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Zoonotic *Bartonella* Species in Fleas Collected on Gray Foxes (*Urocyon cinereoargenteus*)

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Abstract

Bartonella spp. are fastidious, gram-negative, rod-shaped bacteria and are usually vector-borne. However, the vector has not been definitively identified for many recently described species. In northern California, gray foxes (*Urocyon cinereoargenteus*) are infected with two zoonotic *Bartonella* species, *B. rochalimae* and *B. vinsonii subsp. berkhoffii*. Fleas (range 1–8 fleas per fox) were collected from 22 (41.5%) of 54 gray foxes from urban and backcountry zones near Hoopa, California. The flea species were determined, and DNA was individually extracted to establish the *Bartonella* species harbored by these fleas. Of the 108 fleas collected, 99 (92%) were identified as *Pulex simulans*. Overall, 39% (42/108) of the fleas were polymerase chain reaction (PCR)-positive for *Bartonella*, with *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii* identified in 34 (81%) and 8 (19%) of the PCR-positive fleas, respectively. There was no difference between the prevalence of *Bartonella* spp. in *P. simulans* for the urban and backcountry zones. Fourteen (64%) of the 22 foxes were *Bartonella* bacteremic at one or more of the capture dates. In 10 instances, both the foxes and the fleas collected from them at the same blood collection were *Bartonella*-positive. *B. rochalimae* was the predominant species identified in both foxes and fleas. The competency of *Pulex* fleas as a vector of *B. rochalimae* has not been confirmed and will need to be demonstrated experimentally. *Pulex* spp. fleas readily feed on humans and may represent a source of human exposure to zoonotic species of *Bartonella*.

Key Words: *Bartonella vinsonii* subsp. *berkhoffii*—*Bartonella rochalimae*—*Pulex simulans*—*Ctenocephalides felis*—gray fox—*Urocyon cinereoargenteus*—California.

Introduction

Since the reclassification of the genera *Grahamella* and *Rochalimaea* within the genus *Bartonella*, this genus comprises at least 20 species or subspecies, with more than half being zoonotic (Brenner et al. 1993, Birtles et al. 1995, Boulouis et al. 2005, Chomel et al. 2006, Dehio 2004). *Bartonella* spp. are fastidious gram-negative bacteria that infect the erythrocytes and endothelial cells of their hosts (Chomel et al. 2006). Human diseases caused by *Bartonella* species include bartonellosis (*B. bacilliformis*), trench fever (*B. quintana*), cat scratch disease (*B. henselae*), and, in immunocompromised people, bacillary angiomatosis (*B. henselae* or *B. quintana*). A wide array of clinical manifestations, ranging from recurrent bacteremia, endocarditis, and septicemia have also been associated with *Bartonella* infections (Boulouis et al. 2005, Dehio 2004).

Bartonella spp. are usually vector-borne bacteria, transmitted mainly by fleas, lice, or sandflies (Boulouis et al. 2005). *Ctenocephalides felis*, the cat flea, is the main vector for *B. henselae* and presumably for *B. clarridgeiae* (Chomel et al. 1996, Kordick et al. 1997, Finkelstein et al. 2002, Rolain et al. 2003a, Kelly 2005). DNA from several *Bartonella* species has been detected in cat fleas, including *B. henselae*, *B. koehlerae*, *B. clarridgeiae*, and *B. quintana* (Rolain et al. 2003a). *Bartonella* DNA has also been detected in recent years in *Pulex* spp. fleas. *Bartonella quintana* DNA was detected in three *P. irritans* fleas taken from a pet moustached monkey (*Cercopithe-*

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cus cephus) in Gabon (Rolain et al. 2005). In addition, DNA from three unidentified species of *Bartonella* has been detected in *Pulex* spp. fleas collected from the bedding and clothes of schoolchildren and adults in Peru (Parola et al. 2002). A partial sequence from the 16S–23S intergenic spacer (ITS) region from one of these fleas was most similar to *B. clarridgeiae*. The ITS sequence from this flea was later discovered to be 100% identical to the ITS sequence from a *Bartonella* sp. isolated from an American woman with fever, rash, splenomegaly, and a recent history of travel in Peru. This person was also reported to have many insect bites. This human isolate, recently described as a new species of *Bartonella*, has been designated *Bartonella rochalimae* (Eremeeva et al. 2007).

