Brucella microti sp. nov., isolated from the common vole *Microtus arvalis*

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Two Gram-negative, non-motile, non-spore-forming, coccoid bacteria (strains CCM 4915^T and CCM 4916), isolated from clinical specimens of the common vole *Microtus arvalis* during an epizootic in the Czech Republic in 2001, were subjected to a polyphasic taxonomic study. On the basis of 16S rRNA (*rrs*) and *recA* gene sequence similarities, both isolates were allocated to the genus *Brucella*. Affiliation to *Brucella* was confirmed by DNA–DNA hybridization studies. Both strains reacted equally with *Brucella* M-monospecific antiserum and were lysed by the bacteriophages Tb, Wb, F1 and F25. Biochemical profiling revealed a high degree of enzyme activity and metabolic capabilities not observed in other *Brucella* species. The *omp2a* and *omp2b* genes of isolates CCM 4915^T and CCM 4916 were indistinguishable. Whereas *omp2a* was identical to *omp2a* of brucellae from certain pinniped marine mammals, *omp2b* clustered with *omp2b* of terrestrial brucellae. Analysis of the *bp26* gene downstream region identified strains CCM 4915^T and CCM 4916 as *Brucella* of terrestrial origin. Both strains harboured five to six copies of the insertion element IS711, displaying a unique banding pattern as determined by Southern blotting. In comparative multilocus VNTR (variable-number tandem-repeat) analysis (MLVA) with 296 different genotypes, the two isolates grouped together, but formed a separate

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Abbreviations: MLST, multilocus sequence typing; MLVA, multilocus VNTR (variable-number tandem-repeat) analysis; RTD, routine test dilution.

The GenBank/EMBL/DDBJ accession numbers for the gene sequences *omp22*, *omp25*, *omp25b*, *omp31* and *omp31b* of strain CCM 4915^T are AM712379, AM712381, AM712383, AM712385 and AM712387, respectively.

cluster within the genus *Brucella*. Multilocus sequence typing (MLST) analysis using nine different loci also placed the two isolates separately from other brucellae. In the IS711-based AMOS PCR, a 1900 bp fragment was generated with the *Brucella ovis*-specific primers, revealing that the insertion element had integrated between a putative membrane protein and *cboL*, encoding a methyltransferase, an integration site not observed in other brucellae. Isolates CCM 4915^T and CCM 4916 could be clearly distinguished from all known *Brucella* species and their biovars by means of both their phenotypic and molecular properties, and therefore represent a novel species within the genus *Brucella*, for which the name *Brucella microti* sp. nov. with the type strain CCM 4915^T (=BCCN 07-01^T=CAPM 6434^T) is proposed.

At the time of writing, the genus *Brucella* comprises the six species *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella canis*, *Brucella neotomae* and *Brucella ovis* (Corbel & Brinley-Morgan, 1984). Two potential novel species of marine mammal origin, *Brucella pinnipedialis* (formerly '*Brucella pinnipediae*') and *Brucella ceti* (formerly '*Brucella cetaceae*'), have been proposed recently (Cloeckaert *et al.*, 2001; Foster *et al.*, 2007). Due to the high DNA–DNA relatedness of >90 % among these species, it was suggested that the genus should comprise only one species, *B. melitensis*, with the six biovars Melitensis, Abortus, Canis, Neotomae, Ovis and Suis (Verger *et al.*, 1985). However, in 2003, the ICSP Subcommittee on the Taxonomy of *Brucella* agreed unanimously on a return to pre-1986 *Brucella* taxonomy and as a consequence to the reapproval of the six *Brucella* species with the recognized biovars (Osterman & Moriyón, 2006).

Historically, classification of *Brucella* species was based on natural host preference (main isolation source) and phenotypic characteristics, i.e. CO₂ requirement, H₂S production, dye-sensitivity, oxidative metabolic profiles, lysis by *Brucella*-specific bacteriophages and agglutination with monospecific antisera. Indeed, the last proposal for minimal standards to describe novel *Brucella* species was made by Corbel & Brinley-Morgan (1975) and certainly has to be reviewed, in respect of the newly developed molecular typing techniques and evolutionary findings.

