***Bartonella schoenbuchii* sp. nov., isolated from the blood of wild roe deer**

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The genus *Bartonella* comprises two human-specific pathogens and a growing number of zoonotic or animal-specific species. Domesticated as well as wild mammals can serve as reservoir hosts for the zoonotic agents and transmission to humans may occur by blood sucking arthropods or by direct blood to blood contact. Humans may come into intimate contact with free-ranging mammals during hunting, especially during evisceration with bare hands, when accidental blood to blood contact frequently occurs. The objective of this work was to determine the presence and the polymorphism of *Bartonella* strains in wild roe deer (*Capreolus capreolus*) as the most widely spread game in Western Europe. We report the isolation of four *Bartonella* strains from the blood of five roe deer. These strains carry polar flagella similar to *Bartonella bacilliformis* and *Bartonella clarridgeiae*. Based on their phenotypic and genotypic characteristics, three of the four roe deer isolates were different and they were all distinct from previously described *Bartonella* species. They can be distinguished from each other and from other *Bartonella* species by their protein profile, ERIC-PCR pattern, 16S rRNA and citrate synthase (*gltA*) gene sequences, as well as by whole DNA–DNA hybridization. In spite of their considerable heterogeneity, all four strains fulfil the criteria for belonging to a single new species. The name *Bartonella schoenbuchii* is proposed for this new species. The type strain R1^T of *Bartonella schoenbuchii* has been deposited in the National Collection of Type Cultures as NCTC 13165^T and the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 13525^T.

Keywords: *Bartonella schoenbuchii*, roe deer, zoonoses

INTRODUCTION

The genus *Bartonella* comprises small, curved, often piliated or flagellated bacteria, which have been cultured in recent years on axenic media from the blood of many mammalian hosts (Dehio & Sander, 1999). Together with the reclassification of bacteria formerly belonging to the genera *Rochalimaea* and *Grahamella* (Brenner *et al.*, 1993; Birtles *et al.*, 1995) this has resulted in a dramatic expansion of the genus

Bartonella from one described species in 1993 to currently 15 species, among which at least seven have been associated with human disease (Dehio & Sander, 1999). A haemotropic life style together with transmission by blood-sucking arthropods appears to provide a unique parasitic strategy for all *Bartonella* spp. However, each species seems to be highly adapted to one or a few mammalian hosts, causing a haemotropic infection characterized by the ability to parasitize erythrocytes intracellularly. Two species, *Bartonella bacilliformis* and *Bartonella quintana*, are adapted to humans as reservoir host causing oryza fever/verruca peruana and trench fever, respectively (Dehio & Sander, 1999). In contrast, incidental infection of humans as non-reservoir hosts does not appear to lead to erythrocyte invasion but may cause various clinical manifestations (Dehio & Sander, 1999). Transmission to a non-reservoir host can either occur directly via

Abbreviation: ERIC-PCR, enterobacterial repetitive intergenic consensus PCR.

The EMBL accession numbers for the 16S rRNA genes of *Bartonella schoenbuchii* NCTC 13165^T (R1^T), R3, R4 and R6 are AJ278187, AJ278188, AJ278189 and AJ278190, respectively. The EMBL accession numbers for the *gltA* genes of *Bartonella schoenbuchii* NCTC 13165^T (R1^T), R3, R4 and R6 are AJ278183, AJ278184, AJ278185 and AJ278186, respectively.

blood to blood contact or indirectly via haematophagous arthropods such as fleas or lice. For public health management, it is important to evaluate the presence of *Bartonella* species as a potential cause of zoonosis in those animals to which humans are prominently exposed. Cat-associated *Bartonella henselae* and *Bartonella clarridgeiae* (Gurfield *et al.*, 1997; Heller *et al.*, 1997; Kordick *et al.*, 1997), *Bartonella elizabethae* naturally infecting rats (Daly *et al.*, 1993; Ellis *et al.*, 1999), *Bartonella grahamii* (Kerkhoff *et al.*, 1999) and *Bartonella vinsonii* subsp. *arupensis* typically found in mice (Welch *et al.*, 1999), as well as *Bartonella vinsonii* subsp. *berkhoffii* from a dog reservoir (Roux *et al.*, 2000), have all been associated with human infection and therefore represent zoonotic pathogens.

