Association of *Bartonella* with the fleas (Siphonaptera) of rodents and bats using molecular techniques

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ABSTRACT: *Bartonella* spp. are putatively vector-borne bacterial agents of humans and animals. Fleas have been incriminated as vectors of *Bartonella* spp. and are suspected of transmitting *Bartonella* of rodents and bats, but some of these *Bartonella* spp. have not yet been directly detected in wild caught fleas. We report the molecular detection of *Bartonella tribocorum, Bartonella vinsonii* subsp. *vinsonii,* and two novel genotypes of *Bartonella* from the fleas *Xenopsylla cheopis*, *Ctenophthalmus pseudagyrtes, Sternopsylla texanus,* or *Orchopeas howardi. **Journal of Vector Ecology*** **32 (1): 118-122. 2007.**

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INTRODUCTION

Bartonella spp. are Gram-negative, predominantly erythrocyte-adherent bacteria that are putatively transmitted by arthropod vectors (Boulouis et al. 2005). Fleas have been incriminated as the vectors of *Bartonella* spp., such as *Bartonella henselae* and *Bartonella grahamii* (Boulouis et al. 2005). Experimental proof of transmission by fleas in the laboratory remains somewhat questionable and the mechanism of transmission could be mechanical or biological. *Bartonella* spp. pathogenic to humans including *Bartonella henselae,* the cause of cat scratch disease, and *Bartonella quintana*, the cause of trench fever, have all been reported from wild caught fleas (Boulouis et al. 2005, Marie et al. 2006). In addition, fleas have been suggested to be the natural vectors of *Bartonella* spp. of rodents and bats (e.g. Goedbloed et al. 1964, Heller et al. 1998, Stevenson et al. 2003). However, associations between wild caught fleas and *Bartonella* have not been made for many of these suggested vectors. Detection of *Bartonella* in wild caught fleas is essential in establishing a link between potential vectors and pathogens. Detection of DNA is a preliminary step in determining which fleas are potential vectors and should be further studied. We report the molecular detection of four *Bartonella* spp. from fleas of rodents and bats.

MATERIALS AND METHODS

Fleas were collected from small mammals, including southern flying squirrels, Norway rats, Brazilian free-tailed bats, and voles. Fleas were stored in 70% ethanol in a refrigerator. Thirteen Oriental rat fleas, *Xenopsylla cheopis* (Rothchild) (Siphonaptera: Pulicidae), were removed from a Norway rat, *Rattus norvegicus*, trapped on 26 Feb. 2006 in the city of Morton (MS: Scott Co.). Two *Ctenophthalmus pseudagyrtes* Baker (Siphonaptera: Hystrichopsyllidae) were collected from a vole, *Microtus* sp., trapped on 18 Jun. 2004, in the city of Sheridan (WY: Sheridan Co.). Eleven bat fleas, *Sternopsylla texanus* (Fox), (Siphonaptera: Ischnopsyllidae) were collected from Brazilian free-tailed bats, *Tardida brasiliensis*, collected on 20 Jun. 1998 in Mamihaw Cave (OK: Woods Co.). We collected 75 *Orchopeas howardi* (Baker) (Siphonaptera: Ceratophyllidae) from southern flying squirrels, *Glaucomys volans*, collected near Columbus and Statesboro, Georgia 1997-2006 (Muscogee and Bulloch Counties).

Fleas were sorted and rinsed in fresh reagent grade 95% ethanol. Individual fleas were frozen in liquid nitrogen and crushed with a sterile Teflon pestle. Teflon pestles were cleaned in 10% sodium hypochlorite for 3 hours, rinsed in distilled water and autoclaved before each use. Total DNA was extracted from the pulverized fleas with an IsoQuick Nucleic Acid Extraction Kit (ORCA Research Inc., Bothell, WA) and resuspended in nuclease free water. We attempted to detect DNA from *Bartonella* with PCR using the QHEV1 and QHEV4 primers, which amplify a portion of the 16S to 23S rRNA intergenic spacer region, as described by Houpikian and Raoult (2001). These primers were chosen because they have been used in similar studies (Marie et al. 2006) and the *gltA* primers used in other *Bartonella* studies (e.g. Kosoy et al. 2003, Stevenson et al. 2003) amplify DNA from a wide variety of bacteria. We have amplified *gltA* genes from a variety of alphaproteobacteria, deltaproteobacteria, and gammaproteobacteria with these primers (unpublished data) and found them unreliable for *Bartonella* screening. Each PCR reaction contained 12.5 µl of Taq PCR Master Mix Kit (Qiagen, Valencia, CA), 7.5 µl of nuclease free water, 1.25 µl (2.0 µMol) of each primer, and 2.5 µl of DNA template in water. PCR products were