Because of the high DNA–DNA relatedness and phenotypic similarity among the different *Brucella* species, a battery of phenotypic and genotypic tests is required for species delineation and for characterization of potentially novel *Brucella* species. Natural host preference might be used as a further criterion if enough isolates are available.

Recently, molecular approaches, in particular analysis of DNA polymorphism of outer-membrane genes (Cloeckaert *et al.*, 1995, 2001; Vizcaino *et al.*, 2004), a set of species-differentiating PCR (PCR-restriction fragment length polymorphism; PCR-RFLP) assays (Al Dahouk *et al.*, 2005; Garcia-Yoldi *et al.*, 2006; Ferrao-Beck *et al.*, 2006; Ratushna *et al.*, 2006), multilocus sequence typing (MLST) (Whatmore *et al.*, 2007) and multilocus VNTR (variable-number tandem-repeat) analysis (MLVA) typing assays (Le Flèche *et al.*, 2006; Whatmore *et al.*, 2006; Garcia-Yoldi

et al., 2007; Al Dahouk *et al.*, 2007) have been developed that allow differentiation of *Brucella* species and to some extent also at the biovar/biotype level.

Here we describe the classification of two strains, CCM 4915^T and CCM 4916, which had been originally isolated on meat peptone agar (MPA) at 37 °C under aerobic conditions from clinical specimens of diseased wild common voles (*Microtus arvalis*) during a recent epizootic in the Břeclav district in South Moravia, Czech Republic (Hubalek *et al.*, 2007), as a novel species of the genus *Brucella*.

The phenotype of strains CCM 4915^T and CCM 4916 was investigated according to growth behaviour on different media, CO₂ requirement, H₂S production, growth in the presence of dyes (thionine and basic fuchsin), reaction with monospecific A and M antisera, bacteriophage typing (Alton *et al.*, 1988) and light and transmission electron microscopy. Metabolic activity in comparison with other *Brucella* species, *Ochrobactrum anthropi* LMG 3331^T and *Ochrobactrum intermedium* LMG 3301^T was assessed using the Merlin MICRONAUT system (Neubauer *et al.*, 2000). Polar lipids and quinone system were analysed as described by Tindall (1990a, b), Altenburger *et al.* (1996) and Stolz *et al.* (2007), and the polyamine pattern was analysed as described by Busse & Auling (1988) and Stolz *et al.* (2007), despite the fact that the biomass was not collected from liquid culture but cells were harvested from trypticase soy agar (TSA) and inactivated by acetone.

Molecular analyses comprised AMOS PCR (Bricker & Halling, 1994), multiplex PCR (Garcia-Yoldi *et al.*, 2006), sequence analysis of the outer membrane genes *omp2a*, *omp2b* (Cloeckaert *et al.*, 1995, 2001), *omp22*, *omp25*, *omp25b*, *omp31* and *omp31b* (Vizcaino *et al.*, 2004), analysis of the *bp26* gene downstream region to distinguish between brucellae of marine mammal and terrestrial mammal origin (Cloeckaert *et al.*, 2000), IS711 (IS6501)-typing by Southern blotting analysis (Clavareau *et al.*, 1998), MLVA (Le Flèche *et al.*, 2006; Whatmore *et al.*, 2006; Garcia-Yoldi *et al.*, 2007; Al Dahouk *et al.*, 2007), MLST (Whatmore *et al.*, 2007) and DNA–DNA hybridization studies (Ziemke *et al.*, 1998).

Both isolates grew well at 28 and 37 °C on MPA without supplementary CO₂, forming transparent to whitish

Table 1. Differentiating physiological reactions of *B. microti* sp. nov. in comparison with other *Brucella* and *Ochrobactrum* species.

