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Isolation of Potentially Novel *Brucella* spp. from Frogs

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Bacterial isolates from frogs were phenotypically identified as *Ochrobactrum anthropi*, but 16S rRNA sequencing showed up to 100% identity with *Brucella inopinata*. Further analysis of *recA*, *omp2a*, *omp2b*, *bcsp31*, and IS*711* and multilocus sequence analysis (MLSA) verified a close relationship with *Brucella*, suggesting the isolates may actually represent novel members of this growing genus of zoonotic pathogens.

The classical *Brucella* species are *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. neotomae*, and *B. canis*. More recently, *B. pinnipedialis* and *B. ceti* were described (10, 15), followed by *B. inopinata* and *B. microti* (6, 12–14). To date, members of the genus *Brucella* have not been reported in amphibians as natural hosts.

We report the isolation and identification of unusual Gram-negative, non-spore-forming, motile *Brucella*-like coccoid bacteria isolated from wild-caught African bullfrogs (*Pyxicephalus edulis*) originating from Tanzania, which were found dead or moribund in a quarantine unit.

Tissue samples (liver, spleen, lung, heart, kidney, ovaries, gut, and skin) were cultured on Columbia sheep blood, Gassner's agar (Oxoid, Wesel, Germany), and brucella agar (Merck, Darmstadt, Germany) at 20°C and 37°C for up to 96 h in an aerobic and microaerophilic atmosphere (5% H_2, 10% CO_2, 85% N). From 18 isolates suspected of being *Brucella*, 13 were used for phenotypic analysis, and 8 of them were used for further molecular analysis. Field strains were tested with Gram's and Stamp's stain. Biochemical properties were assessed as described by Alton et al. (1). Growth was assessed on *Brucella* agar with and without crystal violet, agglutination with a complement fixation test (CFT) control serum (Institute Pourquier, Montpellier, France), and with mono-specific A, M, and R antisera (Anses, Maisons-Alfort, France) according to Alton et al. (1). Motility was additionally tested microscopically. For further biochemical characterizations, Vitek2 and API-20NE (bioMérieux, Nürtingen, Germany) were used according to the manufacturer's instructions. Phage lysis testing was carried out as described elsewhere (1).

Following DNA purification, analysis was performed to determine the presence of the *Brucella* genus-specific *bscp31* gene (3) and *Brucella*-specific insertion sequence 711 (IS*711*) (4), as well as by AMOS PCR (where "AMOS" represents the first letters of *Brucella* spp. *abortus*, *melitensis*, *ovis*, and *suis*, respectively) (5) and the Bruce-ladder multiplex PCR assay (9).

16S rRNA gene sequences were amplified (11), purified with the E.Z.N.A. MicroPlate Cycle Pure kit (Omega Biotek, Germany), sequenced by SEQLAB (Göttingen, Germany), and subjected to BLASTN analysis (2).

To establish relationships between *Brucella* and *Ochrobactrum*, eight-locus multilocus sequence analysis (MLSA) data (without int-*hyp*) (18) were compared against all 27 sequence types (STs) originally described and equivalent sequences of *Ochrobactrum intermedium* LMG3301^T and *Ochrobactrum anthropi* ATCC 49188^T. Sequences were concatenated, and phylogenetic analysis was performed using the MEGA4 package.

The entire coding regions of *recA*, *omp2a*, *omp2b*, *bcsp31*, and IS*711* (*orfA* and *orfB*) from a single field strain (09RB8471) were extracted and subjected to BLASTN analysis for their relatedness to other sequences deposited in the database.

Isolates grew well without hemolysis on Columbia and *Brucella* agar with or without crystal violet at 20 and 37°C within 24 h, with better growth in an aerobic atmosphere, but not on Gassner's agar. Bacteria were Stamp-positive and Gram-negative small coccoid rods and showed positive reactions for catalase, cytochrome-oxidase, urease (within 20 to 30 min), and agglutination with a CFT control serum, but not with mono-specific A, M, and R antisera, according to Alton et al. (1). The colony morphology of the isolates stained with crystal violet was smooth since they did not take up dye. Oxidative and fermentative glucose metabolism, indole production, and H_2S production in all but two isolates gave negative results. The identification of all 13 strains by Vitek2 and API-20NE was *Ochrobactrum anthropi*, with a probability of 99.9%. All strains turned out to be motile. In electron microscopy, a single polar flagellum and pilus-like structures were visible (data not shown). Detailed data will be presented separately. The bacteria were not lysed by the phages Wb at the routine test dilution (RTD) and Tb at both the RTD and $10^4 \times$ RTD.

Strains were positive for IS*711* and *bcsp31*, but AMOS PCR was negative. Five fragments each (152, 272, 450, 587, and 794 bp) were generated by the Bruce-ladder PCR (9).

The eight isolates subjected to molecular analysis comprised two distinct clusters based on the 16S rRNA gene sequence.

