***Bartonella alsatica* sp. nov., a new *Bartonella* species isolated from the blood of wild rabbits**

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***Bartonella* species are considered as emerging human pathogens, with at least six different species pathogenic or possibly pathogenic for humans. However, little is known about *Bartonella* distribution, species polymorphism and pathogenicity in mammalian species. The objective of this work was to determine the presence, the frequency and the distribution of *Bartonella* species in wild rabbits (*Oryctolagus cuniculus*) caught in warrens in Alsace, France. Humans may come into contact with wild rabbits when hunting, especially when they are picked up with bare hands and at time of evisceration. Of 30 blood samples collected and cultured from wild rabbits, nine (30%) were positive for organisms morphologically similar to *Bartonella* spp. The bacteria appeared as small, fastidious, aerobic, oxidase-negative, Gram-negative rods which could be localized within erythrocytes. Their biochemical properties were similar to those of the genus *Bartonella*. The sequence of the 16S rRNA gene obtained from the rabbit isolates was highly related to the sequences of the different *Bartonella* species (97.8–99.3% similarity). The high DNA hybridization rate (81–90% similarity) between the three strains isolated from rabbit blood confirmed that they belong to the same bacterial species. Hybridization values, obtained with the nuclease–TCA method, when testing type strains of recognized *Bartonella* species (9–14% similarity), support the creation of a new species for the rabbit isolates. The name *Bartonella alsatica* is proposed for these strains isolated from the blood of wild rabbits. The type strain is IBS 382^T (= CIP 105477^T).**

Keywords: *Bartonella*, *Bartonella alsatica*, wild rabbit, *Oryctolagus cuniculus*, zoonoses

INTRODUCTION

The bacterial genus *Bartonella* consisted of one species until 1993 and includes at present 11 species, formerly classified in the genera *Bartonella*, *Grahamella*, *Rickettsia* or *Rochalimaea* (Birtles *et al.*, 1995; Brenner *et al.*, 1993). Nine reference strains isolated from humans or small mammals are available from international bacterial collections and include the following species: *Bartonella bacilliformis*, *Bartonella clarridgeiae*, *Bartonella doshiae*, *Bartonella elizabethae*, *Bartonella grahamii*, *Bartonella henselae*, *Bartonella quintana*, *Bar-*

tonella taylorii and *Bartonella vinsonii*. The two other species, *Bartonella talpae* and *Bartonella peromysci*, initially classified in the genus *Grahamella* (Birtles *et al.*, 1995), were isolated from small mammals, but no reference strains are available and their 16S rRNA gene sequence is also unknown.

Six of the 11 *Bartonella* species described can produce pathogenic disorders in humans (Bass *et al.*, 1997a, b; Kordick *et al.*, 1997; Schwartzman, 1996) and therefore *Bartonella* species are considered as emerging human pathogens. *B. henselae* and *B. clarridgeiae* were isolated from cats and the diseases caused by these bacteria are considered as zoonoses; *B. vinsonii* was isolated from voles and a subspecies, *B. vinsonii* subsp. *berkhoffii*, was isolated from healthy dogs and from a

The EMBL/GenBank accession number for the 16S rRNA gene sequence of strain IBS 382^T is AJ002139.

dog with an endocarditis (Kordick *et al.*, 1996). However, no animal reservoir has been identified for the three other species pathogenic to humans: *B. bacilliformis*, *B. elizabethae* and *B. quintana*. Furthermore, little is known about the distribution, species polymorphism and pathogenicity of *Bartonella* species among various mammalian species.

The objective of this work was to explore potential reservoirs of *Bartonella* species in wild mammals. We therefore tested a population of wild rabbits captured in warrens in the Alsace region, France, to determine the presence, the frequency and the distribution of *Bartonella* species in this population.

METHODS

Reference strains and accession numbers for the 16S rRNA gene sequences. *B. grahamii* NCTC 12860^T (accession no. Z31349), *B. taylorii* NCTC 12861^T (Z31350), *B. doshiae* NCTC 12862^T (Z31351) and *B. vinsonii* ATCC VR-152^T (L01259) were kindly provided by R. Birtles (Bristol, UK). *B. henselae* ATCC 49882^T (M73229) and *B. elizabethae* ATCC 49927^T (L01260) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA).

The EMBL/GenBank accession numbers for the other 16S rRNA gene sequences of the different *Bartonella* species used for sequence comparison are: *B. bacilliformis*, M65249; *B. clarridgeiae* (ATCC 51734^T), X8920; *B. henselae* (type BA-TF), Z11684; *B. quintana* (VR-358^T), M11927.