In this study we have investigated the presence of *Bartonella* in wild roe deer (*Capreolus capreolus*). These animals represent the most widely spread game in Western Europe that is hunted in large numbers (in Germany more than 1 000 000 animals are shot per year). By typical hunting practice, hunters and forestry officials (> 300 000 individuals in Germany) are exposed to direct contact with animal blood, especially during evisceration with bare hands. Accidental injury may bear the risk of direct blood to blood contact and result in infection with *Bartonella* spp. Moreover, a recent study detected *Bartonella* DNA in more than 70% of the ticks collected from roe deer (Schouls *et al.*, 1999), suggesting that ticks may serve as an arthropod vector for transmission of bartonellae. Here we report the isolation of a new *Bartonella* species from the blood of wild roe deer. The prevalence of this species in roe deer of south-western Germany seems to be high and strains belonging to this new species appear to be highly diverse.

METHODS

Culture of *Bartonella* type and reference strains. *Bartonella* spp. were grown on Columbia agar base with 5% defibrinated sheep blood. Plates were incubated at 37 °C in a moist atmosphere containing 5% CO₂ for 4–5 d, except for *Bartonella bacilliformis* which was cultured at 30 °C in a humidified atmosphere for up to 10 d. The following type and reference strains were used for biochemical assays, PCR and DNA relatedness studies: *Bartonella bacilliformis* ATCC 35685^T, *Bartonella clarridgeiae* strain 73 (Heller *et al.*, 1997), *Bartonella doshae* ATCC 700133^T, *Bartonella elizabethae* ATCC 49927^T, *Bartonella henselae* ATCC 49882^T, *Bartonella grahamii* ATCC 700132^T, *Bartonella koehlerae* ATCC 700693^T, *Bartonella quintana* CIP 103739, *Bartonella tribocorum* CIP 105476^T, *Bartonella vinsonii* subsp. *arupensis* ATCC 700727^T, *Bartonella vinsonii* subsp. *berkhoffii* ATCC 51672^T and *Bartonella vinsonii* subsp. *vinsonii*, ATCC VR-152^T.

Isolation of bacterial strains. *Bartonella* isolates were cultured from the blood of wild roe deer. Blood was drawn within 45 min of death of the animal and frozen at –80 °C. Thawed blood (200 µl) was streaked onto blood agar plates made with a Columbia agar base supplemented with 5% defibrinated sheep blood. Incubation of the plates was performed at 37 °C in a moist atmosphere containing 5%

CO₂. The count (c.f.u. ml⁻¹) was determined for each blood agar plate.

Light microscopic examination. Colony phenotypes were observed using a binocular microscope at a magnification of ×5. Gram-staining of the cultured micro-organisms was performed using standard methods (Chapin *et al.*, 2000) and examined with an oil-immersion objective at a magnification of ×1000.

Electron microscopy. Bacteria were prepared for electron microscopy as described by Fussenegger *et al.* (1996). Briefly, plate-grown bacteria were suspended in PBS, spread on a water surface and absorbed to Formvar-coated nickel grids. The grids were stained in 1% uranylacetate and air-dried. The samples were viewed under a Zeiss M109 electron microscope at 80 kV.

Biochemical analysis. Biochemical testing of the roe deer isolates as well as of other *Bartonella* species was performed essentially as described previously (Sander *et al.*, 1997; Sander, 1998; Chapin *et al.*, 2000). *Bartonella* species are biochemically inert in almost all conventional tests and detection of enzyme activities was performed with test kits which are primarily designed for the identification of anaerobes. However, *Bartonella* species are not included in their databases. The RapID ANA II system (Innovative Diagnostic Systems) and Rapid ID 32 A (BioMérieux) were used to test the activity of preformed bacterial enzymes in accordance with the manufacturer's instructions regarding preparation, incubation and biochemical interpretation. Leucyl-aryl-amidase activity was tested in the Api Strep system (BioMérieux).

