March 2006 through 26 May 2006. We tested sera from 56 of these bats. Bats were typically found roosting on the outside of screened attic vents and more rarely on the interior walls of the attic.

Bats were collected directly from diurnal roosts by padded forceps, gloved hand, or butterfly net and were confined in groups in mesh carrying cases or bucket traps until the period of sample collection (Kunz and Kurta 1988). Upon capture, bats were weighed, sexed, and visually inspected for the presence of bat ticks and other ectoparasites.

After cleaning the wing with isopropanol, blood samples were obtained by puncture of the peripheral wing vein with a sterile 23 gauge needle, followed by collection of a maximum of 200 μ l of whole blood with heparinized capillary tubes (Kunz and Nagy 1988). Bleeding either

ceased naturally or was stopped by application of pressure and Kwik-Stop styptic powder (ARC Laboratories, Atlanta, GA). Blood samples were immediately transferred to Microtainer serum separator tubes (Becton-Dickinson Vacutainer Systems, Franklin Lakes, NJ) and stored on cold packs until arrival at the laboratory. Following sample collection, all bats were marked with 2.9 mm split, alloy wing bands (Porzana Ltd., East Sussex, U.K.), and released at the site of capture.

Sera and blood clots were separated by 10 min of centrifugation at 8,000 RPM. Serum was collected and transferred to 2 ml Starstedt tubes for storage at -80°C . Blood clots were stored at -80°C in original serum separator tubes until further processing.

Serology

Fifty-six sera were initially assayed for antibodies reactive with *Rickettsia* by ELISA. Whole-cell *Rickettsia conorii* Malish antigen was produced by pressure cell disruption of gradient-purified rickettsiae, as previously described (Halle and Dasch 1980), and total protein concentration was determined using a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Low-binding EIA microplates (MP Biomedicals, Inc., Irvine, CA) were coated with rickettsial antigen (1.5 ng protein per well), blocked with 3% bovine serum albumin (BSA) (Sigma, St. Louis, MO) in phosphate buffered saline, washed, dried, and stored at 4°C for 2 days. Sera were diluted 1:100 in ELISA buffer [phosphate buffered saline with 1% BSA, 0.01% Tween-20 (Sigma), and 0.02% thimerosol (Sigma)], and 50 μl of diluted serum was applied to duplicate wells of the ELISA plate. After incubation at 37°C for 90 min, plates were washed three times, and 70 ng of goat anti-bat IgG_(H+L) (Bethyl Laboratories, Montgomery, TX) diluted in ELISA buffer was added to each well. After incubation at 37°C for 60 min, plates were washed three times, and 10 ng of HRP conjugated rabbit anti-goat IgG_(H+L) (KPL, Gaithersburg, MD) diluted in ELISA buffer was added to each well. After a final incubation at 37°C for 60 min, plates were washed four times. One hundred μl of one-component ABTS substrate (KPL) was added to each well and, after incubation for 15 min at room temperature, the reaction was stopped by adding 100 μl of 1% SDS (Sigma). The optical density at 405 nm (A_{405}) was recorded using a SpectraMax reader (Molecular Devices, Sunnyvale, CA). Sera producing optical densities greater than 0.60 were considered to be positive by ELISA for antibodies against spotted-fever group rickettsiae.

All sera determined to be positive by ELISA were verified by indirect immunofluorescence assay (IFA) against *Rickettsia akari* (Kaplan strain) grown in egg yolk sac, *Rickettsia conorii* (Malish strain) grown in VERO cells, *Rickettsia typhi* (Wilmington strain) grown in VERO cells, *Rickettsia rickettsii* (Shelia Smith) grown in egg yolk sac, and *Orientia tsutsugamushi* (Karp strain) grown in L929 cells, by modifying the technique described by Schultz et al. (2002). The modification was the use of goat-anti-bat antibodies instead of goat-anti-bear. In addition, all bat sera

were assayed for antibodies reactive with *Borrelia hermsii* by IFA.

Slides were coated with antigen, air dried, fixed with acetone, and frozen at -80°C until used. The slides were warmed to room temperature in a desiccator prior to use. All sera were initially diluted 1:16 in a dilution buffer containing 0.01 M phosphate-buffered saline (PBS) at pH 7.4, 1% bovine serum albumin, and 0.01% thimerosol. Positive, negative, and diluent controls were used in each test. The positive and negative control consisted of human sera known to be reactive with the antigen.