Rabbit population. The wild rabbits (*Oryctolagus cuniculus*) enrolled in the study were caught between October 1996 and January 1997 in a warren in a protected natural zone near the Rhine river in the eastern part of France. Four different episodes of capture were organized. The first three episodes of capture took place in a limited warren of 1 km² (24 rabbits caught); the fourth episode occurred over the same surface range in a warren located 60 km north from the first place (six rabbits caught).

Thirty wild rabbits were caught with the help of a trained ferret and were transferred in capture boxes. They were lightly tranquillized by intramuscular injection of a blend of tiletamine and zolazepam. Approximately 1 ml rabbit blood was collected from an external ear vein and placed into a Pediatric Isolator 1.5 tube (Wampole Laboratories) for haemolysis. One drop of blood of each animal was smeared on a glass slide.

The rabbits were ear-marked with a numeric identification system and then released, except for four of them, which were sent to the Veterinary Departmental Services for histopathological examination.

Isolation of strains. Blood culture was performed as described previously (Heller *et al.*, 1997). Briefly, two different media were used for primary culture: a Columbia agar base containing 5% defibrinated rabbit blood and a Bactec Peds Plus (Becton Dickinson Diagnostic Instrument Systems) liquid medium supplemented with 500 µl Bacto Fildes enrichment (Difco). Incubation of the plates was performed at 35 °C in a moist atmosphere containing 5% CO₂ for up to 2 months. Three randomly selected plates without any sample were incubated in parallel with the inoculated blood agar plates of the same batch as a sterility test. The Bactec

Peds Plus vials were tested for CO₂ production each week for up to 6 months. The CO₂-positive Bactec Peds Plus vials were tested for the presence of bacteria by light microscopic examination as described below. Positive broth medium was subcultured on rabbit-blood Columbia agar medium.

Light microscopic examination. Direct examination of the animal blood smears was performed after staining with the May-Grünwald-Giemsa stain. The stained blood smears were examined by two independent readers through an oil-immersion objective at a magnification of ×1000. If no infected erythrocytes were observed after a 5 min examination, the smear was considered negative.

The cultured micro-organisms were visualized by acridine orange staining and with the enhanced Gram stain by using a counterstain of Kinyoun carbol fuchsin at a 1/20 dilution (Larson *et al.*, 1994) at a magnification of ×1000.

Electron microscopy. Bacteria grown on solid medium were prepared for electron microscopy as previously described by Fussener *et al.* (1996). Briefly, bacteria were suspended in PBS (0.145 M NaCl; 0.15 M sodium phosphate), spread on a water surface and absorbed to Formvar-coated nickel grids. The grids were stained in 1% uranyl acetate and air-dried. The samples were viewed under a Zeiss M109 electron microscope at 80 kV.

Biochemical analysis. The following biochemical tests were performed with diagnostic tablets (Rosco Diagnostica): Voges-Proskauer reaction, tributyrin hydrolysis, pyrazinamidase and trypsin-like activity. The MicroScan Rapid Anaerobe Panel (Baxter Diagnostics) was used to test the activity of preformed bacterial enzymes. These biochemical tests correspond to those used to identify biochemical characteristics described for other *Bartonella* species (Birtles *et al.*, 1995; Brenner *et al.*, 1993).

Amplification of the 16S rRNA gene. Amplification of the 16S rRNA gene was performed as previously described (Heller *et al.*, 1997). Briefly, DNA was extracted from several colonies (3–10 colonies). *In vitro* amplification of a 1500 bp DNA fragment was performed with two 16S rRNA gene eubacterial universal primers: P8 (5' AGAGTTTGATCCTGGCTCAG 3') and Pc1544 (5' AAGGAGGTGATCCAGCCGCA 3'). Standard procedures to prevent sample DNA contamination were undertaken (Kwok & Higuchi, 1989). Negative controls were included in each experiment to check for the absence of cross-contamination between samples and between previously amplified products and field samples.

