The ISME Journal (2010) 4, 955–958
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SHORT COMMUNICATION

Detection of multiple *Bartonella* species in digestive and reproductive tissues of fleas collected from sympatric mammals

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At least 12 species in the genus *Bartonella* are zoonotic pathogens that may be transmitted among mammalian hosts by fleas or other arthropods. Apparent host specificity by some *Bartonella* species to mammalian hosts has been observed, and the detection of multiple *Bartonella* species in mammalian fleas suggests that fleas take bloodmeals from a variety of host species. However, many flea species are observed to parasitize a narrow host range. Therefore, we suspect that fleas may acquire *Bartonella* by a mechanism other than ingesting infectious blood. We found that detection of multiple *Bartonella* genotypes and species is apparently common in fleas and that the majority of fleas tested (5/9) carried *Bartonella* species atypical of their hosts. We also detected *Bartonella* DNA in flea reproductive tissues, suggesting that vertical transmission of this organism in vectors is possible, potentially leading to the accumulation of *Bartonella* diversity over time within fleas.
The ISME Journal (2010) **4,** 955–958; doi:10.1038/ismej.2010.22; published online 11 March 2010
Subject Category: Microbial population and community ecology
Keywords: host specialization; multiple niche polymorphism; pathogen transmission; Siphonaptera

The genus *Bartonella* consists of Gram-negative bacteria that parasitize erythrocytes and endothelial cells of mammalian hosts. Bartonellae are associated with diverse mammalian taxa (Chomel *et al.*, 2009) and 12 *Bartonella* strains or species are classified as zoonotic agents (Chomel *et al.*, 2006; Kosoy *et al.*, 2008). Vector-borne *Bartonella* transmission has been reported in several systems (Chomel *et al.*, 1996; Bown *et al.*, 2004) and a variety of hematophagous arthropods including fleas, lice and sandflies have been implicated in *Bartonella* transmission among host individuals (Chomel *et al.*, 2009).

Bartonellae are commonly detected in mammalian fleas and apparent specialization to particular mammalian hosts has been observed in Asia (Castle *et al.*, 2004) and North America (Bai *et al.*, 2008). In some cases, *Bartonella* detected in a host belongs to different species than the *Bartonella* detected in its fleas, and *Bartonella* can be detected in fleas collected from hosts that were apparently uninfected (Gabriel *et al.*, 2009). This could result from fleas taking bloodmeals from multiple host species and *Bartonella* persisting and replicating in the flea gut (Finkelstein *et al.*, 2002; Gabriel *et al.*, 2009). However, many fleas are highly host-specific and are rarely detected on atypical hosts (Brinkerhoff, 2008). Low rates of host switching by fleas may therefore preclude acquisition of multiple *Bartonella* species unless fleas acquire *Bartonella* by mechanisms additional to the ingestion of infectious blood.

In Colorado grasslands, two dominant rodent species are the black-tailed prairie dog (*Cynomys ludovicianus*) and the North American deer mouse (*Peromyscus maniculatus*), each of which is strongly associated with one *Bartonella* species; *B. washoensis* in *C. ludovicianus* (Bai *et al.*, 2008) and *B. vinsonii* subsp. *arupensis* in *P. maniculatus* (Bai Y, unpublished). Field sampling of over 4500 mammals revealed that the flea assemblages of these hosts are distinct with *P. maniculatus* parasitized primarily by *Aetheca wagneri* (∼93% of *P. maniculatus* fleas) and *C. ludovicianus* parasitized by *Oropsylla hirsuta* (∼98% of *C. ludovicianus* fleas) (Brinkerhoff, 2008). Carnivores in these grasslands acquire a variety of flea species and are commonly parasitized by *Pulex* fleas, which have wide host ranges, including rodents (Brinkerhoff, 2008). To explore relationships between *Bartonella*, fleas and mammalian

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Received 8 December 2009; revised 31 January 2010; accepted 31 January 2010; published online 11 March 2010

hosts, we characterized *Bartonella* DNA sequences detected in fleas and blood collected from three mammalian host species.

