Identification of Novel Zoonotic Activity of *Bartonella* spp., France

Muriel Vayssier-Taussat, Sara Moutailler, Françoise Féménia, Philippe Raymond, Olivier Croce, Bernard La Scola, Pierre-Edouard Fournier, Didier Raoult

Certain *Bartonella* species are known to cause afebrile bacteremia in humans and other mammals, including *B. quintana*, the agent of trench fever, and *B. henselae*, the agent of cat scratch disease. Reports have indicated that animal-associated *Bartonella* species may cause paucisymptomatic bacteremia and endocarditis in humans. We identified potentially zoonotic strains from 6 *Bartonella* species in samples from patients who had chronic, subjective symptoms and who reported tick bites. Three strains were *B. henselae* and 3 were from other animal-associated *Bartonella* spp. (*B. doshiae, B. schoenbuchensis*, and *B. tribocorum*). Genomic analysis of the isolated strains revealed differences from previously sequenced *Bartonella* strains. Our investigation identified 3 novel *Bartonella* spp. strains with human pathogenic potential and showed that *Bartonella* spp. may be the cause of undifferentiated chronic illness in humans who have been bitten by ticks.

*Bartonella* spp. cause varied and multifaceted human diseases, including cat scratch disease (*B. henselae*), Carrion’s disease (*B. bacilliformis*), trench fever (*B. quintana*), endocarditis (*B. quintana* and *B. henselae*) (1,2), bacillary angiomatosis (*B. quintana* and *B. henselae*), and hepatic peliosis (*B. henselae*). *Bartonella* spp. can also cause prolonged intra-erythrocytic bacteremia in both humans and animals (3): in humans, *B. quintana*, *B. bacilliformis*, and *B. rochalimae* are known pathogens, and in animals, *B. henselae*, *B. claridgeiae*, and *B. koehlerae* have been identified in felids; *B. grahamii*, *B. taylorii*, *B. doshiae*, *B. birtlesii*, and others in rodents; and *B. bovis*, *B. chomelii*, *B. schoenbuchensis*, in ruminants. In humans, chronic bacteremia caused by *B. quintana* causes few obvious symptoms apart from generalized fatigue and nonspecific leg pain (1,4).

It has been assumed that each *Bartonella* species infected 1 or a few closely related mammalian reservoir hosts, in which infection caused long-lasting bacteremia. Nonreservoir hosts were considered incidentally infected without bacteria being detected in blood. Recently, these assumptions have been contradicted by studies describing animal-associated *Bartonella* spp. indirectly associated with bacteremia and a spectrum of diverse symptoms in immune-competent persons who had contact with animals, arthropods, or both, which are natural routes of *Bartonella* transmission (5–7). In some cases, the source of infection remains unknown; ticks have been suggested as a possible source of animal-associated *Bartonella* infection in humans (6,8–10).

Related to a patient’s history of tick bites, it is common for physicians to suspect Lyme disease, some rickettsial diseases, or tickborne encephalitis. However, in many cases, the diagnosis is not confirmed by serologic or DNA-based tests. In recent years, alternate interpretations of Lyme disease serology have flourished, leading to considerable discord between formal institutions for infectious disease and patient advocacy associations. Thus, unexplained symptoms after tick bites have become an issue of increasing importance for patients and their physicians (11,12).

In this context, we screened for the presence of *Bartonella* in the blood of patients reporting tick bites and with unexplained and aspecific symptoms. Here we report the isolation and genomic sequencing of 6 *Bartonella* strains obtained by blood culture from 66 patients. Three strains were identified as *B. henselae*, and 3 other strains were identified as different animal–associated species (*B. doshiae, B. tribocorum, and B. schoenbuchensis*).

**Methods**

**Patients**

During January–June 2013, we conducted a study of a cohort of 66 French patients who had consulted their doctors for chronic symptoms appearing after a tick bite. The entire study protocol was approved by the ethics committee of the Institut Federatif de Recherche 48 under reference 13–022–1.