Taxa: 1, *B. microti* CCM 4915^T; 2, *B. microti* CCM 4916; 3, *B. abortus* strains: 544^T (=NCTC 10093^T) (bv. 1), 86/8/59 (=NCTC 10501) (bv. 2), Tulya (=NCTC 10502) (bv. 3), 292 (=NCTC 10503) (bv. 4), B3196 (=NCTC 10504) (bv. 5), 870 (=NCTC 10505) (bv. 6), 63175 (=NCTC 10506) (bv. 7), C68 (=NCTC 10507) (bv. 9); 4, *B. melitensis* strains: 16M^T (=NCTC 10094^T) (bv. 1), 63/9 (=NCTC 10508) (bv. 2), Ether (=NCTC 10509) (bv. 3); 5, *B. suis* strains: 1330 (=NCTC 10316) (bv. 1), Thomsen (=NCTC 10510) (bv. 2), 686 (=NCTC 10511) (bv. 3), Reference 40 (bv. 4), Reference 513 (bv. 5); 6, *B. ovis* strain: 63/290^T (=NCTC 10512^T); 7, *B. canis* strain: RM6/66^T (=NCTC 10854^T); 8, *B. neotomae* strain: 5K33^T (=NCTC 10084^T); 9, *B. pinnipedialis* NCTC 12890^T; 10, *B. ceti* NCTC 12891^T; 11, *O. anthropi* LMG 3331^T; 12, *O. intermedium* LMG 3301^T. oNP, *o*-nitrophenyl; pNP, *p*-nitrophenyl; +, positive (QO₂N values >70); –, negative (QO₂N values <50); v, variable (QO₂N values >50 and <70).

Compound	1	2	3	4	5	6	7	8	9	10	11	12	Substrate class
L-Glutamine	+	+	–	–	–	–	–	–	–	–	+	+	Amino acid
L-Histidine hydrochloride	+	+	–	–	–	–	–	–	–	–	+	+	Amino acid
D-Histidine	+	+	–	–	–	–	–	–	–	–	+	+	Amino acid
L-Citrulline	+	+	–	–	–	–	–	–	–	–	+	+	Amino acid
DAPI	+	+	–	–	–	–	–	–	–	–	+	+	Amino acid
Sarcosine hydrochloride	+	+	–	–	–	–	–	–	–	–	+	+	Amino acid derivative
Fumaric acid	+	+	–	–	–	–	–	–	–	–	+	+	Organic acid
Succinic acid	+	+	–	–	–	–	–	–	–	–	+	+	Organic acid
α-Ketoglutaric acid	+	+	–	–	–	–	–	–	–	–	+	+	Organic acid
Itaconic acid	+	+	–	–	–	–	–	–	–	–	–	–	Organic acid
Mesaconic acid	+	+	–	–	–	–	–	–	–	–	–	–	Organic acid
Glutaric acid	+	+	–	–	–	–	–	–	–	–	+	+	Organic acid
Arginine <i>p</i> -nitroanilide	+	+	–	–	–	–	–	–	–	–	v	+	Amino peptidase
pNP <i>N</i> -acetyl-β-D-galactosaminide (pH 7.5)	+	+	–	–	–	–	–	–	–	–	v	+	Esterase
oNP <i>N</i> -acetyl-α-D-galactosaminide (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Esterase
oNP <i>N</i> -acetyl-β-D-galactosaminide (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Esterase
pNP <i>N</i> -acetyl-β-D-glucosaminide (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Esterase
pNP <i>N</i> -acetyl-L-thio-β-D-glucosaminide (pH 7.5)	v	+	–	–	–	–	–	–	–	–	+	+	Esterase
4-nitro α-D-maltoheptaose-4,6,α-ethylidene (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Esterase
pNP <i>N</i> -acetyl-β-D-glucosamide (pH 5.5)	+	+	–	–	–	–	–	–	–	–	+	–	Esterase
pNP α-L-arabinopyranoside (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Glucosidase
oNP β-D-xylopyranoside (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Glucosidase
pNP L-thio-β-D-galactopyranoside (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Glucosidase
2-Methoxy-4-(2-nitrovinyl)-phenyl β-D-galactopyranoside	+	+	–	–	–	–	–	–	–	–	+	+	Glucosidase
pNP β-L-fucopyranoside (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Glucosidase
pNP β-D-thiofucopyranoside (pH 7.5)	v	+	–	–	–	–	–	–	–	–	+	+	Glucosidase
pNP β-D-mannopyranoside (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Glucosidase
pNP β-D-maltoside (pH 7.5)	+	+	–	–	–	–	–	–	–	–	+	+	Glucosidase
pNP β-D-lactopyranoside (pH 7.5)	v	+	–	–	–	–	–	–	–	–	+	+	Glucosidase
pNP β-D-glucuronide (pH 5.5)	+	+	–	–	–	–	–	–	–	–	+	–	Glucosidase
Aesculin hydrolysis	–	–	–	–	v	–	v	–	–	–	+	+	Classical reaction
Voges–Proskauer reaction	+	+	–	–	–	–	–	–	–	–	+	+	Classical reaction
oNP β-D-galactopyranoside-6-phosphate	+	+	–	–	–	–	–	–	–	–	+	+	Phosphatase
pNP phosphate-di(2-amino-2-ethyl-1,3-propanediol) (pH 5.5)	+	+	–	–	–	–	–	–	–	–	+	+	Phosphatase