Cellular fatty acid analysis. Fatty acid methyl esters were extracted from plate-grown bacteria and chromatographed on a Hewlett Packard series II 5890 gas chromatograph as described by Miller & Berger (1985). The molecules were identified by using a computer-assisted comparison of their retention times with a standard mixture (Microbial Identification System).

Protein profiles (SDS-PAGE analysis). One-dimensional analytical SDS-PAGE was performed by the method of Laemmli (1970) with a 12% separating gel and a 4.5% stacking gel. Low-molecular-mass standards (Bio-Rad) were used to estimate the molecular mass of the separated proteins. The gel was stained with Coomassie blue R-250 (Serva).

Chromosomal DNA preparation. Bacterial DNA was extracted using Qiagen Genomic-tip according to the manufacturer's instructions.

Enterobacterial repetitive intergenic consensus (ERIC)-PCR. ERIC-PCR was performed as described by Sander *et al.* (1998a). The primers ERIC1R (5'-ATGTAAGCTCCTG-GGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTG-GGGTGAGAGCG-3') used in this study have been described by Versalovic *et al.* (1991). The reaction mixture contained 8 ng µl⁻¹ bovine serum albumin (Sigma), 200 µM (each) of the four dNTPs, primers (117 nM each), 2 U *Taq* polymerase (Pharmacia Biotech) and 100 ng genomic DNA in 50 µl Tris borate/EDTA buffer. PCR amplification was performed with initial denaturation (95 °C, 7 min), followed by 30 cycles of denaturation (94 °C, 1 min), annealing (40 °C, 1 min) and extension (65 °C, 8 min) with a single final extension (65 °C, 16 min). The amplified products (20 µl) were electrophoretically separated in a 1% agarose gel at 120 V for 2 h in 0.5 × Tris borate/EDTA buffer,

stained with ethidium bromide, visualized on an UV transilluminator and photographed with Polaroid 665 film.

Amplification of the 16S rRNA and citrate synthase (*glcA*) genes. Amplification of the 16S rRNA and citrate synthase genes was performed as described by Birtles & Raoult (1996) and Heller *et al.* (1997). Briefly, PCR amplification of an approximately 1500 bp DNA fragment was performed with the 16S rRNA gene eubacterial universal oligonucleotide primers P8 and Pc1544 (Heller *et al.*, 1997). For the *glcA* gene, the oligonucleotide primers used for the amplification were CS140f and BhCS.1137n (Birtles & Raoult, 1996). Standard procedures to prevent sample DNA contamination were taken (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 fragments were purified by GeneClean glass beads (Bio101; Dianova). Sequencing reactions were performed by the AmpliTaq BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Sequences were obtained with an ABI 377 DNA sequencer (Applied Biosystems). Sequencing of the complete 16S rRNA gene fragment was performed on coding and complementary strands, with four pairs of oligonucleotide primers as described previously (Heller *et al.*, 1999). Partial sequencing of the *glcA* gene was performed with two pairs of oligonucleotide primer: BhCS.1137n and BhCS.781p (Norman *et al.*, 1995), and prCHD205 (5'-TATAAGCCATAGCAG-CAAGAG-3') and CS140F (Birtles & Raoult, 1996).

Sequence analysis. The sequences obtained were compared with similar sequences from other bacteria, including all *Bartonella* spp. contained in the EMBL/GenBank database. The sequences were aligned by using the CLUSTAL W program (Thompson *et al.*, 1994; <http://www.ebi.ac.uk/clustalw/>) and multiple alignments were edited using Jalview (<http://www.ebi.ac.uk/~michele/jalview/contents.html>). Phylogenetic trees were generated by the neighbour-joining method (Saitou & Nei, 1987) using CLUSTAL W and visualized by the software TREEVIEW (Page, 1996; <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

DNA relatedness determination. DNA relatedness experiments by the hydroxyapatite method have been described by Brenner *et al.* (1982). DNA was labelled *in vitro* with [³²P]dCTP using a nick-translation kit (Gibco). The temperature used for optimal hybridization was 55 °C and for stringent hybridization 70 °C, and divergence was calculated to the nearest 0.5%. All reactions were done at least twice.