All patients associated symptom onset with tick bites that occurred during 2008–2012 (Table 1). At symptom onset, local doctors were consulted, and serologic tests for Lyme borreliosis were performed. All patient samples
tested were seronegative for Lyme borreliosis bacteria; however, since that time, their symptoms had become chronic. The patients completed information forms giving informed consent for the use of their samples in the study. All of the patients lived in the countryside, where ticks were abundant and contact with wild animals was possible. The patients reported that they had not undergone antibacterial drug treatment for ≥3 months before the study.

We collected blood samples from each patient in EDTA-containing sample tubes. For a control population, we used anticoagulated blood samples from 70 anonymous healthy blood donors from Paris (France). All samples (control and patients) were tested simultaneously.

**Bartonella Isolation from Blood**

To specifically isolate *Bartonella* spp., 100 mL of blood samples from patients or healthy donors were directly plated onto sheep blood agar plates and incubated at 35°C in a humidified atmosphere with 5% CO₂ for 45 days. The plates were assessed daily from days 7–45 before the culture was deemed negative (i.e., absence of colony in the absence of contamination) (1). Colony-forming units (CFU) were counted and bacteremia (UFD/mL of blood) evaluated.

**Genome Sequencing, Assembly, and Analysis**

We extracted genomic DNA from each isolated strain by using the EZ1 automated extraction system (QIAGEN, Hilden, Germany), following the manufacturer’s recommendations. Bacterial genomic DNA was sequenced by using the Nextera XT DNA sample prep kit (Illumina Inc., San Diego, CA, USA) and a 2×250 paired-end protocol with the MiSeq pyrosequencer (Illumina), according to the manufacturer’s instructions. We aligned each genome by using Mira version 3.2 software in the mapping mode (13). The resulting contigs were combined by using Opera version 1.2 (14) and GapFiller (15) software. Finally, the genomic assemblies were improved with manual refinement by using the CLC Genomics version 4.7.2 software package (CLC Bio, Aarhus, Denmark). Noncoding genes and miscellaneous features were predicted by using RNAmmer (16) and ARAGORN (17). Coding DNA sequences were predicted by using Prodigal (18), and functional annotation was achieved by using BLAST+ (19) and HMMER3 (20) against the UniProtKB database (21). Coding DNA sequences were also annotated by using the Clusters of Orthologous Groups databases (22) with blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) default parameters.

Single-nucleotide polymorphisms (SNPs) among genomes were identified by using SNIT software (23). SNPs were searched in regions exhibiting >95% nt sequence identity and the SNIT software was used with default parameters except for the Tandem Repeat Finder filter for avoiding ambiguous SNPs in repeat regions. We also performed in silico DNA–DNA hybridization (DDH) between *Bartonella* strains by using GGDC software (24).

**Taxonomic Classification**

To determine the taxonomic classification of the 6 isolates, we used previously proposed criteria (25) in which the *gltA* and *rpoB* gene sequences from each strain were compared to those of validated published *Bartonella* species. These criteria classify *Bartonella* isolates within a particular species if they share >96% and 95.4% nucleotide sequence similarity for the *gltA* and *rpoB* genes, respectively (25).

In our study, *gltA* and *rpoB* sequences were retrieved from the genomes.

**Results**

**Bartonella spp. Isolation**

*Bartonella* spp. were isolated by prolonged culture from blood samples of 6 of the 66 patients who reported chronic symptoms following a tick bite. In contrast, samples from the 70 healthy blood donors remained negative after 45 days of incubation.

Bacteremia in the *Bartonella* infected patients increased from 50 to 850 CFU/mL. For 1 patient (case-patient 2), we had access to 2 blood samples that were taken at a 1-month interval. *B. henselae* was grown from the 2 samples, with similar bacteremia (50 and 60 CFU/mL, respectively), suggesting chronic bacteremia.