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separated by 2% agarose gel electrophoresis and visualized under ultraviolet light with ethidium bromide. Positive control DNA from *Bartonella henselae* and a distilled water negative control were used. We chose to amplify a variety of genes in order to further characterize *Bartonella* genotypes by attempted amplification and sequencing of the *gltA*, *groEL*, *rpoB*, *ribC*, and putative hemin binding protein (PAP) genes using the primers pairs respectively described by Inokuma et al. (2005) (5′ TGCATGCAGATCATGAAC 3′ and 5′ GAGTAAAAITCAACATTIGG 3′), Zeaiter et al. (2002a) (5′-CAGAAGTTGAAGTGAAAGAAAA-3′ and GCIGCTTCTTCACCIGCATT-3′), Drancourt and Raoult (1999) (5’-GCCAATTATCGCAGTTTATGG-3’ and 5’-ACGATTTGCATCATCATTTTC-3’), Johnson et al. (2003) (5’ -TAACCGATATTGGTTGTGTTGAAG-3’) and (5’-TAAAGCTAGAAAGTCTGGCAACATAACG-3’), and Zeaiter et al. (2002b) (5′-TTCTAGGAGTTGAAACCGAT-3′ and 5′-GAAACACCACCAGCAACATA-3’). Some of these primers were published for use on agents other than *Bartonella* but appear to amplify DNA from *Bartonella* in the laboratory. We use them solely for characterization of *Bartonella* and not screening.

PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, California). Duplicate sequencing reactions were performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) using PCR primers, and excess dye was removed with a DyeEx 2.0 column (Qiagen, Valencia, California). Sequences were determined using an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, California). Primer sequences were removed and sequences were assembled with Seqmerge (Accelrys, San Diego, California). Assembled sequences were compared to those in GenBank using the BLAST 2.0 program (NCBI, Bethesda, Maryland). Identification of the *Bartonella* spp. was based on sequence similarity to sequences in GenBank.

In order to reduce the possibility of contamination of the DNA extracts or PCR assays, no cultures of *Bartonella* were allowed in the laboratory where the DNA extraction or PCR was performed. All DNA extractions were performed in a separate area of the laboratory from the PCR setup and gel electrophoresis. All PCR were set up in a containment hood to reduce airflow or contamination. The hood was UV sterilized and wiped clean with ethanol or bleach regularly. The agents detected had not previously been in the laboratory where the research was conducted. In addition negative controls were used in all assays.

Additional slide mounted voucher specimens of each flea species are deposited in the Georgia Museum of Natural History, Athens, Georgia. GenBank accession numbers for the sequences were as follows *Bartonella vinsonii* subsp. *vinsonii*: RNA polymerase beta subunit (*rpoB*) (DQ473480), *Bartonella tribocorum*: *groEL* (DQ480758), 16S-23S rRNA ITS (DQ480757), *Bartonella* sp. TR-9: 16S-23S rRNA ITS (DQ473481), *Bartonella* sp. L-2633: 16S-23S rRNA ITS (DQ336386), putative hemin binding protein (DQ395096).

RESULTS

All positive and negative controls worked as expected and all PCR primers amplified DNA from the positive control. DNA from *Bartonella* was detected in 4/13 *X. cheopis,* 2/2 *C. pseudagyrtes*, 1/11 *S. texanus*, and 1/75 *O. howardi.* There were no mixed infections with more than one genotype of *Bartonella* and each flea species harbored DNA from only one genotype of *Bartonella*. All sequencing reactions produced unambiguous base calls, implying that there was one genotype present in each flea. Not all PCR primers amplified DNA from *Bartonella*. We failed to amplify the *ribC* gene from any of our extracts, and some primers, such as those for the putative hemin binding protein genes, were successful in amplifying DNA from one genotype of *Bartonella* but not others. The reason for PCR failure is unknown but possibilities include inhibition from flea or other microbial DNA and poor primer matching. All PCR products were sequenced and sequence similarities for genes amplified are reported in Table 1. Genes not listed in Table 1 were not amplified.