colonies, 1–2 mm in diameter, after 1–2 days of incubation. H₂S was not produced. Good growth was also observed on blood agar and on standard nutrient agar at 28 and 37 °C. Colonies were slightly concave, smooth, with very light brown exopigment; their edges were continuous. After 72 h of growth at 37 °C, cells appeared as large colonies (6–9 mm) with noticeable brownish pigmentation. Cell

morphology was examined using a Zeiss light microscope at × 1000 magnification with cells grown for 2 days at 37 °C on standard nutrient agar. Gram staining was performed as described by Gerhardt *et al.* (1994) with cells grown for 2 days at 28 °C on MPA. Cells were small (<1 µm) Gram-negative coccobacilli or sporadically formed short rods, arranged individually or in irregular clusters. Flagellation

Fig. 1. Two-dimensional thin-layer chromatogram of total polar lipids of strain CCM 4915^T. PME, Phosphatidylmonomethylethanolamine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; AL1–4, unknown aminolipids; PL, unknown phospholipid; APL1 and 2, unknown aminophospholipids; L1–5, unknown polar lipids.

was determined by transmission electron microscopy (negative staining with 1% uranyl acetate) using a JEM-1010 electron microscope at a magnification of $\times 40\,000$. Cells were unflagellated and morphologically indistinguishable from *B. melitensis* 16M^T, whereas flagella were clearly visible on *O. anthropi* LMG 3331^T (data not shown), which is the type species of the phylogenetically closest genus.

Besides the classical biochemical reactions (Hubalek *et al.*, 2007), a total of 191 different carbohydrates, 190 substrates to determine enzymic activity and 191 different substrates for use as a nitrogen source were tested by using the Merlin MICRONAUT identification system (Neubauer *et al.*, 2000). Each reaction was carried out seven times. The results of the differentiating biochemical reactions of strains CCM 4915^T and CCM 4916 in comparison with the other *Brucella* species and their biovars as well as with *Ochrobactrum* species are summarized in Table 1. The results demonstrate that both isolates exhibit outstanding metabolic capabilities with respect to all other brucellae, sharing a series of reactions with *O. anthropi* LMG 3331^T and *O. intermedium* LMG 3301^T. It is remarkable that both strains were positive in the Voges–Proskauer reaction. Additional results of the phenotypic characterization of strains CCM 4915^T and CCM 4916 by classical methods are given in the species description.

The two strains exhibited a quinone system with ubiquinone Q-10 (91–97%) and Q-9 (3–9%). A similar quinone system was detected in *B. melitensis*, with Q-10 (97%) and Q-9 (3%). Their polar lipid profiles consisted of the major compounds phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and two