The case-patients who tested positive for *Bartonella* (Table 1) reported tick bites occurred 1–5 years before

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**Table 1. Patients whose blood cultures were positive for *Bartonella* spp. that had no previously known zoonotic activity, France**

<table>
<thead>
<tr>
<th>Case-patient no./age, y/sex</th>
<th>Bartonella spp.</th>
<th>Tick bite date</th>
<th>Pets</th>
<th>Wild animal contact</th>
<th>Main complaints</th>
<th>Bacteremia, CFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/49/F</td>
<td><em>B. henselae</em></td>
<td>Multiple since 2008</td>
<td>Cats, dogs, horses</td>
<td>Rats, fish</td>
<td>Fatigue, muscle pain, headache</td>
<td>50</td>
</tr>
<tr>
<td>2/58/M</td>
<td><em>B. henselae</em></td>
<td>2011</td>
<td>Birds, rabbits</td>
<td>No</td>
<td>Fatigue, muscle pain</td>
<td>70</td>
</tr>
<tr>
<td>3/47/F</td>
<td><em>B. henselae</em></td>
<td>2012</td>
<td>Dog, hamster</td>
<td>No</td>
<td>Fatigue, muscle pain, insomnia</td>
<td>80</td>
</tr>
<tr>
<td>4/45/F</td>
<td><em>B. doshiae</em></td>
<td>2009</td>
<td>No</td>
<td>No</td>
<td>Fatigue, blurred vision, arthralgia</td>
<td>50</td>
</tr>
<tr>
<td>5/64/M</td>
<td><em>B. tribocorum</em></td>
<td>2012</td>
<td>Dog</td>
<td>Game animals (hunter)</td>
<td>Fatigue, muscle pain, headache</td>
<td>60</td>
</tr>
<tr>
<td>6/40/F</td>
<td><em>B. schoenbuchensis</em></td>
<td>2011</td>
<td>No</td>
<td>No</td>
<td>Fatigue, muscle pain, fever</td>
<td>850</td>
</tr>
</tbody>
</table>

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*B. henselae* spp., 100 mL of blood samples from patients or healthy donors were directly plated onto sheep blood agar plates and incubated at 35°C in a humidified atmosphere with 5% CO₂ for 45 days. The plates were assessed daily from days 7–45 before the culture was deemed negative (i.e., absence of colony in the absence of contamination) (1). Colony-forming units (CFU) were counted and bacteremia (UFD/mL of blood) evaluated.
blood samples were collected. All of them live in the countryside, in contrast to the healthy blood donors, who were all from Paris, France. The main complaint of the case-patients was chronic fatigue, but they also reported other subjective or nonspecific symptoms (or both), such as headaches and myalgia. A qualifying characteristic of the 70 healthy blood donors was absence of chronic fatigue. Even though potential exposure to ticks is difficult to evaluate, because the anonymous blood donors all lived in Paris, we assumed they were not likely to have frequent tick exposure or wild animal contact.

Taxonomic Classification

Of the 6 *Bartonella* isolates from this study, 3 (MVT01, MVT02, and MVT03) were classified within the *B. henselae* species on the basis of both their phylogenetic position and *gltA* and *rpoB* sequence similarities (Figure; Tables 1, 2). The isolates from samples from case-patient 2 at a 1-month interval shared 100% identity, based on *gltA* and *rpoB* gene comparison. Isolates MVT04, MVT05, and MVT07 were classified within the *B. tribocorum*, *B. doshiiae*, and *B. schoenbuchensis* species, respectively.

The assembly data and main genomic characteristics of each isolated strain are summarized in Table 3; in silico DDH values and SNP numbers are described in Table 4 (http://wwwnc.cdc.gov/EID/article/22/3/15-0269-T4.htm).

All studied strains displayed a similar genomic content when compared with reference genomes. The GGDC software we used proposes that DDH values >70% could classify isolates in the same species. Here, intraspecies values ranged from 80.3% to 100% (Table 4). Nevertheless, from 10 to 1,938 SNPs were identified among *B. henselae* isolates MVT01, MVT02, and MVT03 and from 693 to 2,093 SNPs when comparing these strains to *B. henselae* Houston-1 (Table 4), confirming that each strain was unique and did not result from cross-contamination or contamination from laboratory strains.

