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Zoonotic *Bartonella* Species in Fleas and Blood from Red Foxes in Australia

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Abstract

Bartonella are arthropod-borne, fastidious, Gram-negative, and aerobic bacilli distributed by fleas, lice, sand flies, and, possibly, ticks. The zoonotic *Bartonella* species, *Bartonella henselae* and *Bartonella clarridgeiae*, which are the causes of cat scratch disease and endocarditis in humans, have been reported from cats, cat fleas, and humans in Australia. However, to date, there has been no report of *B. henselae* or *B. clarridgeiae* in Australian wild animals and their ectoparasites. *B. henselae* and *B. clarridgeiae* were detected in fleas (*Ctenocephalides felis*) from red foxes (*Vulpes vulpes*), an introduced pest animal species in Australia, and only *B. clarridgeiae* was detected in blood from one red fox. Phylogenetic analysis of the ribosomal intergenic spacer region revealed that the *B. henselae* detected in the current study were related to *B. henselae* strain Houston-1, a major pathogenic strain in humans in Australia, and confirmed the genetic distinctness of *B. clarridgeiae*. The identification and characterization of *Bartonella* species in red foxes in the Southwest of Western Australia suggests that red foxes may act as reservoirs of infection for animals and humans in this region.

Key Words: *Bartonella* spp.—Fleas—Red fox—Pest animal—Western Australia.

Introduction

Members of the genus *Bartonella* are arthropod-borne, fastidious, Gram-negative, and aerobic bacilli distributed by fleas, lice, sand flies, and, possibly, ticks (Billeter et al. 2008). *Bartonella henselae* and *Bartonella clarridgeiae* have been reported from cats, cat fleas, and humans in Australia (Iredell et al. 2003, Barrs et al. 2010). However, to date, there has been no report of *B. henselae* or *B. clarridgeiae* in Australian wild animals and their ectoparasites.

The European red fox (*Vulpes vulpes*) occupies a wide variety of habitats across the continents of Europe, Asia, and North America. In the Southern Hemisphere, the red fox occurs only in Australia (Rolls 1984), where it was imported during the 19th century by English colonists for the purpose of hunting (Rolls 1984). However, this activity was not sufficient to keep red fox numbers in check, and they have since contributed to major population declines in a number of native Australian fauna species (Saunders and McLeod 2007). They are now considered a serious invasive pest species, and programs to eradicate the animals are conducted throughout Australia (Saunders and McLeod 2007). Previous research has discussed the distribution of some of their diseases and parasites (Glen and Dickman 2005).

Bartonella species have been reported in wild canids in North America and Europe, including coyotes (*Canis latrans*), gray foxes (*Urocyon cinereoargenteus*), and red foxes (*V. vulpes*) (Henn et al. 2009, Gabriel et al. 2009, Marquez et al. 2009); and the possibility that these canid species may act as *Bartonella* reservoirs has been discussed (Henn et al. 2009, Gabriel et al. 2009, Marquez et al. 2009). So far, only *Bartonella rochalimae* has been isolated or detected from red foxes (Henn et al. 2009). *Bartonella* spp. have also been detected in various flea species collected from the wild canids (Marquez et al. 2009), and fleas have been proposed to be major vectors of *Bartonella* spp. among wild canids (Henn et al. 2009, Gabriel et al. 2009).

Here, we report the first detection of zoonotic *Bartonella* species, including *B. henselae* and *B. clarridgeiae*, in both fleas (*Ctenocephalides felis*) and blood collected from European red foxes in southwest Western Australia.

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TABLE 1. NUMBER OF FLEAS, POOLED FLEA DNA, BLOOD SAMPLES, AND *BARTONELLA* SPP. IN TWO LOCATIONS IN WESTERN AUSTRALIA

	Katanning					Boyup Brook				
Sample	Number of samples	DNA samples	PCR positive	*Bartonella henselae*	*Bartonella clarridgeiae*	Number of samples	DNA samples	PCR positive	*Bartonella henselae*	*Bartonella clarridgeiae*
Ctenocephalides felis	117	25	24	4	20	34	9	0	-	-
Blood	10	10	1	0	1	4	4	0	-	-