unknown aminolipids (AL1 and 2), moderate amounts of an unknown phospholipid PL and small to trace amounts of additional unknown aminolipids (AL3 and 4), two unknown aminophospholipids (APL1 and 2) and five unknown polar lipids (Fig. 1). Two additional lipids, one staining positively with ninhydrin (aminolipid) and the other with molybdenum blue (phospholipid), may be present, showing almost the same chromatographic behaviour as AL1, but their presence could not unambiguously confirmed because of insufficient resolution from AL1 in the thin-layer chromatography. *B. abortus* NCTC 10093^T and *B. melitensis* 16M^T exhibited almost the same profile (results not shown), but unknown aminolipid AL2 was not detectable in these two strains and phosphatidylmonomethylethanolamine was present either in traces or lacking. The polyamine patterns of strains CCM 4915^T and CCM 4916 contained the major compound spermidine (77.0–80.2%), putrescine (14.0–16.8%), 1,3-diaminopropane (3.4–5.0%), *sym*-homospermidine (0.8–1.9%) and spermine (0.4–0.5%). Similar polyamine patterns were detected in *B. melitensis* 16M^T (74.2% spermidine, 18.6% putrescine, 3.0% 1,3-diaminopropane, 3.5% *sym*-homospermidine and 0.4% spermine) and representative species of the genus *Ochrobactrum*, including *O. intermedium* LMG 3301^T (results not shown), the nearest phylogenetic relative of the genus *Brucella*. In contrast, species of the genus *Pseudochrobactrum* exhibit a polyamine pattern lacking *sym*-homospermidine (Kämpfer *et al.*, 2006, 2007).

In the IS711-based AMOS multiplex-PCR, a 1900 bp fragment, not amplified from other *Brucella* species, was generated. Using single species-specific primer pairs, it could be demonstrated that the fragment was amplified with the *B. ovis*-specific primers (Hubalek *et al.*, 2007). Sequencing of the PCR product and subsequent comparative BLASTN analyses revealed that the IS element has integrated between the coding sequences of a putative membrane protein (TIGR/KEGG locus tag BR1284/BMEI0717) and *choL* (BR1285/BMEI0716), a methyltransferase gene (data not shown). At the *B. ovis* primer side the partial *radC* (BR1283/BMEI0718) gene, involved in DNA repair, is present. In none of the published *Brucella* genomes is IS711 located at this position. In the multiplex PCR developed by Garcia-Yoldi *et al.* (2006), strains CCM 4915^T and CCM 4916 were indistinguishable from *B. suis* (data not shown).

The entire open reading frames of the *omp22* (639 bp), *omp25* (642 bp) and *omp31* (752 bp) genes were identical to the corresponding genes of *Brucella suis* 1330 (GenBank accession no. AE014291.4), *Brucella ceti* B202R (AY484523.1) and *Brucella pinnipedialis* B2/94^T (AY484522.1). Similarity values of 99% (one to three substitutions) were observed with other brucellae. Within *omp31b* (786 bp), neither deletions, as reported for *B. melitensis*, nor premature stop codons, as observed for *B. canis* and *B. ovis* (Vizcaino *et al.*, 2004), nor inversion of the *omp25b* gene (785 bp), as described for the majority of *B. ceti* strains (Vizcaino *et al.*, 2004), were detected.

Fig. 2. Phylogenetic tree reconstructed with *omp2a* (1104 nt) and *omp2b* (1089 nt) sequences using CLUSTREE neighbour-joining analysis. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. The tree was rooted by outgrouping sequence *omp2a* of *O. anthropi* LMG 3331^T. Bar, 0.1 divergent residues per site.

The *omp2a* genes were 100% identical to *omp2a* of the marine *Brucella pinnipedialis* strain B2/94^T (GenBank accession no. AF300819) and some other *Brucella* strains isolated from marine mammals, i.e. *Brucella pinnipedialis* strain 6516/9 (DQ059380) and *Brucella* sp. JM13/00 (AB126348). In contrast, the *omp2b* genes were mostly related to *omp2b* of *Brucella* strains from terrestrial mammals with one to three mismatches, (99–98% similarity). The combination of *omp2a* from marine mammal and *omp2b* from terrestrial mammal origin has not yet been described.