Of note, *B. tribocorum* isolate MVT04 and *B. schoenbuchensis* isolate MVT07 were the only 2 that exhibited plasmids. However, when compared with reference strain m07a, MVT04 and MVT07 carried a large plasmid and not the small plasmid homologous to the cryptic pBGR plasmid harbored by *B. grahamii* (26).

Discussion

In this study, animal-associated *Bartonella* isolates were individually cultured from the blood of patients who had been bitten by ticks and reported subjective symptoms, whereas no strains were isolated from healthy blood donors. This report describes the isolation of 3 different animal-associated *Bartonella* species from human samples, highlighting their potential novel zoonotic properties. Moreover, we found

![Figure. rpoB gene-based phylogenetic tree showing the relationships of 6 *Bartonella* isolates (underlined). Briefly, rpoB nucleotide sequences were aligned by using ClustalW software (http://www.clustal.org/clustal2/), and phylogenetic relationships were inferred by using the maximum-likelihood strategy and MEGA software (http://www.megasoftware.net). Bootstrap values above 70%, obtained from 500 analyses, are indicated at the nodes. Scale bar represents a 2% nucleotide sequence divergence.](http://wwwnc.cdc.gov/EID/article/22/3/15-0269-T4.htm)
that zoonotic Bartonella spp. can be detected in the blood of febrile patients, as has been shown for human-specific B. quintana and B. bacilliformis and as was recently reported for Candidatus Bartonella ancashi (27). Chronic bacteremia caused by infection by Bartonella spp. is well-described in many mammals, including humans (4,28). The Bartonella–mammalian host association is considered to be species-specific and attributable to co-evolution between host and pathogen (28). However, we show that animal-associated species can also chronically infect human blood, highlighting the possibility of host shift despite apparent host specificity (28,29).

This work is similar to that of E.B. Breitschwerdt et al. (5–7), who also recovered zoonotic Bartonella spp. from human samples using an in-house technique based on results of blood pre-enrichment followed by PCR detection of Bartonella spp.; members of the same team have investigated many cases of persons who had nonspecific symptoms, including arthralgia, muscle pain, fatigue, headaches, visual blurring, neurocognitive symptoms, and, in 2 case-patients, hemangiendothelioma (30). In total, B. henselae DNA was detected in 47 cases (5,30–33); B. koehleri (another common agent of feline bacteremia) DNA in 96 cases, including 2 co-infected with B. henselae (31,32,34,35); and B. vinsonii berkholffii (an agent of canine bacteremia and endocarditis) DNA in 24 cases (31–34), including 16 case-patients with B. henselae and 2 cases of B. melophagi (36). These results have been questioned because minute levels of contamination can result in false positives by PCR. Therefore, we deliberately avoided PCR to overcome this problem, and the resulting strain isolation was consequently straightforward and indisputable. These isolates (Table 2) have been archived in our collection (Collection de Souches de l’Unité des Rickettsies, World Data Center for Microorganisms no. 875, http://www.mediterranee-infection.com/article.php?laref=14&titre=collection-de-souches) and are available upon request under references B546, B547, B548, B549, B550, and B551 for isolates MVT01, MVT02, MVT03, MVT04, MVT05, and MVT07, respectively.

Our findings also confirm studies identifying zoonotic Bartonella in the blood of patients with nonspecific complaints. Among them, B. henselae is well known worldwide

