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Candidatus Bartonella antechini: a novel Bartonella species detected in fleas and ticks from the yellow-footed antechinus (Antechinus flavipes), an Australian marsupial

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Abstract

Bartonella are fastidious, Gram-negative, aerobic bacilli belonging to the Alphaproteobacteria group. In the last ten years, the discovery of new Bartonella species from a variety of mammalian hosts, arthropod vectors and geographical areas has increased. More than 20 species of Bartonella have been identified, of which approximately thirteen are associated with disease in humans and animals. Recently, four novel species of Bartonella were isolated from mammalian hosts in Australia: B. australis from eastern grey kangaroos (Macropus giganteus) and B. rataustraliani, B. queenslandensis and B. coopersplainsensis from rodents. Bartonella-like organisms have also been detected from Ixodes tasmani ticks collected from koalas (Phascolarctos cinereus). However, very little is known about Bartonella spp. in other marsupials in Australia. We report the identification of a novel Bartonella species detected from fleas (Acanthopsylla jordani) and ticks (Ixodes antechini) collected from a small carnivorous marsupial, Antechinus flavipes (Mardos or Yellow-footed antechinus) in the southwest of Western Australia. New nested-PCRs targeting the gltA gene and the ribosomal ITS region were developed as part of the present study. DNA sequencing of the 16S rRNA, gltA, ftsZ and rpoB genes and the ribosomal ITS region revealed that this detection is a distinct Bartonella species and is related to Bartonella australis isolated from kangaroos. This is the first report of two different possible arthropod vectors in Australia (ticks and fleas) being infected with the same species of Bartonella. We propose the name Candidatus Bartonella antechini n. sp. for the recently characterised organism.

Keywords

Bartonella spp., fleas, ticks, marsupials, Western Australia
1. Introduction

The genus *Bartonella*, which is comprised of fastidious, Gram-negative and aerobic bacilli, belongs to the *Alphaproteobacteria* group. Recently, more than 20 species of *Bartonella* have been published and approximately 13 species or subspecies are related to human and animal diseases (Chomel et al., 2004). Cat scratch disease, caused by *B. henselae*, is one of the most important human bartonelloses, and is transmitted by the cat flea (Chomel et al., 1996). Oroya fever caused by *B. bacilliformis* and Trench fever caused by *B. quintana* have been recognized as other important causes of human bartonellosis (Huarcaya et al., 2004). Infectious endocarditis in humans due to *Bartonella* infection has become a significant cause of human endocarditis since the first reports of *B. henselae*, *B. elizabethae* and *B. quintana* causing endocarditis in 1993 (Daly et al., 1993; Hadfield et al., 1993; Spach et al., 1993). Canine bartonellosis is mainly caused by *B. henselae* and *B. vinsonii* subspecies *berkhoffii* (Breitschwerdt and Maggi, 2009).

Recently, four novel species of *Bartonella* were described from animals in Australia. *Bartonella australis* was isolated from blood of the grey kangaroo (*Macropus giganteus*) from central coastal Queensland (Fournier et al., 2007). *Bartonella rattaustrialiani*, *B. queenslandensis* and *B. cooperplainsensis* were characterized in rodent blood from Queensland (Gundi et al., 2009). *Bartonella*-like organisms have also been detected in ticks (*Ixodes tasmani*) collected from koalas (*Phascolarctos cinereus*) (Vilcins et al., 2009). The mardo (*Antechinus flavipes*) is a small carnivorous marsupial belonging to the genus *Antechinus*, order *Dasyuromorphia* (*Dasyuridae*). These native Australian marsupials are found in a variety of habitats across Queensland, New South Wales, Victoria, South Australia and the southwest of Western Australia. All species in this genus have the unusual physiological feature of male semelparity (total male mortality) after a single breeding season.
(Naylor et al., 2008). This event, immediately following mating, is thought to be due to excessive production of testosterone and corticosteroids, resulting in gastrointestinal hemorrhage and failure of the individual’s immune system (McAllean et al., 1998; Naylor et al., 2008). We report here the discovery of a novel species of *Bartonella* that was detected in both fleas (*Acanthopsylla jordani*) and ticks (*Ixodes antechini*) removed from mardos from the southwest of Western Australia.

### 2. Materials and Methods

#### 2.1 Sample collection and vectors identification

Thirty six mardos were trapped in dry sclerophyll forests surrounding the town of Dwellingup in the southwest of Western Australia in 2003 to 2004. Twenty seven ticks and 39 fleas were collected from the animals’ skin and hair coats using a flea comb or tweezers. The ectoparasites were stored in 70% ethanol until required for DNA extraction. Tick morphology was characterized by light microscopy using the standard key for Australian ticks (Robert, 1970). Fleas were also identified using light microscopy according to a standard key for Australian fleas (Dunnet and Mardon, 1974).

#### 2.2 DNA extraction

Ticks and fleas were chopped into small sections using a disposable scalpel blade and DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Maryland, USA).