Materials and Methods

Samples collection

Flea and blood samples were collected from red foxes in March 2010, in the areas surrounding the towns of Katanning (20–21st March) and Boyup Brook (27–28th March) in southwest Western Australia. An excess of 500 red foxes were shot by volunteers and farmers as a part of the "Red Card for the Red Fox" 2010 culling program coordinated by the Department of Agriculture and Food, Western Australia. Carcasses were brought to a central location for recording within 48 h, at which point, flea and blood samples were collected from 164 red foxes. Storage of red fox carcasses together during culling implied that fleas were able to move between carcasses, and as such it was not possible to identify individual fleas originating from specific red fox carcasses. A total of 151 fleas were randomly collected from 164 red foxes by using a flea comb or tweezers. Fleas were stored in 70% ethanol until they were processed for DNA extraction. Blood samples were collected from the peritoneal cavity of 14 red foxes into EDTA tubes and were stored at −20°C. Of these, ten were collected from red foxes in Katanning and four from Boyup Brook. Species of fleas were identified as *C. felis* by light microscopy using the standard key for Australian fleas (Dunnet and Nardon 1974). Between three and five fleas were pooled before DNA extraction, resulting in 34 pooled flea samples from the 151 fleas collected. Of the 117 fleas collected from Katanning, 25 flea pools were prepared and used for DNA extraction, whereas nine flea pools were prepared from 34 fleas collected from Boyup Brook (Table 1).

DNA extraction and PCR

DNA was extracted from pooled fleas and individual blood samples by using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Nested-PCR of

TABLE 2. GENBANK ACCESSION NUMBERS OF *BARTONELLA* SPECIES USED FOR THE CONCATENATED PHYLOGENETIC ANALYSIS

Bartonella *species*	16S rRNA	*ftsZ*	*gltA*	ITS	*rpoB*
Bartonella tamiae	EF672729	EF605281	DQ395177	EF605183	EF672730
Candidatus Bartonella rudakovii	EF682086	EF682092	EF682090	EF682087	EF682088
Bartonella rochalimae	DQ683196	DQ676486	DQ683195	DQ683199	DQ676489
Bartonella rattimassiliensis	AY515120	AY515133	AY515125	AY515121	AY515131
Bartonella rattaustraliani	EU111753	EU111771	EU111797	EU111760	EU111786
Bartonella queenslandensis	EU111758	EU111780	EU111801	EU111765	EU111790
Bartonella phoceensis	AY515119	AY515135	GU056197	AY515123	AY515132
Bartonella coopersplainsensis	EU111759	EU11781	EU111803	EU111770	EU111792
Bartonella chomelii	NR025736	AB290193	AY254308	AB498010	AB290189
Bartonella capreoli	NR025120	AB290192	AF293392	AB498009	AB290188
Bartonella australis	DQ538394	DQ538399	DQ538395	DQ538396	DQ538397
Bartonella alsatica	AJ002139	AF467763	AF204273	AF312506	AF165987
Bartonella bacilliformis	Z11683	AF007266	U28076	L26364	AF165988
Bartonella birtlesii	AF204274	AF467762	AF204272	AY116640	AF165989
Bartonella bovis	AF199502	AF467761	AF293394	AY116638	AF166581
Bartonella clarridgeiae	U64691	AF141018	U84386	AF167989	AF165990
Bartonella doshiae	Z31351	AF467754	AF207827	AJ269786	AF165991
Bartonella elizabethae	L01260	AF467760	U28072	L35103	AF165992
Bartonella grahamii	Z31349	AF467753	Z70016	AJ269785	AF165993
Bartonella henselae	M73229	AF061746	L38987	L35101	AF171070
Bartonella koehlerae	AF076237	AF467755	AF176091	AF312490	AY166580
Bartonella quintana	M11927	AF061747	Z70014	L35100	AF165994
Bartonella schoenbuchensis	AJ278187	AF467765	AJ278183	AY116639	AY167409
Bartonella taylorii	Z31350	AF467756	AF191502	AJ269784	AF165995
Bartonella tribocorum	AJ003070	AF467759	AJ005494	AF312505	AF165996
Bartonella vinsonii subsp.*arupensis*	AF214558	AF467758	AF214557	AF312504	AY166582
Bartonella vinsonii subsp. *berkhoffii*	U26258	AF467764	U28075	AF167988	AF165989
Bartonella vinsonii subsp.*vinsonii*	M73230	AF467757	Z70015	L35102	AF165997