DISCUSSION

Bats and their ectoparasites have been associated with *Bartonella* spp. (e.g. Gardner et al. 1987, Concannon et al. 2005, Loftis et al. 2005, Reeves et al. 2005a). Goedbloed et al. (1964) suggest that fleas of bats are potential vectors of some *Bartonella* spp., but detection of *Bartonella* from bat fleas has not been previously reported. We detected DNA from 1/11 bat fleas, *Sternopsylla texanus.* DNA from *Bartonella* has been detected from ectoparasites of bats, including ticks, mites, cimicid bugs, and bat flies (Loftis et al. 2005, Reeves et al. 2005a, Reeves et al. 2006) but the association between *Bartonella* and bat fleas is novel. Detection of DNA from *Bartonella* in *S. texanus* indicates that this flea potentially fed on infected bats. We were only able to amplify DNA from an ITS region of the *Bartonella* from *S. texanus*. The sequence from this *Bartonella* was unique when compared to those in GenBank but was most similar to *Bartonella* of cervids and rodents (Table 1). The DNA sequence from this new *Bartonella* was not similar to *Bartonella* sequences previously reported from ectoparasites of bats by Reeves et al. (2005a). We ruled out DNA contamination because this DNA sequence was not significantly similar to any sequences previously amplified in our laboratory.

The southern flying squirrel and its ectoparasites have been associated with zoonotic transmission of epidemic typhus, *Rickettsia prowazekii* (e.g. Bozeman et al. 1981). *Orchopeas howardi*, a squirrel flea, is a commonly collected ectoparasite of flying squirrels. Unnamed *Bartonella* spp. have been reported from *O. howardii* collected from gray squirrels, *Sciurus carolinensis*, but not from flying squirrels (Durden et al. 2004, Reeves et al. 2005b). We detected novel DNA sequences of a *Bartonella* (*Bartonella* sp. L-2633) from *O. howardii* collected in Georgia. The ITS sequence was not similar to other *Bartonella* with the exceptions of the tRNA regions, which indicates that this genotype is unique. We

Table 1. Flea species and associated *Bartonella* genotypes with comparisons to the most similar sequences in GenBank.

Source flea	*Bartonella* genotype	Gene sequenced (bp)	% similarity to other *Bartonella* genotypes (GenBank Accession #)
Sternopsylla texanus	*Bartonella* sp. TR-9	16S to 23S rRNA intergenic spacer(ITS)	94% *Bartonella schoenbuchensis* (AY116639) 92% *Bartonella birtlesii* (AY116640)
Orchopeas howardi	*Bartonella* sp. L-2633	ITS (495)	98% similar to the tRNA of *Bartonella quintana* (BX897700) the remainder of the sequence was unique
Orchopeas howardi	*Bartonella* sp. L-2633	putative hemin binding protein (273 bp)	85%-90% similar to portions of the putative hemin binding proteins of *Bartonella henselae* (BX897699)
Xenopsylla cheopsis	*Bartonella tribocorum*	ITS (740 bp)	100% similar to *Bartonella tribocorum* (AF312505)
Xenopsylla cheopsis	*Bartonella tribocorum*	*groEL* (876 bp)	98% similar to *Bartonella tribocorum* (AF304018)
Ctenophthalmus pseudagyrtes	*Bartonella. vinsonii* subsp. *vinsonii*	ITS (596 bp)	98% similar to *Bartonella. vinsonii* subsp. *vinsoni* (L35102)
Ctenophthalmus pseudagyrtes	*Bartonella. vinsonii* subsp. *vinsonii*	RNA polymerase beta subunit (*rpoB*) (498 bp)	99% similar to *Bartonella vinsonii subsp. vinsonii* (AF165997), 96% similar to *Bartonella vinsonii* subsp. *berkhofii* (AF165989), and *Bartonella vinsonii* subsp. *arupensis* (AY166582)

were able to amplify a 273 bp portion of a putative hemin binding protein gene from *Bartonella* sp. L-2633. The DNA sequence similarity for this amplicon was most similar to the putative hemin binding proteins of *B. henselae* (Table 1); but portions of the sequence were divergent. We cannot exclude the possibility that *Bartonella* sp. L-2633 is one of the organisms previously reported by Durden et al. (2004) or Reeves et al. (2005b), as we were unable to amplify DNA using the assays described by those authors. We were unable to amplify the hemin binding protein genes from other *Bartonella* spp. in this study, with the exception of the positive control (*Bartonella henselae*). *Rickettsia prowazekii* was not detected in these fleas (unpublished data).