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**Table 2. Nucleotide similarity of 6 Bartonella isolates from patients in France expressing novel zoonotic activity and their most phylogenetically similar published validated species**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>gltA, %</th>
<th>rpoB, %</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVT01</td>
<td>100</td>
<td>100</td>
<td><em>B. henselae</em></td>
</tr>
<tr>
<td>MVT02</td>
<td>100</td>
<td>100</td>
<td><em>B. henselae</em></td>
</tr>
<tr>
<td>MVT03</td>
<td>99.7</td>
<td>99.6</td>
<td><em>B. henselae</em></td>
</tr>
<tr>
<td>MVT04</td>
<td>100</td>
<td>100</td>
<td><em>B. tribocorum</em></td>
</tr>
<tr>
<td>MVT05</td>
<td>98.7</td>
<td>100</td>
<td><em>B. dohiae</em></td>
</tr>
<tr>
<td>MVT07</td>
<td>100</td>
<td>99.9</td>
<td><em>B. schoenbuchensis</em></td>
</tr>
</tbody>
</table>

*gltA and rpoB sequences were obtained from genomic sequences. Genome sequences of Bartonella strains MVT01, MVT02, and MVT03 were deposited in GenBank under accession numbers HG965802, NZ_LN879429, and HG969191, respectively; the genome sequence of B. tribocorum strain MVT04 was deposited in GenBank under accession numbers HG969192 and HG969193; the genome sequence of *B. dohiae* strain MVT05 was deposited in GenBank under accession numbers CCBL010000001–CCBL010000013; the genome sequence of *B. schoenbuchensis* strain MVT07 was deposited in GenBank under accession numbers CCBL010000013; the genome sequences of *B. henselae* strains HG997193–HG977197; the genome sequences of the reference strains *B. henselae* strain Houston-1; *B. tribocorum* strain CIP 105476; *B. dohiae* strain NCTC 12862; and *B. schoenbuchensis* strain m07a are available in GenBank under accession numbers NG965801, HG969199, HG969201, and HG969209; respectively.*

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**Table 3. Assembly information and main characteristics of 6 sequenced Bartonella genomes from patients in France expressing novel zoonotic activity**

<table>
<thead>
<tr>
<th>Genome characteristics</th>
<th>Species and isolate identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>GenBank accession nos.</td>
<td></td>
</tr>
<tr>
<td>HG965802</td>
<td><em>B. henselae</em></td>
</tr>
<tr>
<td>NZ_LN879429</td>
<td><em>B. henselae</em></td>
</tr>
<tr>
<td>HG969191</td>
<td><em>B. henselae</em></td>
</tr>
<tr>
<td>HG969192–HG969193</td>
<td><em>B. tribocorum</em></td>
</tr>
<tr>
<td>CCBL010000001–CCBL010000013</td>
<td><em>B. dohiae</em></td>
</tr>
<tr>
<td>HG977193–HG977197</td>
<td><em>B. schoenbuchensis</em></td>
</tr>
</tbody>
</table>

- **Size, bp**: 1,902,535 for MVT01, 1,905,383 for MVT02, 1,975,503 for MVT03, 2,609,404 for MVT04, 1,919,109 for MVT05, 1,734,324 for MVT07.
- **No. contigs**: 1 for MVT01, 1 for MVT02, 2 for MVT03, 2 for MVT04, 13 for MVT05, 5 for MVT07.
- **Average read coverage**: 87 for MVT01, 94 for MVT02, 110 for MVT03, 46 for MVT04, 15 for MVT05, 41 for MVT07.
- **Average read length, trimmed**: 183 for MVT01, 190 for MVT02, 192 for MVT03, 192 for MVT04, 188 for MVT05, 193 for MVT07.
- **Total no. reads, trimmed**: 946,882 for MVT01, 1,034,894 for MVT02, 1,263,492 for MVT03, 738,522 for MVT04, 261,085 for MVT05, 666,371 for MVT07.
- **Total no. predicted genes**: 1,659 for MVT01, 1,658 for MVT02, 1,726 for MVT03, 2,335 for MVT04, 1,720 for MVT05, 1,574 for MVT07.
- **Protein-coding genes**: 1,603 for MVT01, 1,602 for MVT02, 1,668 for MVT03, 2,279 for MVT04, 1,654 for MVT05, 1,519 for MVT07.
- **rRNA operons**: 2 for MVT01, 2 for MVT02, 2 for MVT03, 2 for MVT04, 2 for MVT05, 2 for MVT07.
- **tRNAs**: 43 for MVT01, 43 for MVT02, 45 for MVT03, 43 for MVT04, 53 for MVT05, 41 for MVT07.
- **Other RNAs**: 7 for MVT01, 7 for MVT02, 7 for MVT03, 7 for MVT04, 9 for MVT05, 8 for MVT07.
- **GC% content**: 38.18 for MVT01, 38.18 for MVT02, 38.09 for MVT03, 38.84 for MVT04, 37.82 for MVT05, 35.58 for MVT07.
- **Plasmid**: 0 for MVT01, 0 for MVT02, 0 for MVT03, 0 for MVT04, 0 for MVT05, 0 for MVT07.