Samples were collected from mammalian hosts (*C. ludovicianus*, *P. maniculatus* and red fox (*Vulpes vulpes*)) in Boulder County, Colorado, in 2005 and 2006 (methods in Brinkerhoff *et al*., 2008, 2009). Flea tissues were separated by dissection under stereomicroscopic observation. First, fleas were fixed to slides with commercial chemical bond and hemolymph was aspirated from between abdominal tergites. Second, the midgut and proventriculus were removed intact and were washed in sterile phosphate-buffered saline solution. Third, reproductive tissues were separated and washed in sterile phosphate-buffered saline. DNA from all tissues was extracted using the DNeasy tissue kits (Qiagen, Valencia, CA, USA). Amplification of the *Bartonella* citrate synthase gene (*gltA*) by PCR was used to detect the presence of *Bartonella* DNA (Norman *et al*., 1995), and 312 bp of the resulting amplicon was sequenced and aligned with known *Bartonella* species sequences (Inoue *et al*., 2008). For each *Bartonella*-positive tissue, four additional sequences were cloned and amplified from purified plasmid DNA. *Bartonella* in blood samples was cultured and identified using molecular methods (Bai *et al*., 2008).

Bartonella was detected in the blood of two *P. maniculatus* and in at least one tissue in 9 of 11 fleas (Table 1). Identification of *Bartonella* species was based on phylogenetic similarity to known *Bartonella* species; all DNA detected in this study matched to either the *B. washoensis* or the *B. vinsonii arupensis* clade with high bootstrap support (Figure 1). Five fleas carried *Bartonella* in multiple tissues, and DNA from two *Bartonella* species was detected in tissues of two fleas (Table 1, Figure 1). Cloning revealed that some fleas carried DNA of one sequence type, whereas others carried multiple sequence types (Table 1).

B. washoensis is associated with ground squirrels and, in our system, is only known from prairie dogs (Bai *et al*., 2008). We detected *B. washoensis*-like

Table 1 *Bartonella* strains detected in multiple tissues (hemolymph, midgut and reproductive) of fleas collected from five individual mammalian hosts in Boulder County, Colorado

Flea ID	*Flea species*	*Tissue*	*Host species*	*Closest Bartonella species match*
13	*Pulex irritans*	Hemolymph	*Vulpes vulpes* (culture negative)	—
		Midgut		—
		Ovary		—
14	*P. irritans*	Hemolymph	*Vulpes vulpes* (culture negative)	—
		Midgut		*B. washoensis*
		Ovary		—
40	*Cediopsylla inaequalis*	Hemolymph	*Vulpes vulpes* (culture negative)	—
		Midgut		*B. washoensis** (2)
		Ovary		*B. washoensis** (2)
48	*Oropsylla hirsuta*	Hemolymph	*Cynomys ludovicianus*[a] (culture negative)	-
		Midgut		*B. washoensis*
		Ovary		—
49	*Oropsylla hirsuta*	Hemolymph	*C. ludovicianus*[a] (culture negative)	—
		Midgut		*B. washoensis** (2)
		Ovary		*B. washoensis*
51	*Oropsylla hirsuta*	Hemolymph	*C. ludovicianus*[b] (culture negative)	—
		Midgut		*B. v. arupensis*
		Testis		—
52	*Oropsylla hirsuta*	Hemolymph	*C. ludovicianus*[b] (culture negative)	*B. washoensis*
		Midgut		*B. washoensis*
		Testis		*B. washoensis*
65	*Aetheca wagneri*	Hemolymph	*Peromyscus maniculatus*[a] (*B. v. arupensis*)	—
		Midgut		—
		Ovary		*B. washoensis** (2)
66	*A. wagneri*	Hemolymph	*P. maniculatus*[a] (*B. v. arupensis*)	*B. v. arupensis*
		Midgut		*B. v. arupensis*
		Ovary		*B. v. arupensis** (3), *B. washoensis*
78	*Orchopeas leucopus*	Hemolymph	*P. maniculatus*[b] (*B. v. arupensis*)	—
		Midgut		—
79	*Orchopeas leucopus*	Hemolymph	*P. maniculatus*[b] (*B. v. arupensis*)	—
		Midgut		*B. washoensis*
		Ovary		*B. v. arupensis*

Hosts were screened for *Bartonella* infection by culture and the resulting infections are indicated in parentheses for each individual. Individual *C. ludovicianus* and *P. maniculatus* are designated by superscript letters (a and b). All flea tissues that yielded *Bartonella* DNA were cloned and sequenced (initial PCR plus four clones); asterisks indicate samples that produced multiple distinct amplicons with the number in parentheses indicating the number of unique amplicons per sample. We failed to successfully separate reproductive tissue from one flea (no. 78).

