RESEARCH ARTICLE

The Change of a Medically Important Genus: Worldwide Occurrence of Genetically Diverse Novel Brucella Species in Exotic Frogs

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Abstract

The genus Brucella comprises various species of both veterinary and human medical importance. All species are genetically highly related to each other, sharing intra-species average nucleotide identities (ANI) of > 99%. Infections occur among various warm-blooded animal species, marine mammals, and humans. Until recently, amphibians had not been recognized as a host for Brucella. In this study, however, we show that novel Brucella species are distributed among exotic frogs worldwide. Comparative recA gene analysis of 36 frog isolates revealed an unexpected high genetic diversity, not observed among classical Brucella species. In phylogenetic reconstructions, the isolates consequently formed various clusters and grouped together with atypical more distantly related brucellae, like B. inopinata, strain BO2, and Australian isolates from rodents, some of which were isolated as human pathogens. Of one frog isolate (10RB9215) the genome sequence was determined. Comparative genome analysis of this isolate and the classical Brucella species revealed additional genetic material, absent from classical Brucella species but present in Ochrobactrum, the closest genetic neighbor of Brucella, and in other soil associated genera of the Alphaproteobacteria. The presence of gene clusters encoding for additional metabolic functions, flanked by tRNAs and mobile genetic elements, as well as by bacteriophages is suggestive for a different ecology compared to classical Brucella species. Furthermore it suggests that amphibian isolates may represent a link between free living soil saprophytes and the pathogenic Brucella with a preferred intracellular habitat. We therefore assume that brucellae from frogs have a reservoir in soil and, in contrast to classical brucellae, undergo extensive horizontal gene transfer.
Introduction

Brucellosis, caused by Brucella spp., is a zoonosis and a disease of both veterinary and public health significance worldwide. The majority of human infections are acquired through transmission from infected animals, either by direct contact or by the consumption of contaminated food products like meat of milk.

The genus comprises several important highly pathogenic species and can be divided into the classical brucellae (B. melitensis, B. abortus, B. suis, B. canis, B. ovis, B. neotomae), brucellae infecting marine mammals (B. ceti and B. pinnipedialis), and the more recently identified species (B. microti, B. inopinata, B. papionis, and B. vulpis). Additional strains from various human and animal sources are currently awaiting final genus affiliation. Brucella microti, B. inopinata, and B. vulpis belong to the ‘atypical’ group of brucellae, exhibiting either atypical phenotypic traits (B. microti) or represent genetically more distantly related species (B. inopinata and B. vulpis).

Human infections are mainly caused by classical Brucella species, in particular B. melitensis, which contributes to 98% of all human brucellosis cases. Within the ‘atypical’ brucellae, only B. inopinata and the non-classified Brucella strain BO2 were isolated from human infections [1, 2]. Despite a pronounced natural host preference, Brucella species can infect various warm-blooded animal species. Brucella ceti and B. pinnipedialis are known to infect marine mammals but human infections caused by these two species are very rare [3]. The natural hosts of B. inopinata and strain BO2 are still unknown.

All classical Brucella species are genetically highly related to each other with average core genome nucleotide identities (ANI) of >99% and harbor identical 16S rRNA and recA gene sequences [4]. ‘Atypical’ Brucella species like B. inopinata and B. vulpis still have ANI values of 98% compared with the type species, B. melitensis, but show a few differences (2–5 nucleotides) in their 16S rRNA and recA gene sequences [5, 6]. The genomes of some of the ‘atypical’ Brucella species (B. inopinata, B. vulpis, strain BO2) carry additional genetic material not found in classical Brucella species but present in other soil associated bacteria of the Alphaproteobacteria. Most of the accessory genes encode for additional metabolic functions or represent bacteriophages and mobile genetic elements, which indicates a different ecology in comparison to the classical host-adapted Brucella species.