#### 2.3 PCR screening and genotyping analysis

Nested-PCRs for the ribosomal ITS region and the *gltA* gene (citrate synthase) were performed initially to detect *Bartonella* DNA in tick and flea samples. Outer primers for the *gltA* gene and the ITS region were designed from the DNA sequence of *B. henselae*
The inner primers for nested-PCR of the ITS region and *gltA* gene were as previously described (Jensen et al., 2000, Norman et al., 1995). A new reverse primer targeting the ITS region was also designed for amplification and sequencing of a large fragment of the ITS region (Table 1). Following successful amplification from a sample, 3 other loci were amplified using single-step PCRs targeting the 16S rRNA, *ftsZ* and *rpoB* genes. New primers for the 16s rRNA were designed from the DNA sequence of *B. henselae* (GenBank, M73229) and degenerate primers used for the *ftsZ* and *rpoB* genes were modified from previous studies (Zeaiter et al., 2002; Renesto et al., 2001). All primers used are described in Table 1. PCR products from all genes were purified from agarose gel slices using UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories Inc. West Carlsbad, California, USA). Sequencing was performed using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystems 3730 DNA Analyzer, following the manufacturer’s instructions. Nucleotide sequences generated for all 5 loci were analysed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned with reference sequences from *Bartonella* spp. from GenBank using Clustal W (http://www.clustalw.genome.jp). Phylogenetic trees of the concatenated sequences of all 5 loci were constructed using Distance and Maximum-parsimony methods using Mega version 4 (Mega4: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, U.S.A.) (Tamura et al., 2007). Phylogenetic analysis was conducted on ten *Bartonella* PCR positive DNA samples including 6 from fleas and 4 from ticks.
3. Results

The genetic similarity of the concatenated sequences compared with sequences from other Bartonella spp., shown in Figures 1 and 2, revealed that the Bartonella spp. from the mardo was 94.5% to 98.8% similar to other Bartonella spp. at the 16S rRNA locus, 80.3% to 87.8% similar at the gltA locus, 84.6% to 89.2% similar at the ftsZ locus and 82.5% to 87.9% similar at the rpoB locus (Table 2). The proposed cut-off values for differentiation of a new Bartonella species are genetic identities of less than 96.0% and 95.4% for gltA and rpoB respectively (La Scola et al., 2003). On this basis, the Bartonella isolate described here from mardo ticks and fleas should be considered a new species and we propose the name Candidatus Bartonella antechini n. sp. The concatenated phylogenetic analysis in this study showed the close relationship between this new species and B. australis isolated from grey kangaroos (Macropus giganteus) in Queensland (Fig. 1 and 2). Separate clustering of Bartonella species harbored by marsupials or marsupial vectors, is also evident. Bartonella DNA was also detected from the tick samples using nested-PCR targeting the gltA and ITS loci, but we were unable to amplify PCR products from the same tick samples using a single step PCR for the 16S rRNA, ftsZ, and rpoB genes. Amplification of Bartonella DNA from all five loci was successful for the flea extracts. The gltA fragment and ITS sequences generated from flea samples were identical to the corresponding Bartonella DNA sequences from ticks. Partial sequences for the five loci corresponding to this new species were submitted to GenBank under the accession numbers GU168958; ftsZ, GU168959; 16S-23S rRNA intergenic spacer (ITS), GU168960; 16S rRNA, GU168961; rpoB, GU168962; gltA.
4. Discussion

The novel *Bartonella* species described here is named *Candidatus Bartonella antechini* after the genus of marsupial host (*Antechinus*) from which the ectoparasites were collected. Genetic characterization of this new species detected from fleas (*Acanthopsylla jordani*) and ticks (*Ixodes antechini*), based on multi-locus sequence analysis of the 16S rRNA, *gltA*, *ftsZ*, *rpoB* loci and the ITS region, have confirmed its genetic distinctness. The results of the present study have confirmed that two different types of possible vectors, ticks and fleas, from one mammalian host in a geographical area, are able to harbor the same species of *Bartonella*. While fleas and ticks have both been identified as potential vectors of *Bartonella henselae*, the reports were made from different studies involving different animal species (Chomel et al., 1996; Cotte` et al., 2008).

It is not known why the amplification of 16S rRNA, *ftsZ* and *rpoB* genes in the tick-positive samples was not successful but this may be due to the different PCR sensitivities between single step and nested-PCR. Differences in *Bartonella* multiplication rates inside ticks and fleas have been observed in experimental *Bartonella* infections in ticks and fleas (Cotte` et al., 2008; Chomel et al., 2009). Single step PCR may lack the sensitivity required to detect *Bartonella* spp. in some stages of infection in tick vectors.