- **m07a** was used as a reference for assembly (accession nos.) B. henselae Houston-1 (NC_005956), B. henselae Houston-1 (NC_005956), B. henselae Houston-1 (NC_005956), B. tribocorum CIP 105476 (NC_010161, NC_010160), B. dohiae NCTC 12862 (NZ_JH725094–NZ_JH725100), B. schoenbuchensis m07a (NZ_KB915627–NZ_KB915629, NZ_CM001846, NZ_CM001845).
as a zoonotic agent infecting both cats and their fleas and has also been found in ticks (10). B. henselae has been detected in the blood of a patient without apparent symptoms 4 months after recovering from cat scratch disease. For this particular case, the sequence of manifestation of cat scratch disease, then bacteremia, followed by endocarditis was proposed because it has been known to occur for B. quintana bacteremia. One of the case-patients in this study owns a cat and may have been infected by this pet.

The 3 other animal-associated species we detected should now be considered zoonotic Bartonella spp. B. doshiae and B. tribocorum are both rodent-associated species; in France and worldwide, these species have mainly been recovered from rats (Microtus arvalis for B. doshiae and Rattus rattus for B. tribocorum). B. schoenbuchensis is normally found in deer, elk, and cattle (37,38).

The zoonotic agents we isolated from patients from France have also been detected in animals in France. Similarly, in the United States and Thailand, Bartonella species known to be prevalent in animals have also been identified in humans: (B. henselae, B. vinsonii berkholffii, and B. koehlerae in the United States (33,35) and B. tribocorum and B. rattimassiliensis in Thailand (39). Therefore, the zoonotic Bartonella species discovered in humans in this study generally appear to be related to the prevalence among animals.

The significance of these Bartonella spp. in the genesis of the clinical picture is difficult to determine. Bartonella spp. are present in ticks, and we have previously reported Bartonella infections following tick bites, such as SENLAT (scalp eschar and neck lymphadenopathy after tick bite [40]). However, the causal link between the conditions observed here, Bartonella and tick bite, cannot yet be concretely established, especially for persons with tick bites occurring up to 5 years previously, which introduces innumerable potential confounding exposures within the same period, including bites by other arthropods. For instance, 1 of the 3 patients with B. henselae bacteremia reported contact with cats; this contact was a more plausible source of infection than tick bites. Furthermore, it is crucial to determine whether Bartonella played a notable role in the observed pathologies, because treatment for chronic Bartonella bacteremia (as for B. quintana) is particularly arduous and may require 6 weeks of doxycycline treatment together with 3 weeks of gentamicin, as these are the only antimicrobial drugs known to be effective in eradication of Bartonella (1). Many Bartonella spp. can also cause endocarditis, including B. quintana and B. henselae; therefore, reports of rare cases of endocarditis attributed to zoonotic Bartonella such as B. kohlerae, B. alaska, Candidatus B. mayotimonensis, B. vinsonii, or B. elizabethae may actually be the final manifestation of asymptomatic bacteremia, similar to that reported by our infected patients (28).

In summary, our major finding is the isolation of zoonotic Bartonella other than B. quintana in the blood of patients with poorly qualified syndromes. These results indicate that zoonotic Bartonella spp. infection may cause undifferentiated chronic illness in humans.

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References


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