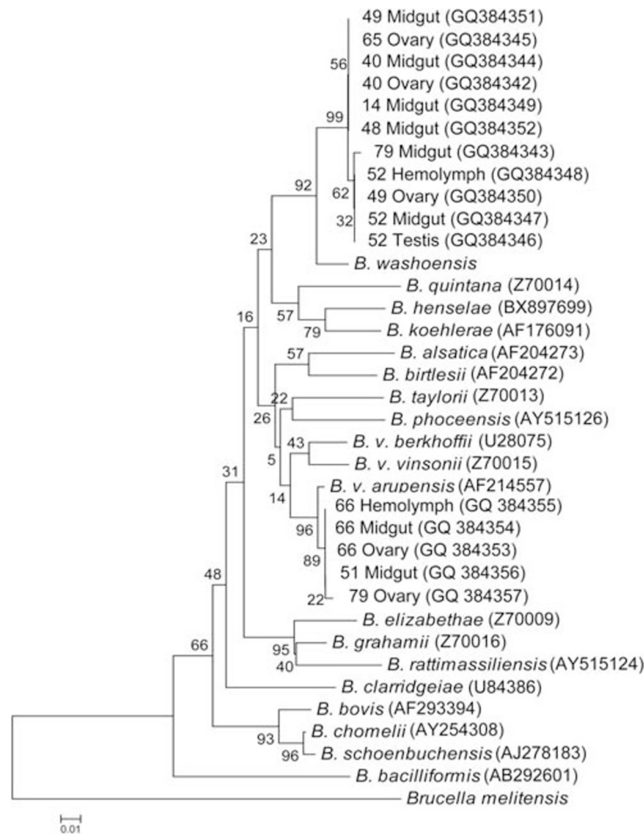

Figure 1 Neighbor-joining tree showing inferred relationships between *Bartonella* citrate synthase (*gltA*) amplicons derived from field-collected mammalian fleas and known *Bartonella*-type species. This phylogeny was constructed using the Kimura two-parameter model, and bootstrap values were calculated after 1000 replicates. Analysis was conducted using MEGA 4.0 software (Tamura and Dudley, 2007).

DNA in fleas collected from prairie dogs, deer mice and fox (Table 1). *B. v. arupensis* is associated with *Peromyscus* mice (Jardine *et al.*, 2005) and was detected in *P. maniculatus* blood samples, yet was also detected in prairie dog and deer mouse fleas (Table 1). Furthermore, *Bartonella* DNA was detected in five of six fleas collected from culture-negative hosts. These results suggest that fleas harbor richer *Bartonella* assemblages than their hosts, and that mammals may be exposed to a wider spectrum of *Bartonella* than those causing bacteremia. Abbot *et al.* (2007) reported similar patterns of coinfection in rodent fleas and suggested that host specialization may account for mammal–*Bartonella* relationships. Bartonellae can 'jump' among host species (Jardine *et al.*, 2005; Bai *et al.*, 2007) and fleas have been implicated in facilitating such events (Bai *et al.*, 2007). Our results show that fleas can acquire *Bartonellae* that are characteristic of multiple host species; however, flea-mediated transmission of *Bartonella* between prairie dogs and deer mice seems unlikely as there is little flea exchange between these hosts (Brinkerhoff, 2008; Salkeld and Stapp, 2008). Note, however, that PCR detection of *Bartonella* in fleas does not necessarily indicate active infection in the host just as lack of *Bartonella* detection by culture of host blood does not rule out low-level infection.

Bartonella detection in reproductive tissue suggests that vertical transmission is a possible alternative mechanism to horizontal transmission in fleas. If vertical transmission of *Bartonella* within flea lineages exists, rare host-switching events could lead to the accumulation and maintenance of atypical *Bartonella* species in fleas. In a study of blood-sucking flies, 100% of pupae tested positive for *Bartonella* DNA (Halos *et al.*, 2004), but we are unaware of any such reports in fleas. *Bartonellae* may also be transmitted through arthropod feces; louse feces have been shown to support *B. quintana* and are implicated in inoculating humans with this pathogen (Raoult and Roux, 1999). Adult flea feces can support *Bartonella* (Finkelstein *et al.*, 2002) and are an important food source for larvae.

This project was funded by a fellowship to RJB from the East Asia and Pacific Summer Institutes program of the National Science Foundation (award no. OISE-0714395) and by the Japan Society for the Promotion of Science. A number of undergraduate students at the Nihon University College of Bioresource Sciences assisted with various aspects of this research. In particular, we would like to thank Yasuhito Izumi for demonstrating to us the technique for flea tissue separation.

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