Bartonella vinsonii subsp. *berkhoffii*, a pathogen of dogs and humans, has been suspected to be carried by ticks, based both on seroepidemiological studies and DNA detection in different tick species, but transmission by ticks has not been confirmed (Chang et al. 1999, 2001, Chomel et al. 2006). Detection of *B. vinsonii* subsp. *berkhoffii* DNA has not been reported to date from cat fleas. However, DNA extracted from a *Pulex* flea collected from a human in Peru showed a sequence closely related to *B. vinsonii* subsp. *berkhoffii* (Parola et al. 2002). In domestic dogs (*Canis familaris*), *B. vinsonii* subsp. *berkhoffii* infection has been associated with endocarditis, myocarditis, arrhythmia, and other diverse clinical outcomes (Breitschwerdt and Kordick 2000, Chomel et al. 2006). At least one human case of endocarditis-associated *B. vinsonii* subsp. *berkhoffii* has also been reported (Roux et al. 2000).

Wildlife may function as natural reservoirs or maintenance hosts for *Bartonella* species, and peridomestic wildlife may bring arthropod vectors in close contact with domestic animals and humans. Coyotes (*Canis latrans*) are suggested to be the main sylvatic reservoir of *B. vinsonii* subsp. *berkhoffii* in California due to the high percentage (28%) of bacteremic animals reported in one study (Chang et al. 1999). In northwestern California, gray foxes (*Urocyon cinereoargenteus*) may serve as a reservoir for the recently described *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii* (Henn et al. 2006, 2007). Among 53 gray foxes from Humboldt county, 26 (49%) were *Bartonella* bacteremic, 22 (42%) with *B. rochalimae* (formerly *B. clarridgeiae*-like), and 5 (9.4%) with *B. vinsonii* subsp. *berkhoffii* (Henn et al. 2006, 2007). *Bartonella rochalimae*, previously designated *B. clarridgeiae*-like, has also been associated with a case of endocarditis in a dog (MacDonald et al. 2004).

Gray foxes are a cosmopolitan species, which can often occur in high densities (Trapp and Hallberg 1975, Fritzell and Haroldson,1982, Cypher 2003), and commonly harbor ectoparasites that could be vector-competent for *Bartonella* species. Moreover, gray foxes are frequently observed near human dwellings, can interact with pets, and eat unattended outdoor pet food, garden crops, or refuse left outside of these dwellings (Harrison 1993, Cypher 2003). The purpose of the present study was to identify fleas collected from gray foxes in a rural area of northern California to determine if *Bartonella* spp. DNA could be amplified from these fleas. The gray foxes had been previously tested for *Bartonella* (Henn et al. 2007), allowing for comparison of *Bartonella* spp. DNA identified in the fleas with the results of blood culture performed on their hosts.

Materials and Methods

Sampling was conducted on the Hoopa Valley Indian Reservation in northeastern Humboldt County, California (UTM 10 04 43624 E, 45 44 450 N). Gray foxes were live-trapped from June 2003 to mid-October 2004 in 81 × 25 × 31–cm wire mesh traps (Model 108 Tomahawk Live Trap Company, Tomahawk, WI) and the trapping protocol followed the methods described previously (Gabriel 2006, Henn et al. 2007). A total of 54 foxes were trapped 70 times, as several foxes were trapped more than once (Henn et al. 2007).

A thorough systematic examination (≥5 minutes) of the fur on each individual fox was conducted. All fleas observed were collected in 70% ethanol for later identification. Foxes were designated as an "urban fox capture" or a "backcountry fox capture" (Gabriel 2006). The urban zone included the valley floor as it follows the Trinity River over 16.4 km in a south–north direction with elevations varying between 76 m and 152 m above sea level (Singer and Begg 1975), where the majority of the people live (Fig. 1). The backcountry zone included the remaining area of the Hoopa Valley Indian Reservation with elevations ranging from 152–1170 m. This area is managed for its natural resources and cultural preservation. Fleas that were removed from the foxes were also assigned the same code number and locality designation than the fox from which it was removed.