RESULTS

Roe deer population and bacterial culture

Blood samples from five wild roe deer (designated R1^T, R2, R3, R4 and R6) were collected aseptically within 45 min of death. The roe deer had been shot between September and December 1999 either in the Schönbuch Nature Park or the woodland area of the Spitzberg, both territories being situated in the vicinity of the city of Tübingen in south-western Germany. Before killing, all animals appeared healthy and no particular abnormalities in inner organs (i.e. liver, spleen, heart and lymph nodes) were observed during

Fig. 1. Colony dimorphism of strain R1^T at second-passage on blood agar after 8 d of growth. Bar, 2 mm.

evisceration, especially in relation to *Bartonella* infection.

Bacterial culture

Plates streaked with blood from R2 remained sterile for more than 2 weeks, while on the plates streaked with the four remaining blood samples (of R1^T, R3, R4 and R6), bacterial colonies appeared 4–6 d after inoculation and continued to grow for several days. The titre of colony-forming organisms in the various blood samples ranged from approximate 1000 to 10000 c.f.u. (ml blood)⁻¹. Primary colonies had a characteristic firm, adherent, deeply invaginated, cauliflower-like morphology. After the first passage, a shiny, smooth colony phenotype appeared in addition (Fig. 1), which, due to more rapid growth, outgrew the primary colony phenotype upon multiple passages and was therefore used for further analysis.

Phenotypic identification

Light microscopic examination of the Gram-stained micro-organisms showed small, pleomorphic Gram-negative rods. They were often very short and slightly curved. Electron microscopic examination showed small rods with multiple, unipolar flagella (Fig. 2).

Biochemical analysis

In biochemical testing, the four new isolates tested were negative for catalase, oxidase, indole and urea hydrolysis. All the new strains were positive for leucyl-aryl-amidase activity (LAP) in the Api Strep system. In the Rapid ANA II system the reaction profiles of the new isolates were 000 671, which is identical to that of *Bartonella henselae*, *Bartonella quintana* and several other *Bartonella* spp. In detail, enzymic hydrolysis of D-disaccharide (BLTS), L-arabinoside (aARA), D-galactoside (ONPG), D-glucoside (aGLU), D-galactoside (BGLU), L-fucoside (aFUC), D-glucosaminide (NAG), p-nitrophenylphosphate (PO4) and pyrrolidonyl (PYR) were negative. A strong

Fig. 2. Negative staining of strain R1^T. Bar, 1 µm.

positive reaction was seen in the enzymic hydrolysis of leucyl-glycine (LGY), glycine (GLY), arginine (ARG) and serine (SER). The hydrolysis of proline (PRO) and arginine (ARG) was less pronounced but clearly positive. Using this test system, *Bartonella clarridgeiae* and *Bartonella doshiae* are the only strains showing enzymic hydrolysis of D-glucosaminide (NAG).

The biotype code of the new isolates in the Rapid ID 32A system was 0000 0737 05 for strains R1^T, R3 and R4, which are therefore indistinguishable from that of *Bartonella henselae*, *Bartonella quintana* and several other *Bartonella* species. Amino acid arylamidase activity was obtained with the following amino acids: arginine (ArgA), proline (ProA), leucyl-glycine (LGA), phenylalanine (PheA), leucine (LeuA), tyrosine (TyrA), alanine (AlaA), glycine (GlyA), histidine (HisA) and serine (SerA). In contrast, isolate R6 was lacking LGA and is therefore biochemically different from the other strains (code 0000 0337 05).