Purification of the amplified product and DNA sequencing. The amplified DNA fragment was purified by phenol extraction and 2-propanol precipitation (Brow, 1990). Sequencing was performed with a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deazadGTP (Amersham Life Science) according to the manufacturer's instructions. Sequence was obtained with an ALF DNA Sequencer (Pharmacia Biotech) according to the manufacturer's instructions. Sequencing of the complete 16S rRNA gene fragment was performed on the coding and the complementary strands, for the type strain and two other strains, with four couples of primers: P8 and Pc535 (5' GTATTACCGCGGCTGCTGGCAG 3'), P515 (5' GTGCAGCAGCCGCGGTAACAC 3') and Pc804 (5' GACTACCAGGGTATCTAATCC 3'), P784 (5' GGATTAGATACCCTGGTAGTC 3') and Pc1198 (5' ACTTGACGTATCCCCACCTTCC 3'), P1174 (5' GAGGAAGGTGGGATGACGTC 3') and Pc1544. All primers were 5'

labelled with fluorescein isothiocyanate. For the other isolates, only sequencing of the region of the first 430 bases of the 16S rRNA gene was performed by using the internal primer Pc535. This region corresponds to the most informative region when compared with other known 16S rRNA gene sequences.

16S rDNA sequence analysis. The sequences obtained were compared with the homologous sequences of other bacteria including all *Bartonella* spp. contained in the EMBL/GenBank database and the sequences of the other *Bartonella* isolates obtained from rabbits. The sequences were aligned by using the CLUSTAL method (Higgins & Sharp, 1988) with DNASTar software.

DNA–DNA hybridization. Colonies were harvested from blood agar plates and DNA was extracted as previously described (Riegel *et al.*, 1994). Hybridization between the labelled DNA and the fragmented-DNA preparation was carried out at 58 °C for 16 h in 0.42 M NaCl by the nuclease–TCA method (Grimont *et al.*, 1980; Riegel *et al.*, 1994). This corresponds to stringent conditions.

Determination of DNA base composition. The G + C content of DNA was determined by capillary electrophoresis as described previously (Riegel *et al.*, 1995).

RESULTS

Rabbit population

A total of 30 wild rabbits were sampled. Two of the four rabbits which were sent to the Veterinary Departmental Services for histopathological examination were positive in culture for *Bartonella* sp. All four rabbits looked healthy and no particular histopathological abnormalities were observed, especially in relation to *Bartonella* infection.

Culture and identification

Of the 30 wild rabbits tested, nine (30%) were culture-positive for *Bartonella* spp. Eight (33%) strains were isolated from the rabbits captured in the first warren and one (17%) was isolated from the rabbits captured in the second warren. The colonies were seen on blood agar after 10 d post-inoculation and culture was detectable in liquid medium after 4 weeks. For six of the nine positive rabbits, culture was obtained both on solid and liquid media, while the three remaining isolates were cultured only from liquid medium on primary isolation. On blood agar, the number of c.f.u. ml⁻¹ ranged from 100 to 2500 with a mean of 866 c.f.u. ml⁻¹.

The colonies which grew after 10 d on blood agar appeared as small, white, smooth, regular colonies (diameter of about 1 mm). When the plates were incubated for a longer time period, the colonies became bigger, turned brown and were adherent to the medium. Microscopic examination after enhanced Gram staining showed small Gram-negative rods. Electron microscopic examination showed small bacilli (0.5–0.8 µm wide and 1–2 µm long) with polar pili

Fig. 1. Negative staining of strain IBS 382^T showing the presence of polar pili. Bar, 0.25 µm.

but without flagella (Fig. 1). The three isolates tested (IBS 382^T, IBS 379 and IBS 460) were catalase-negative, exhibited a negative Voges–Proskauer reaction, had negative pyrazinamidase and urease activities and did not produce acid from trehalose. They did not hydrolyse bis-*p*-nitrophenyl phosphate or *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, and had amino acid arylamidase activity with the following amino acids: leucine, methionine, lysine (alkaline as well as acid), glycine, proline, arginine and tryptophan. They had glycylglycylarylamidase and trypsin-like activity but no pyrrolidonylarylamidase activity.

Sequencing of the complete 16S rRNA gene was performed for the three different isolates previously examined (isolates IBS 382^T, IBS 379 and IBS 460). The three sequences obtained were identical to each another but differed from those previously known for other *Bartonella* species. The closest 16S rRNA gene sequence was the one of *B. vinsonii*, which had 10 different bases (0.7% difference), and the most distant was *B. bacilliformis*, with 30 different bases (2.2% difference). Sequencing of the six other isolates was performed only for the first 430 bases and the six sequences were strictly identical to those obtained for the three isolates whose 16S rRNA gene was entirely sequenced. Half of the nucleotide differences between the sequences of the rabbit isolates and the sequences of the other *Bartonella* species are localized in the first 430 bases of the 16S rRNA gene. The G + C content of

Table 1. DNA–DNA hybridization and 16S rRNA gene similarity between *Bartonella* species and strain IBS 382^T

<i>Bartonella</i> strain	Similarity (%) for the 16S rRNA gene	Hybridization (%) with strain IBS 382 ^T
IBS 382 ^T	100.0	100
IBS 379	100.0	90
IBS 460	100.0	81
<i>B. vinsonii</i>	99.3	14
<i>B. doshiae</i>	99.1	12
<i>B. grahamii</i>	99.0	9
<i>B. taylorii</i>	98.9	14
<i>B. henselae</i>	98.9	12
<i>B. elizabethae</i>	98.5	9
<i>B. quintana</i>	98.2	NT
<i>B. clarridgeiae</i>	98.0	NT
<i>B. bacilliformis</i>	97.8	NT

NT, Not tested.

