

A heterogeneous population of motile brucellae out of the frog pond

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From systemically diseased African bullfrogs (*Pyxicephalus edulis*), 21 small Gram-negative motile coccobacilli were isolated and primarily identified as *Ochrobactrum anthropi* by API 20NE and VITEK2. In contradiction to this tentative identification, isolates were positive for the *Brucella* specific genetic marker *bcs31* and the insertion sequence IS711 in PCR analysis. Subsequent 16S rRNA gene sequencing revealed two different genotypes. Type A was 100% identical to the sequence of *Brucella inopinata*. Type B was closely related to *B. inopinata* (99% similarity) but additionally carried an insertion of 44 nucleotides with the closest match (95% similarity) to *Ochrobactrum* spp. Phylogenetic reconstructions using eight markers in a Multi Locus Sequence Analysis (MLSA)- and 16 loci in a Multi Locus Variable Number Tandem Repeat Analysis (MLVA) both placed the isolates in a cluster related to *B. inopinata* and other non-classical and potentially novel *Brucella* species. Molecular analyses clearly illustrated five distinct lineages with three major clades and two singleton isolates. In-depth phenotypic characterization also showed that the amphibian strains are markedly distinct from hitherto known *Brucella* species. Comparative whole genome analysis of the most diverse strains from the previous analyses verified affiliation to the genus *Brucella* but also revealed significant genetic differences within the group. Four representative but molecularly and phenotypically diverse strains were used for *in vitro* and *in vivo* infection experiments. They all readily multiplied in macrophage-like murine J774-cells and their overall intramacrophagic growth rate was comparable to that of *B. inopinata*, and slightly higher than that of *B. microti*. In the Balb/c murine model of infection the amphibian strains replicated both in spleen and liver but were less efficient than *B. suis* 1330. However, they were able to survive in the mammalian host up to 12 weeks depending on the strain investigated.

The heterogeneity of the described novel strains might hamper a single species description but the features of the amphibian isolates suggest that these novel lineages represent missing evolutionary links between a soil associated ancestor and the modern host-adapted pathogenic *Brucella* species.