Within the last three years, novel ‘atypical’ brucellae emerged from exotic frogs, a host not previously recognized as a reservoir for Brucella. Notably this concerns various frog species from different continents, including Africa, South and Central America, Asia, and Australia. To date, four cases of Brucella infections in frogs have been published. The first report describes the isolation of Brucella sp. from wild-caught African bullfrogs (Pyxicephalus edulis) imported from Tanzania in a quarantine centre of a zoo in Germany [7]. The bullfrogs displayed localized or systemic granulomatous lesions that could not reliably be distinguished from co-occurring mycobacterial and/or fungal infections (T. Eisenberg, unpublished). The second publication reports the isolation of a Brucella inopinata-like strain from subcutaneous abscess material of a big-eyed tree frog (Leptopelis vermiculatus) bought from a pet shop in Germany [8], whilst the third case [9] was reported from the UK in a White’s tree frog (Litoria caerulea) with fluid-filled skin lesions. The most recent case of Brucella infection was described in a Pac-Man frog (Ceratophrys ornata) at a veterinary hospital in Texas; USA [10].

Meanwhile ‘atypical’ brucellae were also identified in tomato frogs (Dyscophus antongilii), a red-eyed tree frog (Agalychnis calidryas) and Amazonian milk frogs (Trachycephalus resinifictrix) from two zoos in Germany (this study) as well as in cane toads (Chaunus [Bufo] marinus) from Australia [11] (this study).
In order to determine the genetic diversity among brucellae isolated from frogs we characterized 36 ‘atypical’ *Brucella* strains, isolated from various frog species of different geographical origins by *recA* gene sequence analysis. In the case of one strain the whole genome sequence was determined and compared to classical *Brucella* species.

**Material and Methods**

**Brucella isolates and frog species**

A total of 36 atypical *Brucella* isolates were investigated. The majority of the isolates were obtained from African bullfrogs (*P. edulis*) imported from Tanzania and kept in quarantine in a zoo in Germany in 2009 [7]. Details on all *Brucella* strains including host species with geographical origin and cross-pathology, and country and year of isolation are provided in Table 1. Seven isolates from Australian cane toads were originally identified as *Ochrobactrum anthropi* [11] and could be reclassified as *Brucella* sp. in this study.

More detailed information about pathological findings among the various frog species can be retrieved from a recent publication by Muehldorfer et al. [12].

**RecA gene analysis**

The *recA* genes of all 36 isolates from amphibians were amplified, sequenced and compared phylogenetically as described previously [13]. Briefly, the primer pair *recA*-BrucOchro-f (5′-atgtctcaaaatctggtgcgc-3′) / *recA*-BrucOchro-r (5′-AGCATCTTCTTCGGTCCGC-3′) was used to amplify a 1065 bp *recA* gene fragment. Trimmed partial sequences (628 bp) were aligned using MUSCLE implemented in MEGA v. 6 [14]. Phylogenetic reconstructions were performed using the neighbor joining (Kimura 2-parameter substitution model) and maximum likelihood (Jukes-Cantor) methods of MEGA with 500 repetitions. The type strains of *O. anthropi* and *O. intermedium* served as outgroup. The *recA* gene sequences from strains *B. inopinata* BO1, BO2, 83–13, and NF 2653 were extracted from the genome sequences available at (http://www.broadinstitute.org/) or (https://www.patricbrc.org).

**Genome sequencing**

The genome sequence of strain (10RB9215), isolated from an African bullfrog, was determined using the PacBio (Pacific Biosciences, Menio Park, USA) sequencing platform. Briefly, DNA was extracted using the Qiagen spin column kit (Qiagen, Hilden, Germany). Genome sequencing was carried out by a sequencing company (GATC, Konstanz, Germany). Assembly of the PacBio generated reads (1 SMRT cell) was done using the freely available SMRT Analysis software (v. 2.3.0) (http://www.pacb.com/devnet/), and the HGAP3 algorithm with a read length minimum of 2500 bp. Genes that have no orthologues (singletons) in classical *Brucella* species were calculated using the singletons option of the EDGAR platform (available at https://edgar.computational.bio.uni-giessen.de) and an in-house *Brucella* genome database consisting of all known classical *Brucella* species. Identified singletons were further compared to the RefSeq database using the BLAST+ implementation of BLASTP with an initial evalue cutoff of 1e-10. Results were filtered for the best two hits of every query and the annotation and source organism for these best hits were extracted from the database.