In North America, *B. tribocorum* was previously reported from *R. norvegicus* from Maryland (Ellis et al. 1999). In the past, this agent has not been detected in ectoparasitic arthropods, and Gundi et al. (2004) reported a failure to culture *B. tribocorum* from an unspecified number of rat fleas. We report detection of DNA from *B. tribocorum* in *X. cheopis*. We based our identification of this *Bartonella* on the sequence similarity between the ITS and *groEL* amplicons from these fleas and that of *B. tribocorum* (Table 1). Heller et al. (1998) hypothesized that *X. cheopis* was a probable vector of *B. tribocorum*; however, they did not isolate this bacterium from fleas. *Bartonella tribocorum* has been a model organism for *Bartonella* research, but the agent is transmitted to laboratory animals by intravenous injection of infective bacteria (e.g. Schulein and Dehio, 2002). *Xenopsylla cheopis* and *R. norvegicus* can be maintained in the laboratory (e.g. Farhang-Azad et al. 1985) and further study would indicate if rats and fleas could be ideal organisms for modeling flea borne transmission of *Bartonella*.

We detected DNA from *B. vinsonii* subsp. *vinsonii* from *C. pseudagyrtes*, a flea of insectivores and small rodents. The ITS sequences of the amplicons from our *Bartonella* were almost identical (98%) to the type strain of *B. vinsonii* subsp. *vinsonii* (Table 1). They were not similar to other subspecies of *B. vinsonii*. *Bartonella vinsonii* has been an enigmatic rickettsial agent since its original description. It was originally isolated from a chigger, *Neotrombicula microti* (Ewing) (reported as *Trombicula microti* (Ewing)), removed from voles, *Microtus pennsylvanicus*, from Grosse Isle, Quebec, Canada (Baker, 1946). Baker (1946) identified the isolate as a variant of scrub typhus, *Orientia tsutsugamushi*. Weiss et al. (1978) determined that the isolate from Grosse Isle (named the "vole agent") was a strain of *Bartonella quintana* (*Rochalimaea quintana* at the time). Weiss and Dasch (1982) later determined that *B. vinsonii* was a separate species from *B. quintana*. The subspecies designation *B. vinsonii* subsp. *vinsonii* was made by Kordick et al. (1996) when they described a new subspecies *B. vinsonii* subsp. *berkhoffii* from dogs. Isolates of *B. vinsonii* subsp. *vinsonii* have been made from rodents from Saskatchewan, Canada and Amager, Denmark (Jardine et al. 2005, Engbaek and Lawson 2004), but ectoparasites have not been implicated as potential vectors since the original work by Baker (1946). Our molecular detection of *B. vinsonii* subsp. *vinsonii* from *C. pseudagyrtes* is noteworthy because Baker (1946) reported *C. pseudagyrtes* from voles on Grosse Isle. He was unsuccessful in isolating *Bartonella* from these fleas but did not clarify if his isolates failed due to contamination or failure to grow agents. Our detection of *B. vinsonii* subsp. *vinsonii* from fleas in Wyoming is both the first detection of this agent in the United States and from a flea.

We detected several unique genotypes of *Bartonella* but must emphasize that a unique genotype is by definition not a new species. There are genotypic variations within bacterial species. Our data suggest that fleas of wild rodents and bats are associated with *Bartonella* spp. Fleas have been incriminated as vectors of some *Bartonella* spp.; however, relatively few fleas of wildlife have been examined. Detection of DNA from *Bartonella* in a flea does not demonstrate vector competence or even the capacity for fleas to serve as vectors. However, detection of DNA from *Bartonella* in fleas demonstrates that these ectoparasites are exposed to these agents, an initial step in determining which ectoparasites might be vectors of various *Bartonella* spp.

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