A phylogenetic neighbour-joining tree constructed with *omp2a* and *omp2b* of strains CCM 4915^T and CCM 4916, *B. suis* biovars (bv) 2, 4, 5 and sequences available from the EMBL nucleotide database is shown in Fig. 2. The tree was constructed using the online accessible bioinformatics tools of HUSAR, available from <http://genius.emblnet.dkfz-heidelberg.de/menu/w2h/w2hdkfz>. It was constructed from the alignments obtained with CLUSTREE and the

implemented Kimura two-parameter model. In order to assess probabilities of the nodes, 1000 bootstrap resamplings were performed. The tree was rooted with *omp2a* of *Ochrobactrum anthropi* LMG 3331^T as the outgroup.

The PCR fragment size of the *bp26* gene downstream region was 1029 bp, which is characteristic for terrestrial mammal brucellae (data not shown).

The MLVA data obtained with strains CCM 4915^T and CCM 4916 were compared with 424 *Brucella* isolates corresponding to 296 genotypes that have previously been analysed by Le Flèche *et al.* (2006), Garcia-Yoldi *et al.* (2007) and Al Dahouk *et al.* (2007). Cluster analysis was performed as described by Al Dahouk *et al.* (2007) using eight-minisatellite (panel 1) and seven-microsatellite VNTR markers (panel 2) and the categorical distance coefficient and the unweighted pair-group method using arithmetic averages (UPGMA) clustering method. Briefly, the seven microsatellites are divided in two groups, taking

into account their mutation rate (Garcia-Yoldi *et al.*, 2007). Panel 2A comprises the most stable microsatellites Bruce 18, 19, 21 and 30. Panel 2B comprises the highly variable markers Bruce 07, 09 and 16. In the clustering analysis, markers from panel 1, 2A and 2B were given an individual weight of respectively 2, 1 and 0.3. In this analysis, the two strains clustered together (and differed from each other by 4 out of 16 VNTR loci, including one marker from panel 2A and the three markers from panel 2B), but grouped separately from all other known *Brucella* species and their biovars (Fig. 3). Because of the large number of strains, clusters were condensed. *B. neotomae* reference strain 5K33 and *B. pinnipedialis* strain B2/94 appear as closest neighbours in this analysis, whereas the two *B. suis* biovar 5 strains are more distantly related.

In MLST analysis (Whatmore *et al.*, 2007), the two strains clustered separately from other *Brucella* strains most closely related to *B. suis* biovar 5 (ST19) and marine mammal ST25, from which they differ at two out of nine loci (Fig. 4). The MLST profile was 1-11-4-2-1-3-5-2-1. In the IS711 insertion element typing analysis using the Southern blotting technique, the two isolates were indistinguishable from each other, but exhibited a unique banding pattern when compared with other *Brucella* species, corresponding to five to six copies (not shown).

Results from DNA–DNA hybridizations using labelled DNA of *B. melitensis* 16M^T showed $84.1\% \pm 0.7$ DNA relatedness with strain CCM 4915^T. These results are in agreement with previous reports (Verger *et al.*, 1985) which showed that, if the 70 % DNA-relatedness threshold value is applied, species of this genus cannot be separated based on results from DNA–DNA hybridizations. The DNA relatedness to the phylogenetically closest neighbour, *O. intermedium* LMG 3301^T, was 55.5 %.

In summary, from the results of the phenotypic and genotypic analyses, it is evident that isolates CCM 4915^T and CCM 4916 are members of the genus *Brucella*, but can be clearly differentiated from all established species of this genus, including their biovars. Hence, by applying the *Brucella* species concept suggested by the ICSP Subcommittee on the taxonomy of *Brucella* (Osterman & Moriyón, 2006), the two strains represent a novel species of the genus *Brucella*, for which we propose the name *Brucella microti* sp. nov.

Future investigations such as whole-genome sequencing and studies on host cell interactions will provide further insight in the evolution of brucellae and host–pathogen interactions.

Description of *Brucella microti* sp. nov.

Brucella microti (mi.cro'ti. L. gen. n. *microti* of *Microtus*, after the vole genus from which the first strains of this species have been isolated).