DNA, determined by capillary electrophoresis, was 37 mol %.

Blood smear examination

No blood smear from any of the 30 wild rabbits was found positive. Nevertheless, we injected the isolate IBS 382^T experimentally to three laboratory rabbits and observed bacteria in their erythrocytes, as determined by blood smear examination. The number of bacteria observed in each infected erythrocyte ranged between 10 and 40 organisms, but very few erythrocytes were infected ($<10^{-4}$). The bacteraemia in experimentally infected rabbits persisted for 2–3 months.

DNA–DNA hybridization

The DNA of isolate IBS 382^T was labelled and tested by DNA–DNA hybridization against unlabelled DNA of two other rabbit isolates (IBS 379 and IBS 460) and against unlabelled DNA of type strains of phylogenetically related *Bartonella* species (less than 1.5 % difference in the 16S rRNA gene sequence). The results are shown in Table 1 in parallel with the 16S rRNA gene sequence homology values. Isolates IBS 379 and IBS 460 showed more than 80 % DNA–DNA similarity with isolate IBS 382^T whereas low relatedness (less than 15 %) was found between isolate IBS 382^T and type strains of *Bartonella* species tested, thus confirming that the three wild rabbit isolates represent a single species distinct from other *Bartonella* species.

DISCUSSION

The bacteria isolated from the blood of wild rabbits grew slowly and required enriched media. They appeared as small, fastidious, aerobic, oxidase-negative,

Gram-negative rods, which could be localized within erythrocytes. They grew best on blood-enriched media in an atmosphere containing 5 % CO₂. Their biochemical properties were similar to those of other species of the genus *Bartonella*. Their 16S rRNA sequence was also highly related to the 16S rRNA sequence of *Bartonella*, since the closest one was the sequence of *B. vinsonii* with 99.3 % homology and the most divergent one, among the genus *Bartonella*, was the sequence of *B. bacilliformis* with 97.9 % homology. The high hybridization rate of the three isolates (81–90 %) tested by DNA–DNA hybridization confirmed that they belong to the same bacterial species. Only species with a 16S rRNA gene sequence similarity over 98.5 % were tested for DNA–DNA hybridization; this threshold was chosen because in the genus *Bartonella* the intraspecies differences in the 16S rRNA gene are known to be very low (Birtles & Raoult, 1996), so slight differences can be considered to be significant. The low rate of hybridization of the rabbit isolate IBS 382^T with the other type strains of *Bartonella* species tested demonstrates that this isolate belongs to a new *Bartonella* species. Our hybridization values obtained between the type strains of *Bartonella* species were low in comparison to the hybridization values obtained by other workers. These results are due to the hybridization methods used. In contrast to most of the previous studies on *Bartonella* where the hydroxyapatite method was used (Birtles *et al.*, 1995; Daly *et al.*, 1993; Welch *et al.*, 1992), we used in this study the nuclease–TCA method, which is known to give lower hybridization rates (Grimont *et al.*, 1980; Riegel *et al.*, 1994) as the experimentation is performed under more stringent conditions. Based on criteria defining a species (Wayne *et al.*, 1987), we conclude that the corresponding strains constitute a new species within the genus *Bartonella* for which the name *Bartonella alsatica* is proposed.

For characterization of the different strains, 16S rRNA gene amplification followed by sequencing was used. This method was selected as it allows a universal characterization of tested strains, especially for unknown bacteria. Other methods using restriction enzymes such as PCR–RFLP are more useful only when the tested species are well-defined, as the results depend greatly on the restriction enzyme used.