ITS, intergenic spacer.

the ribosomal intergenic spacer (ITS) region and the *gltA* gene was initially performed to detect *Bartonella* DNA in both flea pools and blood samples. External primers for the *gltA* gene and the ITS region were designed from the DNA sequences of *B. henselae* held in GenBank (*gltA*: L38987, ITS: L35101) (Table 2). The internal primers for nested-PCR of the ITS region and the *gltA* gene were as previously described, respectively (Norman et al. 1995, Jensen et al. 2000). An internal reverse primer targeting the ITS region was also designed for amplification and sequencing of a large fragment of the ITS region. DNA amplifications and sequencings of other loci including the 16S rRNA, *ftsZ*, and *rpoB* genes were performed to confirm the species status of *Bartonella* species detected in this study. All primers used in this study were described in a previous study (Kaewmongkol et al. 2011). PCR products from all genes were purified from agarose gel slices by using an UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories, Inc.). Sequencing was performed by using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Biosystems) on an Applied Biosystems 3730 DNA Analyzer, following the manufacturer's instructions. Nucleotide sequences generated for all 5 loci were analyzed by using Chromas lite version 2.0 (www.technelysium.com.au) and aligned with reference sequences from *Bartonella* spp. from GenBank by using Clustal W (www.clustalw.genome.jp). Phylogenetic analysis was conducted on both the large fragment of the ITS region and on concatenated sequences from the 16S rRNA, *gltA*, *ftsZ*, *rpoB* genes, and the ITS region. GenBank accession numbers of *Bartonella* species used for the concatenated phylogenetic analysis were shown in Table 2. Distance estimation was conducted using MEGA version 4.1 (MEGA4.1: Molecular Evolutionary Genetics Analysis software), based on evolutionary distances calculated with the Kimura's distance and grouped using neighbor joining. Bootstrap analyses were conducted by using 10,000 replicates to assess the reliability of inferred tree topologies.

Results

Bartonella species were detected in 24 of the 34 DNA samples (70.5%) from pooled fleas using nested PCR of the ITS region and *gltA* gene. All 24 positive samples were from fleas collected from the area surrounding the town of Katanning (only one sample from fleas from this area was negative for *Bartonella* spp.). DNA sequencing of the 16S rRNA, *gltA*, *ftsZ*, and *rpoB* genes and the ITS region in all 24 *Bartonella* PCR positive samples revealed that 20 PCR positive samples were *B. clarridgeiae*, and 4 were *B. henselae*. A concatenated phylogenetic tree of all 5 loci was constructed to identify *Bartonella* species in this study (Fig. 1). *B. clarridgeiae* was also detected in 1 of the 10 blood samples collected from red foxes in Katanning (Table 1). The ITS region sequence of *B. clarridgeiae* amplified from the blood of this fox was identical to the corresponding *B. clarridgeiae* DNA sequences from fleas. Partial sequences for the five loci corresponding to these *B. henselae* and *B. clarridgeiae* detections were submitted to GenBank under the accession numbers HM990954, HM990959 (16S rRNA), HM990955, HM990960 (*gltA*), HM990956, HM990961 (*rpoB*), HM990957, HM990962 (16S-23S rRNA ITS), HM990958, and HM990963 (*ftsZ*). All of the sequences for a particular *Bartonella* species were identical across all samples amplified.