Flea identification

All fleas in vials that had five or fewer fleas per fox capture were identified to species. In the vials that had more than five fleas (up to eight fleas maximum), only five individual fleas that showed superficially visible morphological differences (i.e., pronotol combs, genal combs) were identified from each capture and were processed. Fleas were maintained in 70% ethanol, removed from ethanol individually onto a clean glass slide and allowed to sit until the ethanol had evaporated. An incision was made with a sterile scalpel blade across the tergites to release body contents, then the entire flea was incubated in a 1.5 mL Eppendorf tube with 180 μL of ATL buffer (QuiAmp, Valencia, CA) and 20 μL of proteinase K at 55°C for 6 hours. Subsequently, the exoskeleton was removed for mounting for identification by immersion in potassium hydroxide for 24 hours and then transfer through dehydration series (70%, 80%, 95%, and 100%) of ethanol for 30 minutes per step. Specimens were then transferred to methyl salicylate for 30 minutes, then xylene for 1 hour, and were mounted in Canada balsam. Each flea was identified to species using western North American taxonomic keys (Hubbard 1968, Lewis et al. 1988). All remaining material in the tube was processed for DNA extraction as per the Qiagen kit tissue extraction protocol (QuiAmp, Valencia, CA).

DNA extraction and polymerase chain reaction–restriction fragment length polymorphism procedures

DNA was extracted from the material remaining after the flea was removed from incubation in proteinase K using a commercial DNA extraction kit (QuiAmp). Flea DNA samples were analyzed by polymerase chain reaction (PCR) of the 16S–23S ITS region (Rolain et al. 2003b) and the *fts*Z (Zeaiter et al. 2002) gene. PCR reaction vials were set up as

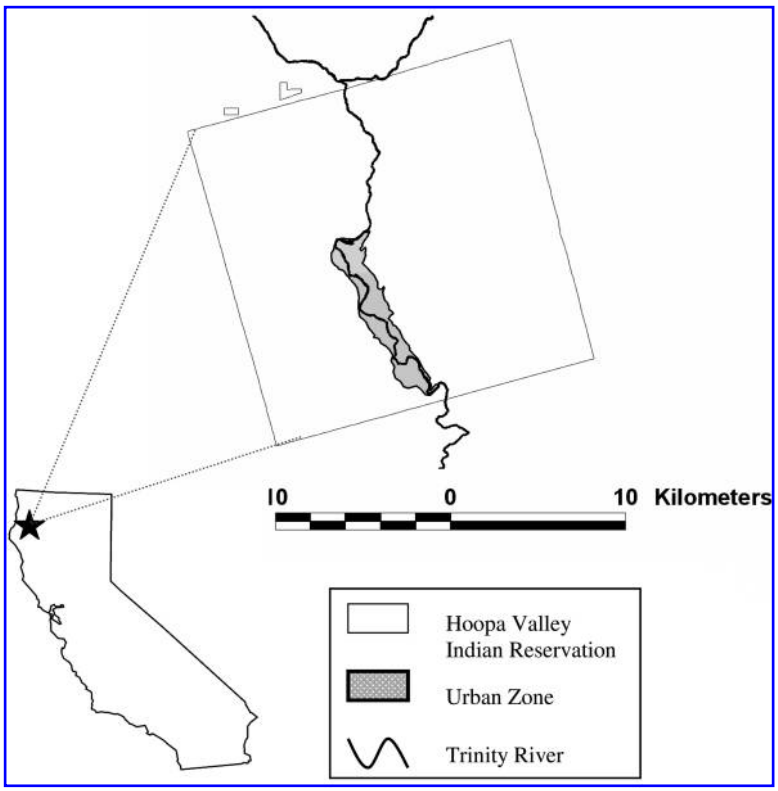

FIG. 1. Map of the Hoopa Valley Indian Reservation, Humboldt County, California.

previously described (Chang et al. 2000). Briefly, DNA was diluted 1:10 in buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid) and 0.25 μM of each primer was used. The primers used for the ITS region were 5′-CTTCGTTTCTCTTTCTTCA-3′ and 5′-CTTCTCTTCACAATTTCAAT-3′ and the primers for the *fts*Z gene were 5′-ATTAATCTGCAYCGGCCAGA-3′ and 5′-ACVGADACACGAATAACACC-3′. Amplified PCR products were identified by ethidium bromide fluorescence after electrophoresis in 2% agarose gels (SeaKem LE agarose, Cambrex Bio Science Rockland Inc., Rockland, ME). The amplified product of the ITS region was digested with *Hae*III restriction endonuclease (Promega, Madison, WI). Banding patterns were compared with the patterns observed for *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii* isolates from the gray foxes from which the fleas were collected.