Cellular fatty acid analysis

The major fatty acids of the four new *Bartonella* isolates are *cis*-11-octadecanoic acid (C_{18:1} ω7c), which accounted for 42·39–49·85% of total acids, octadecanoic acid (C_{18:0}), 34·45–37·04% of total acids, and hexadecanoic acid (C_{16:0}), 11·76–15·99% of total acids. Very small amounts of C_{12:0} 3OH (0·55–0·69%) and C_{17:0} (0·00–0·45%) were present but no cyclo-

Fig. 3. SDS-PAGE analysis of total bacterial protein extracts of the four roe deer isolates as well as other *Bartonella* species. Extracts of total bacterial protein were separated by SDS-PAGE and stained with Coomassie brilliant blue. Lanes: M, molecular mass markers; 1, R1^T; 2, R3; 3, R4; 4, R6; 5, *Bartonella henselae*; 6, *Bartonella quintana*; 7, *Bartonella bacilliformis*; 8, *Bartonella elizabethae*; 9, *Bartonella clarridgeiae*; 10, *Bartonella alsatica*; 11, *Bartonella tribocorum*; 12, *Bartonella grahamii*.

propane acids were detected. These fatty acid patterns are similar to those observed for other *Bartonella* species (Sander, 1998).

SDS-PAGE analysis

Coomassie blue-stained SDS-polyacrylamide gels of total bacterial protein extracts of the four roe deer isolates showed three distinct protein profiles: R3 and R4 were similar, but R1^T and R6 were different (Fig. 3). Distinct protein profiles were found for all the different *Bartonella* species tested (Fig. 3).

ERIC-PCR

The analysis of the four roe deer isolates by ERIC-PCR confirmed the results obtained by SDS-PAGE: strains R3 and R4 showed an identical banding pattern which was different from those obtained from strains R1^T or R6. The fingerprint patterns of all other known *Bartonella* species are clearly different from those of the four roe deer isolates (Fig. 4).

16S rRNA and *gltA* gene sequence analysis

A 1446 bp region of the 16S rRNA gene sequence, corresponding to *Escherichia coli* positions 23–1521, was determined for the four roe deer isolates. Compared to strain R1^T, strains R3, R4 and R6 had a single substitution of an adenosine by a cytosine residue at position 1021 (*E. coli* numbering system) (corresponding to 99·9% sequence identity). The sequences of the roe deer isolates were aligned with the 16S rRNA gene sequences of all other *Bartonella* species and with *Brucella abortus* as an outgroup. The GenBank accession numbers and strain designations for the

Fig. 4. DNA fingerprint analysis of the four new isolates and other *Bartonella* species by ERIC-PCR. Lanes: M, molecular mass markers; 1, negative control; 2, R1^T; 3, R3; 4, R4; 5, R6; 6, *Bartonella henselae*; 7, *Bartonella quintana*; 8, *Bartonella bacilliformis*; 9, *Bartonella elizabethae*; 10, *Bartonella claridgeiae*; 11, *Bartonella alsatica*; 12, *Bartonella tribocorum*; 13, *Bartonella grahamii*; 14, *Bartonella doshiae*; 15, *Bartonella vinsonii* spp. *arupensis*; 16, *Bartonella vinsonii* spp. *berkhoffii*; 17, *Bartonella vinsonii* spp. *vinsonii*; 18, *Bartonella koehlerae*.

sequences used in the alignment are given in Table 1. Sequences in the alignment extending the fully aligned core region were deleted, resulting in a 1379 base sequence consisting of region 28–1459 (*E. coli* numbering). The multiple alignment was used to generate a phylogenetic tree (Fig. 5a), which indicated that the roe deer sequences were most similar to *Bartonella bacilliformis* (corresponding to 98.0% sequence identity with R1^T).

The *gltA* gene sequence was determined for a region of 1013 bp (strain R1^T), 1026 bp (strain R3), 1025 bp (strain R4) or 1034 bp (strain R6). A phylogenetic tree was generated based on a sequence alignment for the roe deer isolates together with all available *gltA* sequences from other *Bartonella* species which cover a similar size range, as well as *Sinorhizobium meliloti* which served as the outgroup (Fig. 5b). The GenBank accession numbers and strain designations for the sequences used in the alignment are given in Table 1. Sequences in the alignment extending the shortest sequence included (*Bartonella grahamii*) were deleted, resulting in a 903 base sequence consisting of region 557–1459 of the *Sinorhizobium meliloti* sequence. For the roe deer isolates this tree reflects a much higher sequence diversity in the *gltA* gene than observed for the 16S rRNA gene (sequence identities: 98.0% between R1^T and R3/R4; 96.8% between R1^T and R6; and 96.6% between R3/R4 and R6), while the four strains form a discrete cluster most closely related to *Bartonella bacilliformis* (86.6% sequence similarity with R1^T).