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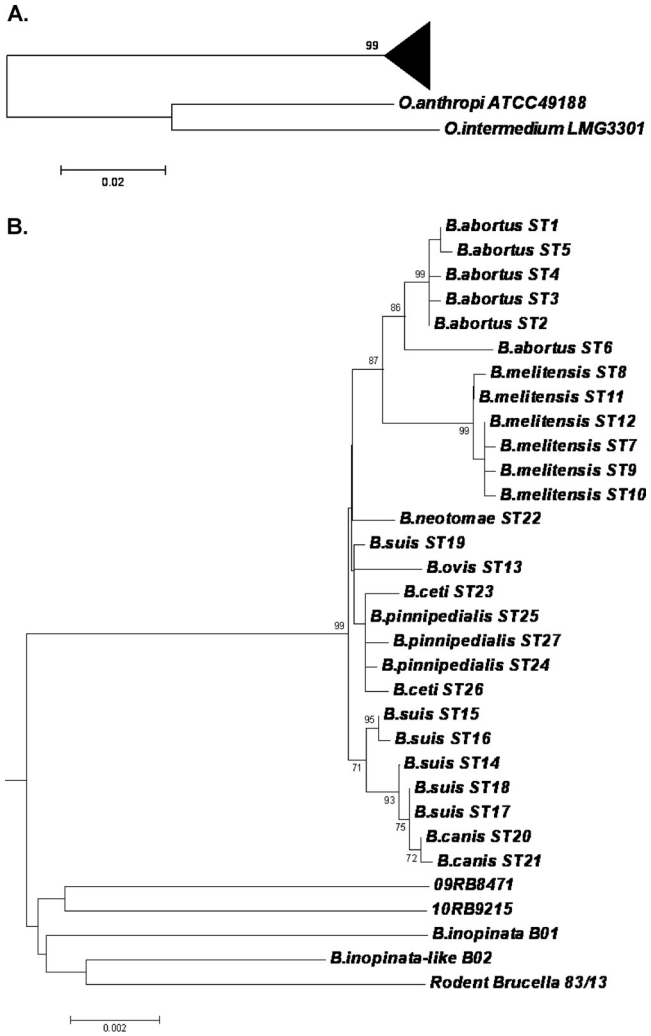

FIG 1 Relationships of two bullfrog isolates (09RB8471 and 10RB9215) with *Brucella* and *Ochrobactrum* isolates based on eight-locus MLSA. Sequences were concatenated, and the phylogenetic tree was constructed by the neighbor-joining approach. For ease of presentation, the *Brucella* clade is reduced in panel A to show relationships with the *Ochrobactrum*, and the subtree is presented separately in panel B to show the relationships among the *Brucella* complex in more detail. Numbers at nodes correspond to proportions of 100 resamplings that support the topology shown, with only values of >70% indicated. The bar indicates the number of substitutions per nucleotide position.

BLASTN revealed the isolate *Brucella inopinata* BO1 (NCBI GenBank EU053207) (7) as the closest relative for one group (type A) with 100% sequence identity (1,212 bp). Type B isolates are most closely related with *Ochrobactrum* sp. strain ROi52 (NCBI GenBank EF219049) (15), and uncultured bacterium clone RO328 (NCBI GenBank EF219037) (15) with 98.6% identity (1,204 bp) due to an insertion of 44 nucleotides (nt) (CCTTT AAAATTCCAAGAAACATAAAATGCCTTGGCATTTTAT GG) with highest similarity to *Ochrobactrum*. Two isolates were examined by MLSA and had novel alleles at all loci compared to all previously described strains and compared to each other. Phylogenetic reconstruction (Fig. 1) revealed that the two isolates cluster within the extended *Brucella* group that includes *B. inopinata* and other recently described *Brucella* isolates, such as isolate BO2 (16) and Australian rodent isolates (17), and are much more distantly related to *Ochrobactrum* species. Analysis of *recA*, *bcsp31*, and IS*711* unequivocally demonstrated that the isolate examined is genetically most closely related to *Brucella*.

We isolated for the first time potentially novel *Brucella* spp. from a cold-blooded amphibian host. These findings significantly enhance the understanding of the ecological potential of the genus *Brucella*. Like *B. inopinata* and *B. microti*, the isolates of this study are fast-growing bacteria with a biochemical profile similar to that of *Ochrobactrum* (11). The presence of *recA*, *bcsp31*, and IS*711* and MLSA analysis unequivocally demonstrated that the isolates are genetically most closely related to *Brucella*. The unusual agglutination properties may reflect an atypical lipopolysaccharide (LPS) gene cluster, as has been reported in strain BO2 (16), another atypical *Brucella* isolate. Additionally, motility is exceptional in *Brucella*, and these strains might represent the first motile *Brucella* species reported to date. All *Brucella* species are equipped with genes of the flagellar apparatus, and amotility is only based upon deletions and insertion within the flagellin operon. Under certain conditions, *Brucella* is indeed capable of formation of a sheathed flagellum (8). Therefore, investigations are under way to find the reason for this motility. Besides these taxonomical/phylogenetic aspects, the isolation of potential novel *Brucella* spp. from amphibians significantly extends the ecologic range of the genus and suggests that all vertebrate classes, including fish (7) and reptiles, may be able to serve as hosts for brucellae. Comparative whole-genome sequencing will provide further insight into the genetic relatedness of these atypical brucellae in comparison with the classical *Brucella* species.

Nucleotide sequence accession numbers. The EMBL accession numbers for the gene sequences of *recA*, *omp2a*, *omp2b*, *bcsp31*, and IS*711* (*orfA* and *orfB*), partial 16S rRNA, and MLSA loci (18) are HE603354 to HE603361 and HE663465 to HE663480, respectively.

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