Cocci, coccobacilli or short rods, 0.5–0.8 µm in diameter and 0.6–1.4 µm in length. Arranged individually or in

Fig. 3. Condensed dendrogram of clustered MLVA-16 genotypes obtained with 424 *Brucella* isolates corresponding to 296 different genotypes. Bar, percentage of sequence divergence.

irregular clusters. Gram-negative. Aerobic, non-fermentative, non-motile, non-spore-forming. Oxidase-, catalase- and urease-positive. Nitrate and nitrite are reduced (with gas formation from nitrate). No production of H₂S,

Fig. 4. Unrooted phylogenetic reconstruction of the relationships between sequence types (ST). This tree was constructed with the concatenated sequence data of the nine loci (4396 bp) using the neighbour-joining approach. The Jukes-Cantor model, which is based on the assumption that all nucleotide substitutions are equally likely, was used to determine genetic distances. Percentage bootstrap confidence levels of internal branches were calculated from 1000 resamplings of the original data. bv, Biovar; MM, marine mammals. Bar, 0.0005 substitutions per site.

arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, fluorescein (King's B medium). Negative on Simmons' citrate, malonate and acetamide and no growth in broth with 6.5% NaCl. Positive (API 20NE) for D-glucose, maltose, L-arabinose, D-mannose, N-acetylglucosamine and adipic acid. Negative for D-mannitol, citric acid, gluconate, capric acid, malic acid and phenylacetic acid. Acid is produced from glucose, maltose, fructose and xylose in OF medium (Difco). Positive (API ZYM) for acid phosphatase, alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, β -glucosidase and esterase lipase. Enzymes absent on API ZYM are esterase, lipase, α - and β -galactosidase, β -glucosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Starch, casein, DNA, tyrosine, aesculin, ONPG, Tween 80, lecithin and gelatin are not hydrolysed. Differentiating physiological reactions (MICRONAUT) of strains CCM 4915^T and CCM 4916 with respect to other *Brucella* species and biovars and *Ochrobactrum anthropi* LMG 3331^T are given in Table 1. Cells are sensitive to gentamicin, tobramycin, cotrimoxazole (trimethoprim plus sulphamethoxazole) and ofloxacin, but resistant to colistin, piperacillin, ceftazidime and tazobactam. Non-fastidious; good growth occurs on MPA, TSA, blood agar and standard nutrient agar at 25–42 °C. Beige, translucent to whitish colonies with light-brown exopigment and entire edges are formed within 24 h, with a diameter of approximately 2 mm. Good growth does not require CO₂, supplementary serum or blood. No haemolysis is observed. Isolates grow with thionine at dilutions of 1/25 000, 1/50 000 and 1/100 000, and with basic fuchsin at dilutions of

1/50 000 and 1/100 000. Cultures are lysed by Tb, F1 and F25 phages at routine test dilution (RTD) 10⁴ but not at RTD and by Weybridge phage (Wb) at both dilutions. Both strains agglutinate with monospecific anti-M serum up to a dilution of 1 : 80. The quinone system is composed of the major compound ubiquinone Q-10 and minor amounts of Q-9. Predominant polar lipids are phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and two unknown aminolipids, moderate amounts of an unknown phospholipid PL and small to trace amounts of additional unknown aminolipids (AL3 and 4), two unknown aminophospholipids (APL1 and 2) and five unknown polar lipids. The polyamine pattern contains the major compound spermidine, moderate amounts of putrescine and small amounts of 1,3-diaminopropane, sym-homospermidine and spermine.

The type strain is CCM 4915^T (=BCCN 07-01^T=CAPM 6434^T) isolated in 2000 from the liver of *Microtus arvalis*, South Moravia, Czech Republic. Another strain of this species is CCM 4916.

Acknowledgements

We are grateful to Csilla Lodri, Stefan Schatz and Angelika Draeger for excellent technical assistance. We thank Philippe Le Flèche for the MLVA typing and Mark S. Koylass for MLST analysis of strains CCM 4915^T and CCM 4916. The development of genotyping methods for the precise strain identification of dangerous pathogens as part of microbial forensics is supported by the French DGA (Délégation Générale pour l'Armement). The work of B.H. and H.-J.B. was

supported by a Contract-Research-Project for the Bundeswehr Medical Service.

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