DNA hybridization studies

The results of DNA-relatedness studies are given in Table 2. The levels of DNA hybridization between the four roe deer isolates ranged from 76 to 100% under optimal hybridization conditions (55 °C) and from 56 to 100% under stringent (70 °C) hybridization conditions with divergence levels of 0.0–2.5 °C. The percentage relatedness between the roe deer isolates and the type strains of other *Bartonella* spp. was less than 69% under optimal conditions and less than 43%

Table 1. Strain designation and GenBank accession numbers for sequences in 16S rRNA gene and *gltA* gene trees

Organism	Strain	GenBank accession no.	
		16S rRNA	<i>gltA</i>
<i>Bartonella alsatica</i>	CIP 105477 ^T	AJ002139	
<i>Bartonella bacilliformis</i>	LA6.3	Z70003	Z70021
<i>Bartonella claridgeiae</i>	ATCC 51734 ^T	X89208	U84386
<i>Bartonella doshiae</i>	ATCC700133 ^T	Z31351	Z70017
<i>Bartonella elizabethae</i>	ATCC 49927 ^T	L012560	Z70009
<i>Bartonella henselae</i>	ATCC 49882 ^T	M73229	L38987
<i>Bartonella grahamii</i>	ATCC 700132 ^T	Z31349	Z70016
<i>Bartonella koehlerae</i>	ATCC 700693 ^T	AF076237	
<i>Bartonella quintana</i>	ATCC VR-358 ^T	M11927	Z70014
<i>Bartonella taylorii</i>	NCTC 12861 ^T	Z31350	Z70013
<i>Bartonella tribocorum</i>	CIP 105476 ^T	AJ003070	AJ005494
<i>Bartonella vinsonii</i> subsp. <i>vinsonii</i>	ATCC VR-152 ^T	L01259	Z70015
<i>Brucella abortus</i>	11-19	X13695	
<i>Sinorhizobium meliloti</i>	104A14		U75365
	R1 ^T	AJ278187	AJ278183
	R3	AJ278188	AJ278184
	R4	AJ278189	AJ278185
	R6	AJ278190	AJ278186

Fig. 5. Phylogenetic tree based on 16S rRNA sequences (a) and *gltA* sequences (b) showing the position of strains R1^T, R3, R4 and R6 in relation to the known *Bartonella* spp. The tree was rooted by using *Brucella abortus* (a) and *Sinorhizobium meliloti* (b) as the outgroup.

under stringent conditions, with divergence levels greater than 8.0%. Since no strains of *Bartonella taylorii*, *Bartonella talpae* or *Bartonella peromysci* are available, it was not possible to include these species in the DNA hybridization studies. Among the *Bartonella* species tested, *Bartonella henselae* was found to be closely related to the roe deer strains under optimal hybridization conditions (68% at 55 °C), while *Bartonella bacilliformis* was found to be most closely related under stringent hybridization conditions (42% at 70 °C).

DISCUSSION

Bartonella species have been identified as human-specific pathogens (i.e. *Bartonella bacilliformis* and *Bartonella quintana*; Bass *et al.*, 1997a) as well as important zoonotic agents (i.e. *Bartonella henselae* which naturally infects cats; Bass *et al.*, 1997b). In the last 5 years, new *Bartonella* species have been isolated from a wide range of mammalian reservoirs, including rodents (Birtles *et al.*, 1995; Heller *et al.*, 1998), lagomorphs (Heller *et al.*, 1999) and carnivores (Kordick *et al.*, 1996), and a number of them have

already been demonstrated to bear a significant zoonotic potential (Kordick *et al.*, 1997; Ellis *et al.*, 1999; Kerkhoff *et al.*, 1999; Welch *et al.*, 1999; Roux *et al.*, 2000). In this study we have investigated the presence of bartonellae in roe deer. A previous study detected *Bartonella* DNA in more than 70% of the ticks collected from roe deer (Schouls *et al.*, 1999), suggesting that this animal is frequently infected by bartonellae. Indeed, the recovery of four *Bartonella* strains from the blood of five roe deer from southwestern Germany indicates a high prevalence of bartonellae infection in this cervid. In North America, *Bartonella* isolates have been obtained in high frequency (90% prevalence of bacteraemia) from mule deer, another free-ranging wild cervid (Chang *et al.*, 2000).