Little is known about *Bartonella* spp. occurring in marsupials and their arthropod vectors in Australia. The identification and characterisation of *Bartonella* spp. in both marsupials and marsupial vector populations is important for understanding the epidemiology of *Bartonella* infections in Australia. Multilocus sequence analysis of the *Bartonella* will help to inform the debate over the evolution, ecology, host interactions and virulence of these bacteria in native Australian fauna.
Acknowledgments

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References


Glen, A.S., Dickman, C.R., 2005. Complex interactions among mammalian carnivores in


Oligonucleotide primers used for nested-PCR and single step PCR amplification of the 16S rRNA, *gltA*, *ftsZ*, *rpoB* loci and the ribosomal ITS region

**Table 2**

The percentage genetic similarity of the concatenated sequences from *Candidatus Bartonella antechini* n. sp. compared with other confirmed *Bartonella* spp.

**Fig. 1.** Neighbor-Joining concatenated phylogenetic tree of 16S rRNA, *gltA*, *ftsZ*, *rpoB*, and the ITS region of Australian marsupial isolates and validated species and subspecies of *Bartonella*. Percentage bootstrap support (>50%) from 1000 pseudoreplicates is indicated at the left of the supported node.

**Fig. 2.** Maximum-Parsimony concatenated phylogenetic tree of 16S rRNA, *gltA*, *ftsZ*, *rpoB*, and the ITS region of Australian marsupial isolates (*B. australis* and *Candidatus B. antechini*) and validated species and subspecies of *Bartonella*. Percentage bootstrap support (>50%) from 1000 pseudoreplicates is indicated at the left of the supported node.
# Table 1

Oligonucleotide primers used for nested-PCR and single step PCR amplification of the 16S rRNA, gltA, ftsZ, rpoB loci and the ribosomal ITS region

<table>
<thead>
<tr>
<th>Primer</th>
<th>Locus</th>
<th>Nucleotide sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>gltA-F2†</td>
<td>gltA</td>
<td>GCTTCGTTGTGAATCGAAAATCA</td>
<td>This study</td>
</tr>
<tr>
<td>gltA-R2†</td>
<td>gltA</td>
<td>GCGGTTAAGCTTCCAATCATA</td>
<td></td>
</tr>
<tr>
<td>BhCS.781F (gltA)*</td>
<td>gltA</td>
<td>GGGGACCAGCTCATGGTGG</td>
<td>(Norman et al., 1995)</td>
</tr>
<tr>
<td>BhCS.1137R(gltA)*</td>
<td></td>
<td>AATGCAAAAAAGAACAGTTAAAACA</td>
<td></td>
</tr>
<tr>
<td>WITS-F†</td>
<td>ITS</td>
<td>ACCTCCTTTTCTAAGGATGAT</td>
<td>This study</td>
</tr>
<tr>
<td>WITS-R†</td>
<td>ITS</td>
<td>AAAGACCAGCTTCTGAGAT</td>
<td></td>
</tr>
<tr>
<td>Bh311-332F (ITS) *</td>
<td>ITS</td>
<td>CTCTTTTCTCCAGATGATGATCC</td>
<td>(Jensen et al., 2000)</td>
</tr>
<tr>
<td>Bh473-452R (ITS) *</td>
<td></td>
<td>AACCAAACGTGAGCTACAAGGCCCT</td>
<td></td>
</tr>
<tr>
<td>Inner ITS-R (ITS)*</td>
<td></td>
<td>GCGGTTAAGCTTCCAATCATA</td>
<td>This study</td>
</tr>
<tr>
<td>W16S-F</td>
<td>16S rRNA</td>
<td>AGTAACGCGTGGAATCTAC</td>
<td>This study</td>
</tr>
<tr>
<td>W16S-R</td>
<td></td>
<td>CACAGCACCTTGTTCCGCAATACGA</td>
<td></td>
</tr>
<tr>
<td>RpoB-F</td>
<td>rpoB</td>
<td>CGCATTTGGAATTCTCGCATG</td>
<td>(Renesto et al., 2001)</td>
</tr>
<tr>
<td>RpoB-R</td>
<td></td>
<td>GTRGAYTGATTTGAAACGTYG</td>
<td></td>
</tr>
<tr>
<td>FtsZ-F</td>
<td>ftsZ</td>
<td>ATTAATCTGCAAYCGGAGAGA</td>
<td>(Zeaiter et al., 2002)</td>
</tr>
<tr>
<td>FtsZ-R</td>
<td></td>
<td>ACBGAHACACGAATAACACC</td>
<td></td>
</tr>
</tbody>
</table>

*Inner primers, †Outer primers.
Table 2

The percentage genetic similarity of the concatenated sequences from *Candidatus Bartonella antechini* n. sp. compared with other confirmed *Bartonella* spp.

<table>
<thead>
<tr>
<th>Percent genetic similarity to other <em>Bartonella</em> sp.</th>
<th>16S rRNA</th>
<th>ftsZ</th>
<th>rpoB</th>
<th>gltA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candidatus</em>  <em>B. antechini</em></td>
<td>94.5-98.8</td>
<td>84.6-89.2</td>
<td>82.5-87.9</td>
<td>80.3-87.8</td>
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</tbody>
</table>