Only one type of 16S rRNA gene sequence was found for all *Bartonella* strains isolated from wild rabbits. In contrast, different *Bartonella* species may be isolated (Heller *et al.*, 1997) from cat blood and co-infections have been observed (Gurfield *et al.*, 1997). The two rabbit populations tested were living at a distance of approximately 60 km from each another, but the strains isolated from these rabbits had an identical 16S rRNA gene sequence and a high DNA homology, as shown by strains IBS 382^T and IBS 379 isolated from rabbits from the first area, and strain IBS 460 isolated from a rabbit captured in the second area. The blood of other small mammals (field mice or field voles) trapped in the same areas was also tested but failed to

yield the same *Bartonella* species. This work reports for the first time the isolation of *Bartonella* from wild rabbits. Further studies need to be conducted to determine the distribution of this new *Bartonella* species among rabbit populations worldwide.

The fact that (i) the *Bartonella* species isolated from the wild rabbits was different from all the *Bartonella* species hitherto known, (ii) only one type of 16S rRNA gene sequence could be found for the different isolates and (iii) no clinical or histopathological abnormalities could be detected strongly supports a long-term adaptation of this new bacterial species to its rabbit reservoir.

The rate of infected erythrocytes seems to be very low in natural conditions. No infected red blood cells could be observed on the wild rabbit blood smears, contrary to Kordick & Breitschwerdt (1995), who found 2.9–6.2% infected erythrocytes in the blood of cats. However, culture of wild rabbit blood had a maximum yield of 2500 c.f.u. ml⁻¹ in primary isolation on blood agar and three strains were isolated only from liquid media. This may explain why *Bartonella* species were not previously described for wild rabbits (Weinman & Kreier, 1977).

Since we used domestic rabbit blood to make the agar medium on which *Bartonella* was cultivated, systematic controls were performed to test the sterility of the plates. For each batch, three randomly selected plates without any sample were incubated in parallel with the inoculated blood agar plates, to assess that the *Bartonella* growing on the inoculated plates did not originate from the rabbit blood incorporated in the agar medium. None of the control plates tested yielded *Bartonella* cultures. Moreover, for each batch, 0.2 ml of the domestic rabbit blood used for preparing blood agar plates was also cultured in parallel on these blood agar plates, and no growth of *Bartonella* species was ever observed.

More than a half of the wild rabbits sampled were infested by fleas or ticks. These ectoparasites have been implicated in *Bartonella* transmission, especially fleas for the cats (Chomel *et al.*, 1996; Higgins *et al.*, 1996), and could also be implicated in the transmission of *Bartonella* in wild rabbits. Furthermore, infested wild rabbits could be a potential source of human contamination when hunted and skinned, as the ectoparasites could eventually bite humans. We have no evidence about whether the *Bartonella* species carried by these rabbits can be transmitted to humans and eventually be implicated in human pathology. However, several human cases reported as 'cat scratch disease' are not serologically related to *B. henselae*, and some cases have occurred without any cat contact history. The rate for these cases was estimated by some authors to be as high as 60% of clinical cat scratch disease cases (Dupon *et al.*, 1996; Yoshida *et al.*, 1996). Many *Bartonella* species appear to be present in our environment and should be carefully investigated. This investigation should focus on updating diagnostic

tools to determine the importance of these *Bartonella* species in human and animal pathology.

Description of *Bartonella alsatica* sp. nov.

Bartonella alsatica (al.sa'ti.ca. L. adj. *alsaticus* from Alsace, the region in the eastern part of France near the Rhine river where the wild rabbits were trapped and where the strains were isolated and identified).

Colonies grown on blood agar appeared after 10 d as small, white, smooth, regular colonies (diameter of about 1 mm). Electron microscopic examination showed small bacilli without flagella and with a size of approximately 1–2 µm in length by 0.5–0.8 µm in width. *B. alsatica* is a Gram-negative rod, aerobic, oxidase-negative. It grows best on blood-enriched media in a moist atmosphere containing 5% CO₂. The three strains tested were all catalase-negative, produced negative Voges–Proskauer reaction, had negative pyrazinamidase and urease activities and did not acidify trehalose. They did not hydrolyse bis-*p*-nitrophenyl phosphate or *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, and had amino acid arylamidase activity with the following amino acids: leucine, methionine, lysine (alkaline as well as acid), glycine, proline, arginine and tryptophan. They had glycylglycylaryl- and trypsin-like activity but no pyrrolidonyl-aryl- and arylamidase activity. The new species is distinguished from the other *Bartonella* species by its 16S rRNA gene sequence and by the whole DNA hybridization. The type strain is IBS 382^T, which was isolated from the blood of a wild rabbit and deposited in the Collection de l'Institut Pasteur under the number CIP 105477^T. Its G+C DNA content is 37 mol% (as determined by the capillary electrophoresis method). Its 16S rRNA gene sequence has been deposited in the EMBL database under the number AJ002139.

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