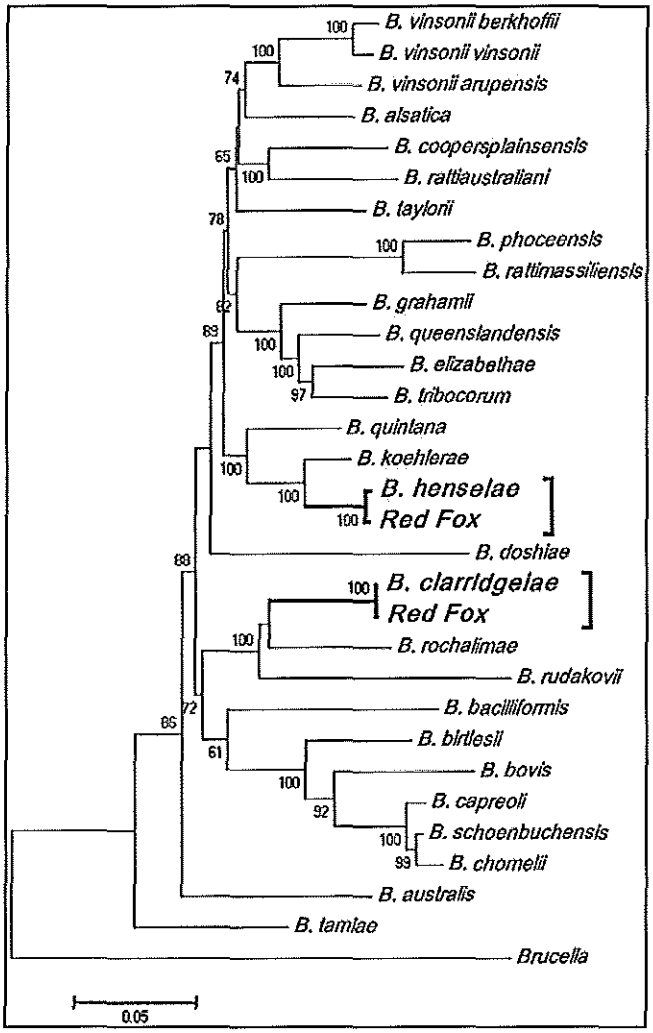

FIG. 1. Neighbor-joining concatenated phylogenetic tree of 16S rRNA, *gltA*, *ftsZ*, *rpoB*, and the intergenic spacer (ITS) region of *Bartonella henselae* and *Bartonella clarridgeiae* detected in fleas from red foxes. Percentage bootstrap support (>60%) from 10,000 pseudoreplicates is indicated at the left of the supported node.

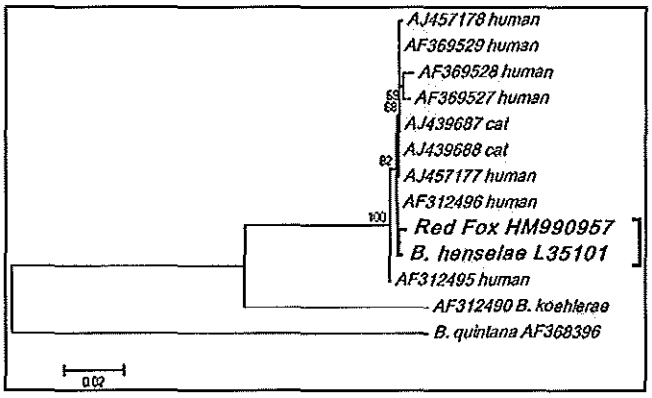

FIG. 2. Neighbor-joining phylogenetic tree of the ITS region of *B. henselae* isolates. Percentage bootstrap support (>60%) from 10,000 pseudoreplicates is indicated at the left of the supported node. The tree is rooted by using *Bartonella quintana* as an outgroup (GenBank accession numbers AF368396).

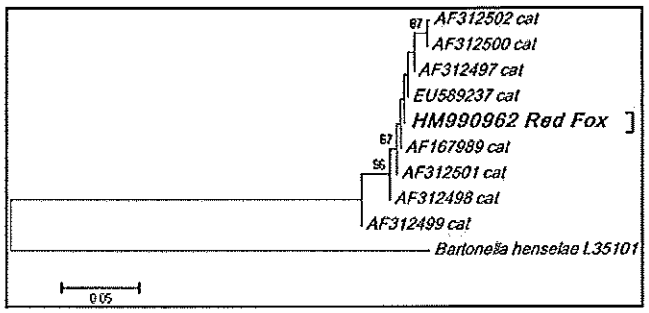

FIG. 3. Neighbor-joining phylogenetic tree of the ITS region of *B. clarridgeiae* isolates. Percentage bootstrap support (>60%) from 10,000 pseudoreplicates is indicated at the left of the supported node. The tree is rooted by using *B. henselae* as an outgroup (GenBank accession numbers L35101).