DNA sequencing

PCR products used for DNA sequencing were purified with QIAquick PCR purification kit (QIAGEN Sciences, Germantown, MD), and sequencing was done using a fluorescence-based automated sequencing system (Davis Sequencing, Davis, CA).

Statistical analysis

All statistical analyses (chi-square for trend) were conducted using statistical software from Number Cruncher Statistical Software (NCSS 2001). Results were considered significant if p values were ≤ 0.05.

Results

Of the 53 foxes for which a blood culture was performed, 22 (41.5%) had fleas and 108 fleas were removed for further analysis. On average, there were five fleas collected per infested fox (range 1–8 fleas). Ninety-nine (94%) fleas were identified as *P. simulans*, three (2.8%) as *Ctenocephalides felis*, and one each as *Ctenocephalides canis*, *Cediopsylla inequalis interrupta*, and *Orchopeas laens*. Seventy-five (76%) *P. simulans* were female and 24 (24%) were males. Two of the three *C. felis* were females and the single *C. canis* was a female. Three fleas could not be identified to species due to damage from mounting of the specimen leading to absence of distinctive morphological characters.

Forty-two (39%) of the 108 flea DNA samples tested were PCR-positive for *Bartonella* spp. based on the ITS region. Thirty-nine (93%) of the 42 *Bartonella* PCR-positive fleas were identified as *P. simulans*, with 28 (37%) positive females and 11 (46%) positive males. Despite a higher prevalence of infection in male *P. simulans* fleas, the difference was not statistically significant ($\chi^2 = 0.55$, df = 1, $p = 0.45$). One *C. felis*, one *C. inequalis*, and one unidentified flea species were PCR-positive for *Bartonella*. Thirty-three (78.6%) of the 42 *Bartonella* PCR-positive samples had an ITS RFLP profile characteristic of *B. rochalimae*, and 8 (19%) had a banding pattern similar to that observed for the *B. vinsonii* subsp. *berkhoffii* strains isolated from gray foxes in Humboldt county. Banding patterns could not be determined for the remaining two *Bartonella* PCR-positive fleas.

Six flea samples with RFLP profiles from *Hae*III digests of ITS PCR products matching *B. rochalimae* and four flea sam-

ples with RFLP profiles matching *B. vinsonii* subsp. *berkhoffii* were selected for nucleic acid sequencing. Whenever enough DNA was available, sequencing was performed for both the ITS region and *ftsZ* gene. For the six fleas with RFLP profiles matching *B. rochalimae*, partial sequences from the ITS region and *ftsZ* gene were ≥99.6 % identical to *B. rochalimae* previously isolated from the gray foxes. For two of the four flea products sequenced with RFLP profiles similar to *B. vinsonii* subsp. *berkhoffii*, ITS and *ftsZ* DNA sequences were 100% identical to *B. vinsonii* subsp. *berkhoffii* previously isolated from the gray foxes. For the third flea, the ITS sequence was identical to *B. vinsonii* subsp. *berkhoffii*, but the partial sequence from the *ftsZ* gene amplicon was identical to *B. rochalimae*. For the fourth flea, the RFLP profile for the initially amplified PCR product had suggested *B. vinsonii* subsp. *berkhoffii*, but when DNA was amplified a second time and sent for sequencing, the ITS sequence was identical to *B. rochalimae*; there was not enough DNA available for partial sequencing of the *ftsZ* gene. Finally, for the two *Bartonella*-positive flea samples that did not have clear RFLP profiles due to low amounts of DNA in the ITS PCR product, sequences from the *ftsZ* PCR product for these samples were identical to *B. rochalimae*. Overall, 39% (42/108) of the fleas were PCR-positive for *Bartonella*, with *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii* identified in 33 (78.5%) and 8 (19%) of the PCR-positive fleas, including two fleas that appeared to be coinfected.