Primary cultures of the new isolates from roe deer were obtained on blood-enriched medium, as in the culture of other bartonellae. Primary colonies displayed a rough phenotype which changed to a smooth, fast-growing phenotype during passages, a dimorphism which has been previously reported for *Bartonella henselae* (Regnery *et al.*, 1992). In contrast to the often slow growth of primary cultures of other *Bartonella* species, primary colonies of *Bartonella* isolates from roe deer have been obtained within 4–6 d of incubation.

Biochemical profiles cannot be used routinely and reliably for the differentiation of *Bartonella* species because of the relatively inert nature of bartonellae. Among the four roe deer isolates, only strain R6 could be distinguished from *Bartonella henselae* or other *Bartonella* species by biochemical testing using preformed enzyme assays. Whole-cell fatty acid analysis provides a useful tool for the identification of *Bartonella* at the genus level but is a poor method for differentiating species. Consistently, cellular fatty acid of the four new isolates were found to be very similar to those of all other *Bartonella* species (high amounts of C_{18:1} ω7c, C_{18:0} and C_{16:0}).

Repetitive element sequence-based PCR such as ERIC-PCR, has been successfully used for the identification of *Bartonella* strains on the species level as well to discriminate between isolates of the same species (Rodriguez-Barradas *et al.*, 1995; Sander *et al.*, 1998a). Using ERIC-PCR, we obtained from the four strains three different banding patterns, which were clearly different from all other *Bartonella* species. Three distinct protein profiles of the four new isolates were also found by SDS-PAGE. Consistently, DNA sequence analysis of the *gltA* gene again revealed significant heterogeneity within the three distinct sequences. In contrast, 16S rRNA sequences were less divergent, displaying a single base substitution in strain R1^T compared with the three others. These results provide evidence, not only for a clear distinction of the four strains from previously described species, but also for a significant heterogeneity among these strains, isolated from animals shot within an area of less than

Table 2. DNA relatedness of *Bartonella* isolates from roe deer to other *Bartonella* species

Strain	R1 ^T			R4			R6		
	55 °C*	D†	70 °C‡	55 °C*	D†	70 °C‡	55 °C*	D†	70 °C‡
R1 ^T	100	0.0	100	78	2.5	75	84	2.5	76
R3	77	2.5	61	99	0.5	96	95	1.5	84
R4	76	2.5	56	100	0.0	100	97	1.0	85
R6	89	2.0	82	99	0.5	97	100	0.0	100
<i>Bartonella henselae</i>	68	8.5	34	ND	9.5	31	ND	9.0	33
<i>Bartonella quintana</i>	66	10.0	40	ND	10.0	16	ND	10.0	26
<i>Bartonella clarridgeiae</i>	61	10.0	35	ND	10.0	19	ND	8.5	25
<i>Bartonella doshiae</i>	54	11.0	27	ND	10.0	18	ND	10.5	25
<i>Bartonella vinsonii</i>	62	9.0	35	ND	ND	ND	ND	ND	ND
<i>Bartonella grahamii</i>	58	9.5	28	ND	ND	ND	ND	ND	ND
<i>Bartonella koehlerae</i>	59	10.0	35	ND	ND	ND	ND	ND	ND
<i>Bartonella elizabethae</i>	56	10.5	27	ND	ND	ND	ND	ND	ND
<i>Bartonella alsatica</i>	59	10.5	35	ND	ND	ND	ND	ND	ND
<i>Bartonella tribocorum</i>	63	10.0	40	ND	ND	ND	ND	ND	ND
<i>Bartonella bacilliformis</i>	62	8.5	42	ND	ND	ND	ND	ND	ND

ND, Not determined.

