A heterogeneous population of motile brucellae out of the frog pond

Sascha Al Dahouk[1,2*], Stephan Köhler[3,4,5], Alessandra Occhialini[3,4,5], María Pilar Jiménez de Bagüés[6], Jens-André Hammerl[1], Gilles Vergnaud[7,8], Axel Cloeckaert[9], Michel S. Zygmunt[9], Adrian M. Whatmore[10], Falk Melzer[11], Alice R. Wattam[13], Kevin P. Drees[14], Jeffrey T. Foster[14], Holger C. Scholz[15]

[1] Federal Institute for Risk Assessment (BfR), Department of Biological Safety, Berlin, Germany
[2] RWTH Aachen University, Department of Internal Medicine III, Aachen, Germany
[3] Université Montpellier I, Centre d'études d'agents Pathogènes et Biotechnologie pour la Santé (CPBS)
[4] CNRS, UMR 5236, CPBS, Montpellier, France
[5] Université Montpellier II, CPBS, Montpellier, France
[6] Unidad de Producción y Sanidad Animal, Centro de Investigación y Tecnología Agroalimentaria (CITA), , **Instituto Agroalimentario de Aragón – IA2 (CITA-Universidad de Zaragoza),** Zaragoza, Spain
[7] DGA/D4S - Mission pour la Recherche et l'Innovation Scientifique, Bagneux, France
[8] Université Paris-Sud, Institut de Génétique et Microbiologie, CNRS, Orsay, France.
[9] INRA, UMR1282, Infectiologie et Santé Publique, Nouzilly, France
[10] Veterinary Laboratories Agency, Woodham Lane, Addlestone, United Kingdom
[11] Friedrich-Loeffler-Institut, German National Reference Centre for Human and Animal Brucellosis, Jena, Germany
[13] Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, VA, USA
[14] University of New Hampshire, Department of Molecular, Cellular, and Biomedical Sciences, Durham, NH, USA
[15] Bundeswehr Institute of Microbiology, Munich, Germany

Abstract

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 *bcsp31* and the insertion sequence IS*711* 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. Comparative sequence analyses of the *recA*, *omp2a*, *omp2b* genes confirmed *Brucella* as the closest genetic relative with sequence similarities of 97% to 99%, however, demonstrating sequence heterogeneity among the various isolates. Multi Locus Sequence Analysis (MLSA)- and Multi Locus Variable Number Tandem Repeat Analysis (MLVA)-based phylogenetic reconstruction using eight different MLST markers and 16 VNTR loci placed the isolates as a separate cluster related to *B. inopinata* and other non-classical and potentially novel *Brucella* species. Comparative whole genome analysis of pre-selected representative strains verified affiliation to the genus *Brucella* but also revealed significant genetic differences within the group. 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. 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 less efficiently 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 will hamper species description but the features of the amphibian isolates suggest that this novel species represents the missing evolutionary link between a soil associated ancestor and the modern host-adapted pathogenic *Brucella* species.