Sixty-six *P. simulans* were removed from foxes in the urban zone, and 33 *P. simulans* were removed from foxes in the backcountry zone. Two *C. felis*, the *C. inequalis*, and *C. canis* were removed from a fox in the urban zone. One *C. felis* and the *O. laens* flea were removed from a fox in the backcountry zone. Twenty-three (34.8%) of the 66 urban *P. simulans* were PCR-positive, and 16 (48.5%) of the 33 backcountry *P. simulans* were positive. The difference between the prevalence of *Bartonella* spp. in *P. simulans* between the urban area and the backcountry was not statistically significant (χ^2 = 1.71, df = 1, p = 0.19).

Seven (32%) of the 22 foxes that had fleas removed from them were trapped more than once during the course of the study (Table 1). Fourteen (64%) of the 22 foxes were found to be infected with *Bartonella* spp. on one or more capture dates when tested as part of a previous study (Henn et al. 2007). There were 10 (71.4%) instances in which both the foxes and the fleas collected from them on the same capture date were positive for *Bartonella* DNA. In 7 (72.7%) of these 11 pairs (foxes #6, 8, 12, 13, 14, 18, 21), both the foxes and fleas were positive for *B. rochalimae*. For two pairs, the foxes (#1 and #3) were bacteremic with *B. vinsonii* subsp. *berkhoffii* and had fleas on them that were PCR-positive for both *B.*

Table 1. *Bartonella* PCR and Blood Culture Results for *Pulex simulans* and Their Gray Fox Hosts

	1st capture			*Recapture 1*			*Recapture 2*			*Recapture 3*		
Fox #	*Flea PCR+*	*Bart type*	*Fox cult type*	*Flea PCR+*	*Bart type*	*Fox cult type*	*Flea PCR+*	*Bart type*	*Fox cult type*	*Flea PCR+*	*Bart type*	*Fox cult type*
1	0/1	Neg	Neg	1/2	1Bvb	Neg	2/2	1Bvb 1Br	Bvb			
2	0/4	Neg	Br									
3	4/5	3Bvb 1Br	Bvb									
4	2/5	2Br	Neg									
5	1/4	1Br	Neg									
6	2/4	2Br	Br									
7	2/6	2Br	Neg									
8	3/6	3Br	Br									
9	0/1	Neg	Br									
10	0/3	Neg	Neg									
11	0/0	NA	Bvb	0/1	Neg	Neg	0/0	NA	Br	4/6	4 Br	Neg
12	1/7	Br	Br									
13	0/1	Neg	Neg	2/4	2Br	Br	3/8	2Br 1Bvb	Br			
14	2/5	2Br	Br									
15	2/4	2Br	Neg									
16	0/0	NA	Neg	0/1	Neg	ND						
17	3/7	2Br 1Bart (Unknown sequence)	Neg	0/2	Neg	Br						
18	1/2	1Br	Br									
19	3/6	3Br	Neg									
20	2/3	1Br 1Bvb	Neg									
21	1/2	1Br	Br	0/1	Neg	Neg						
22	0/0	NA	Br	1/5	1Bvb	Br						

The Flea PCR + column describes the number of fleas that were *Bartonella* PCR-positive out of the total number of fleas tested from that fox. PCR, polymerase chain reaction; NA, not applicable; ND, not done; Neg, negative; Br, *Bartonella rochalimae*; Bvb, *Bartonella vinsonii berkhoffii*.

vinsonii subsp. *berkhoffii* and *B. rochalimae*. The last pair was composed of a fox (#22) infected with *B. rochalimae*, but with one out of five fleas infected with *B. vinsonii* subsp. *berkhoffii*. There were three foxes (#2, 9, 17) infected with *B. rochalimae* that had no *Bartonella*-positive fleas at the time of the positive blood culture. One fox (#11) was sequentially bacteremic with *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae*, but the only PCR-positive fleas (infected with *B. rochalimae*) were collected on the last recapture, when the animal was already abacteremic. Finally, there were eight foxes (#4, 5, 7, 10, 15, 16, 19, 20) that were *Bartonella* culture-negative that had 32 fleas collected from them, 12 (37.5%) of which were *Bartonella* PCR-positive. Eleven (91.7%) of these fleas were positive for *B. rochalimae*.