Ten μl of sera were placed on each well and slides were incubated in a dark humid chamber at 37°C for 30 min then rinsed in PBS three times (5 min per wash). Unlabeled goat-anti-bat (Bethyl Laboratories, Montgomery, TX) was added to each well at a dilution of 1:100. Slides were incubated and washed as before. Fluorescein conjugated rabbit anti-goat IgG(H+L) immunoglobulin (KPL, Gaithersburg, MD) was diluted 1:150 and centrifuged at 10,000 G for 5 min to remove precipitants. Conjugate dilution was optimized by checkerboard titration. Ten μl of diluted conjugate were applied to each well. Slides were incubated in the dark for 30 min at 37°C and washed with PBS as previously described. During the second wash, 8 drops of 1.65% aqueous Eriochrome black in water were added to the PBS as a counterstain. After the third wash, 10 μl of mounting medium (10% PBS + 90% glycerol + 0.3 M DABCO) was added to each well prior to covering the slide. Slides were allowed to settle in the dark for at least 30 min and were examined with a Zeiss Axiophot ultraviolet epifluorescence microscope at 400X magnification for distinct fluorescence of individual organisms of *Borrelia*, *Orientia*, or *Rickettsia*. All sera were assayed at 1:100 dilution and those deemed positive were retested at serial dilutions from 1:16 to 1:256 for *Rickettsia* and *Orientia* and 1:16 to 1:1012 for *Borrelia hermsii*. Sera that was reactive at 1:256 or higher dilution were determined to be reactive with the antigen.

Guinea pig

A specific pathogen-free guinea pig (*Cavia porcellus*, HsdPoc:DH, female) was obtained from Harlan (Indianapolis, IN) and maintained in accordance with Institutional Animal Care and Use Committee protocols. The guinea pig was infested with approximately 100 larvae of *C. kelleyi*, using previously described techniques (Loftis and Levin 2004). The larvae were hatched from eggs laid by a female tick that was shown to be naturally infected with the spotted fever group *Rickettsia* described by Loftis et al. (2005). Engorged larvae were collected, allowed to molt to the second nymphal (first blood-feeding) stage, and then DNA was extracted from individual nymphs for PCR. Blood was collected from the guinea pig, for PCR detection of *Rickettsia* spp. and serology, prior to exposure and on days 3, 7, 10, 14, 21, and 35. The guinea pig was euthanized on day 35, and samples of the blood, liver, spleen, lung, and kidney were aseptically collected for PCR. Extraction of DNA from tissues and ticks was performed as previously described (Loftis and Levin 2004). Nested PCR for the

17kD antigenic gene of *Rickettsia* spp. was performed as previously described (Loftis et al., 2005). For the IFA, sera were diluted 1:16, 1:32, 1:64, and 1:128 and applied to glass slides coated with acetone-fixed, *Rickettsia rickettsii*-infected VERO cells. Detection of antibodies reactive with *Rickettsia* was achieved using FITC conjugated goat anti-guinea pig IgG_(H+L) (KPL, Inc., Gaithersburg, MD) diluted 1:100.

Culturing of *Bartonella*

We attempted to culture *Bartonella* from 50 frozen bat bloods using the techniques described by Kosoy et al. (2004). Briefly, up to 200 µl of clotted bat blood was mixed with an equal volume of sterile brain heart infusion medium. Aliquots of 0.2 ml of the mixture were applied to agar plates supplemented with heart infusion and 5% rabbit blood. Plates were incubated at 34° C in an aerobic atmosphere with 5% CO₂ and held for 24-30 days. The cultures were examined every three days for bacterial growth. Colonies that morphologically resembled *Bartonella* spp. were picked with a sterile pipette tip, mixed with 0.2 ml of sterile brain heart infusion medium, and inoculated onto a new agar plate. DNA was extracted from the remnants of the original colonies by mixing the colony in 50 µl of sterile water and heating at 95° C for 9 min. A portion of the 16S rRNA gene was amplified by PCR and sequenced using the RickF1 and RickR4 primers as described by Reeves (2005). PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Duplicate sequencing reactions were performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using PCR primers, and excess dye was removed with a DyeEx 2.0 column (Qiagen, Valencia, CA). Sequences were determined using an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA). Primer sequences were removed and sequences were

assembled with Seqmerge (Accelrys, San Diego, CA). Assembled sequences were compared to those in GenBank using the BLAST 2.0 program (NCBI, Bethesda, MD).