Phylogenetic analysis of *B. henselae* was conducted by using an 862-bp fragment of the ITS region. Distance analysis of the ITS region showed the close relationship between *B. henselae* detected in fleas from red foxes and *B. henselae* strain Houston-1 (0.3% genetic distance) (Fig. 2). An 893-bp fragment of the ITS region of *B. clarridgeiae* from the current study was also compared with other isolates of *B. clarridgeiae* by using distance analysis, and the resultant tree revealed that the *B. clarridgeiae* detected in red foxes was genetically distinct from previously published sequences of *B. clarridgeiae* (0.1%–2.8% genetic distance) (Fig. 3).

Discussion

This is the first report of *B. henselae* and *B. clarridgeiae*, two zoonotic species of *Bartonella*, from red foxes and their fleas in Australia; and this is the first time that *B. clarridgeiae* has been identified in a red fox. Until now, only *B. rochalimae* has been isolated or detected from red foxes from France (Henn et al. 2009). In the current study, concatenated phylogenetic analysis of all 5 loci confirmed the species status of *B. clarridgeiae* detected from fleas from red foxes, which exhibited 9.5% genetic distance from *B. rochalimae* (Fig. 1). Single-step PCRs for the 16S rRNA, *ftsZ*, and *rpoB* loci were unable to amplify *B. clarridgeiae* DNA in the blood of a red fox. However, *B. clarridgeiae* detected in the blood of a red fox was identical to the corresponding *B. clarridgeiae* sequences from flea extracts at the ITS region. Mixed sequences between *B. henselae* and *B. clarridgeiae* were not detected in this study.

B. clarridgeiae was detected in only one blood sample from a red fox. The true prevalence of *Bartonella* in the fox host may be higher than this result suggests; a pre-enrichment procedure followed by PCR detection has been shown to greatly improve the sensitivity of detecting *Bartonella* DNA in dog blood samples (Duncan et al. 2007). However, the red fox may not be the only natural reservoir for *B. henselae* and *B. clarridgeiae* in this region. Investigation of other wildlife reservoirs living in the same area, including native marsupials, feral cats, rabbits, and pet dogs, should be performed to further elucidate the ecology of the organisms.

The distance between the towns of Katanning and Boyup Brook in the southwest of Western Australia is approximately 120 kilometers. However, all positive samples were detected only in fleas collected from red foxes in the area of Katanning. It is not known why *Bartonella* species were not detected in flea samples from Boyup Brook, and further work needs to be performed to elucidate the ecology of *Bartonella* across the region. Multi-locus sequence typing has previously been conducted to differentiate *B. henselae* strains detected in cats and humans in Australia (Iredell et al. 2003). *B. henselae* sequence type 1 (ST 1), also known as strain Houston-1, has been identified as the principal strain causing human bartonellosis, and it is distributed widely in the cat population in Australia and North America (Iredell et al. 2003, Arvand et al. 2007). Distance analysis of the ITS region revealed that the *B. henselae* strains detected in the current study are closely related to *B. henselae* strain Houston-1 (ST 1) (0.3% genetic distance). The distribution of this strain in other mammalian hosts and their fleas should be defined, particularly in domestic cats in southwest Western Australia.

B. clarridgeiae detected in the current study seems to be distinct from other isolates using distance analysis of a large fragment of the ITS region. Although a prevalence study of *B. clarridgeiae* in cats and cat fleas from Eastern Australia has been previously reported (Barrs et al. 2010), there is little information on the ITS sequences of *B. clarridgeiae* in Australia in GenBank. Two separate ITS trees were produced due to the high level of variation at the ITS region, the phylogenetic analysis of *B. henselae* and *B. clarridgeiae* were resolved much better by conducting two separate analyses.

In conclusion, the identification and characterization of *Bartonella* species in red foxes in the SW of Western Australia suggests that foxes may act as reservoirs of infection for other animals, both wild and domesticated, and humans in this region.

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Disclosure Statement

No competing financial interests exist.

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