Discussion

This is the first report of the presence of two zoonotic bartonellae, *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii*, in fleas collected from gray foxes. Most of these fleas were identified as *P. simulans*, which are commonly found on gray foxes (Lewis et al. 1988, Hubbard 1968). Overall, 42 (39%) of the 108 fleas were *Bartonella* PCR-positive. *B. rochalimae* was the predominant (81%) species identified in these fleas. Two of the eight samples that were initially identified as *B. vinsonii* subsp. *berkhoffii* based on the restriction fragment length polymorphism profile also appeared to have *B. rochalimae* DNA present when PCR amplification was performed a second time before sequencing. This unexpected finding suggests that these fleas were harboring both *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae*.

The prevalence of *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii* identified in fleas was similar to the prevalence of the two species identified in foxes by blood culture. *Bartonella rochalimae* was isolated from 22 (42%) of the 53 gray foxes tested, and *B. vinsonii* subsp. *berkhoffii* was isolated from only 5 (9.4%) gray foxes (Henn et al. 2007). Fleas from six of the eight nonbacteremic foxes were PCR-positive for *B. rochalimae*, and a flea on one of those six foxes was PCR-positive for *B. vinsonii* subsp. *berkhoffii*. This suggests that the amplification of *Bartonella* DNA from these fleas could reflect more than just a recent blood meal on a bacteremic host and that the organism may be multiplying in the flea gut, as has been demonstrated with *B. henselae* (Higgins et al. 1996). Furthermore, it may also reflect earlier infection of the fleas when feeding on another bacteremic fox or our lack of detecting a very low level of bacteremia in the culture-negative foxes.

Bartonella clarridgeiae-like and *B. rochalimae* DNA has also been detected in *Pulex* fleas from other parts of the world. *Bartonella* DNA was detected in *Pulex* fleas collected from people in Peru, including a sample described as similar to *B. clarridgeiae* (Parola et al. 2002) and later found to be identical to a human *B. rochalimae* isolate based on partial sequence of the 16S–23S ITS region (Ereemeva et al. 2007). In Chile, 5 (15%) of 33 *P. irritans* fleas collected on dogs were found to be *Bartonella* PCR-positive, and partial sequencing of the *rpo*B gene identified a *Bartonella* similar to *B. clarridgeiae* (95% identity; Gonzales-Acuna et al. 2006). Furthermore, *Bartonella* DNA was detected in 4 of 19 pools (total 95 *P. irritans*) collected from four different red foxes (*Vulpes vulpes*) in Hungary (Sreter-Lancz et al. 2006). The *groEL* sequences of all four positive pools were identical and similar to the sequence of a *Bartonella* detected in rat fleas (*Xenopsylla cheopis*) from Egypt and similar to *B. clarridgeiae* in the maximum parsimony tree published by Loftis et al. (2006). These data are suggestive that DNA from a *B. clarridgeiae*-like group, possibly *B. rochalimae*, could be identified in *P. irritans* fleas collected on red foxes in central Europe and in rat fleas in Egypt, two flea species that can infest humans. Furthermore, strains closed to *B. clarridgeiae* were recently identified in *Ctenophthalmus lushuiensis* fleas collected in vole nests from Yunnan, China (Li et al. 2007), and strains close to *B. clarridgeiae* and *B. rochalimae* were described from two fleas (*Polygenis gwyni*) collected from cotton rats (*Sigmodon hispidus*; Abbot et al. 2007).

There were no differences between the *Bartonella* prevalence in fleas collected from foxes in the urban zone and in the backcountry, suggesting that this pathogen is well distributed among the fox population in the Hoopa Valley. Gray foxes are likely to represent one of the main reservoirs of *B. rochalimae* in this area of northern California. The competency of *P. simulans* as a vector of *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii* has not been established, and experimental studies will be needed to demonstrate transmission of these organisms by *P. simulans* to pathogen-free hosts. As *Pulex* spp. will readily feed on humans, these fleas may represent a source of human exposure to zoonotic species of *Bartonella*.

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