* Relative binding ratio [(percentage heterologous DNA bound to hydroxyapatite/percentage homologous DNA bound by hydroxyapatite) × 100] at 55 °C.

† Divergence (D) is the decrease in thermal stability (in degrees Celsius) of heterologous DNA duplexes compared with those of homologous DNA duplexes.

‡ Relative binding ratio [(percentage heterologous DNA bound to hydroxyapatite/percentage homologous DNA bound by hydroxyapatite) × 100] at 70 °C.

100 km². Although differences between strains belonging to one species have previously been noted for other *Bartonella* species (Sander *et al.*, 1998b), the heterogeneity observed in the roe deer isolates appears to be very prominent. However, the limited sample size of this study did not allow epidemiological observations. Further studies need to be conducted to determine the heterogeneity of *Bartonella* isolates among roe deer populations.

DNA–DNA hybridization remains the method of choice for defining a species. It has been recommended that a species be considered to consist of strains whose DNAs are 70% or more related under optimal DNA reassociation conditions and 55% or more related under stringent DNA reassociation conditions, with 5% or less divergence within related sequences (Brenner *et al.*, 1982; Wayne *et al.*, 1987). According to these conditions, all four isolates belong to a single new species, although the strains are so divergent that we could have described two different subspecies (i.e. for R1^T and for R3, R4 and R6) as has been done previously in the genus *Bartonella* (Kordick *et al.*, 1996; Welch *et al.*, 1999). This novel species is proposed and described below. Based on stringent DNA hybridization and phylogenetic trees obtained for the 16S rRNA and the *gltA* gene sequences, the new *Bartonella* species is most closely related to *Bartonella bacilliformis*. Moreover, *Bartonella bacilliformis* together with *Bartonella clarridgeiae* are the

only described *Bartonella* species which carry flagella as described here for all the roe deer isolates. Regarding the relatedness of the new *Bartonella* species to *Bartonella bacilliformis* as well as its considerable heterogeneity we speculate that this pathogen not only has the potential to cause zoonosis (i.e. in hunters and forestry personnel who are exposed to the blood of roe deer or who are infested by ticks), but also that variants may adapt to humans, eventually developing into a human-specific pathogen such as *Bartonella bacilliformis*.

Description of *Bartonella schoenbuchii* sp. nov.

Bartonella schoenbuchii (schoen.buch'i.i. N.L. gen. n. *schoenbuchii* from Schönbuch, a nature park near Tübingen in south-west Germany where most of the roe deer analysed in this study were shot).

Growth obtained on Columbia agar base supplemented with 5% defibrinated sheep blood in a moist atmosphere containing 5% CO₂. Colonies appear in primary culture within 4–6 d as firm adherent, deeply invaginated, cauliflower-like colonies which upon passaging may switch to a more rapidly growing, shiny and smooth colony phenotype. The Gram-negative, monopolar politrachous flagellated rods are approximately 1–2 × 0.5 µm, aerobic and negative for oxidase, indole, catalase and urea hydrolysis. The code for preformed enzymes obtained in the RapID ANA II

system is 000 671. The biotype code using Rapid ID 32A was 0000 0737 05 or 0000 0337 05, depending on the strain analysed. The major fatty acids are *cis*-11-octadecanoic acid (C_{18:1} ω7c), octadecanoic acid (C_{18:0}) and hexadecanoic acid (C_{16:0}), similar as observed for other *Bartonella* species. The four strains thus far isolated from the blood of healthy roe deer display phenotypic and genotypic differences but belong to a single new species. This new species is distinguished from previously known *Bartonella* species by the total protein profiles in SDS-PAGE, the banding patterns in the ERIC-PCR, the *gltA* and 16S rRNA gene sequences, and by whole-DNA hybridization analysis. Type strain is R1^T, isolated from the blood of a 4-month-old female wild roe deer. Deposited in the National Collection of Type Cultures as NCTC 13165^T and the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 13525^T.

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