RESULTS

Results of the assays on serum from *E. fuscus* are presented in Table 1. The guinea pig developed a titer of 1:16 against *R. rickettsii* 10 days post-infestation and a peak titer of 1:32 against *R. rickettsii* 14 days post-infestation. After day 14 the titer did not change and the animal was killed 35 days post-infestation. No DNA from *Rickettsia* was detected in the tissues sampled. Bacteria were isolated from the blood of *E. fuscus*, but DNA sequencing indicated that the isolates were not *Bartonella*. Isolates included species of *Listeria*, *Streptococcus*, and unnamed bacteria.

DISCUSSION

Sera from *E. fuscus* were reactive with *B. hermsii* or *R. conorii* and *R. rickettsii* (Table 1). Of the sera tested, 3/56 were reactive with *B. hermsii* and 1/56 reacted with *Rickettsia*. Our data supports the hypothesis that *E. fuscus* are exposed to both a relapsing fever *Borrelia* and spotted fever group *Rickettsia*. Serology can not be used to conclusively speciate what pathogen to which an animal has been exposed. The pathology to bats by these agents are unknown as are the human health risks. Further study should be conducted based on the fact that bat ticks will feed on people (Gill et al. 2004) and possibly expose them to these agents. Isolation of these agents from bats and experimental transmission by *C. kelleyi* are needed to demonstrate if either agent can be transmitted by ticks.

The specific pathogen-free guinea pig fed on by laboratory hatched larvae of *C. kelleyi* had a weak seroconversion to *R. rickettsii* starting 10 days after

Table 1. Results of immunofluorescent assays of 56 sera from *Eptesicus fuscus* collected in Georgia, U.S.A. from 12 July 2005 to 17 March 2006 against *Rickettsia akari*, *R. conorii*, *R. typhi*, *R. rickettsii*, *Orientia tsutsugamushi*, and *Borrelia hermsii*.

Number of bats	Date sampled	County	Sex ratio Male/Female	Serologic results
11	12 July 2005	Forsyth	4/7	2 female bats 1:256 for <i>B. hermsii</i>
5	16 July 2005	Spalding	2/3	Negative
10	18 July 2005	Fulton	2/8	1 female bat 1:1012 for <i>B. hermsii</i>
12	29 August 2005	Henry	4/8	Negative
6	7 September 2005	Fulton	1/5	Negative
2	15 September 2005	Fulton	2/0	Negative
7	21 September 2005	Henry	0/7	1 female bat 1:256 for <i>R. conorii</i> and <i>R. rickettsii</i>

infestation with a peak titer of 1:32 on day 14. The titer remained at 1:32 until day 35 when the animal was killed. DNA from *Rickettsia* spp. was not detected in any of the tissue samples from the guinea pig. This data could indicate that only a small quantity of *Rickettsia* antigen was inoculated by the larval ticks and that the *Rickettsia* did not infect the guinea pigs. We believe the *Rickettsia* in the larvae was viable because 18 of these larvae molted to the second nymphal stage and 12 of these nymphs were PCR-positive for rickettsiae. Loftis et al. (2005) determined that *Rickettsia* from *C. kelleyi* was closely related to *R. rickettsii*; however *R. rickettsii* might not be an appropriate substitute antigen for IFA. If the *Rickettsia* of *C. kelleyi* can be cultured, it would be the optimal antigen for further study.

Although *Bartonella* spp. have been detected from ectoparasitic arthropods associated with *E. fuscus* and other bat species (Loftis et al. 2005), we failed to culture *Bartonella* from the blood of *E. fuscus*. Several bacterial species were isolated, including *Listeria* and *Streptococcus* species, but none of these were *Bartonella*. Gardner et al. (1987) were also unable to detect *Bartonella* in the blood of *Eptesicus serotinus*, a related species of bat.

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