**Phylogenetic reconstruction**

Core genome-based phylogenetic trees were constructed as described in Blom et al. [15] using the EDGAR platform. Briefly, the core genome with *B. melitensis* 16M as the reference genome and the type strains and biovars of various *Brucella* species was calculated using the implemented
Table 1. History and origin of ‘atypical’ *Brucella* strains isolated from amphibian host species. (wc): wild caught, (cb): captive bred.

| Strain designation | Host species | Geographical origin | Gross pathology | Country, year of isolation | Reference |
|--------------------|--------------|---------------------|-----------------|---------------------------|-----------|
| 070194 A           | Cane toad\(^1\) (wc) | Tropical Americas, invasive species in Australia | spinal arthropathy | AUS, 2008 | (11), this study |
| 070064 B           | Cane toad\(^1\) (wc) | Tropical Americas, invasive species in Australia | spinal arthropathy | AUS, 2008 | (11), this study |
| 070064 C           | Cane toad\(^1\) (wc) | Tropical Americas, invasive species in Australia | spinal arthropathy | AUS, 2008 | (11), this study |
| 070194 C           | Cane toad\(^1\) (wc) | Tropical Americas, invasive species in Australia | spinal arthropathy | AUS, 2008 | (11), this study |
| 070064 E           | Cane toad\(^1\) (wc) | Tropical Americas, invasive species in Australia | spinal arthropathy | AUS, 2008 | (11), this study |
| 070194 E           | Cane toad\(^1\) (wc) | Tropical Americas, invasive species in Australia | spinal arthropathy | AUS, 2008 | (11), this study |
| 09RB8471           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | (7) |
| 09RB8908           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 09RB8909           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 09RB8910           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 09RB8913           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 09RB8914           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 09RB8915           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 09RB8918           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9205           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9206           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9207           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9208           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9209           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9210           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9211           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9212           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9213           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9214           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |
| 10RB9215           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | (7) |
| 10RB9216           | African bull frog\(^2\) (wc) | Southeast, Central and Western Africa | granulomatous / purulent dermatitis | GER, 2009\(^*\)A | this study |

(Continued)
function of EDGAR. The core genome consisted of 2466 coding sequences (CDS) per genome. Multiple alignments of the nucleotide coding sequences or their translated products were created for all core genes using MUSCLE [16]. The gene set alignments were concatenated to one large multiple alignment. Finally, phylogenetic trees (nucleic acid- and protein based) were generated using the F84 (DNA) or Kimura (AA) distance matrix and the neighbour joining method with 200 repetitions as implemented in PHYLIP [17]. Genome sequences used in this project were retrieved from either (http://www.ebi.ac.uk/genomes/) or (https://www.patricbrc.org).

Results
Genetic diversity

Thirty-six Brucella isolates collected from seven different exotic frog species were investigated by recA gene analysis to obtain a better understanding of their genetic diversity and their
phylogenetic relationships to classical Brucella species infecting humans. Although recA provides no genetic resolution among the classical Brucella species, it is a powerful phylogenetic marker to delineate ‘atypical’ Brucella species and provides highly discriminatory resolution among the various Ochrobactrum species, the closest phylogenetic neighbors of Brucella [13].

RecA gene cluster analysis revealed an unexpectedly high genetic heterogeneity among the various frog strains, not observed in classical Brucella species (Fig 1). Notably, isolates from the same frog species and origin were allocated to different recA- clusters whereas other clusters with identical recA gene sequences were formed by various frog species of different geographical origin. The 21 isolates from African bullfrogs formed three major clusters, consisting of nine, six, and four isolates, respectively. Two of the 21 strains (10RB9213 and 10RB9215) formed single lineages. The UK isolate from the white tree frog represented a singleton isolate most closely related to the largest of these groups. In contrast, the classical Brucella species and also B. microti and B. papionis were indistinguishable by means of their recA gene sequences. Notably, B. inopinata BO1 and unclassified strain BO2, both isolated from human infections, clustered within the frog isolates, indicating a close relationship and a possible source of infection. Four of the Australian isolates formed a novel clade. One strain (07/0194C) from a cane toad grouped together with B. inopinata BO1. The Australian rodent isolates 83–13 and NF 2653 had identical recA sequences and grouped separately within the frog isolates. Strain B13-0095, recently isolated from a Pac-Man frog (origin South America) clustered together with tomato frogs from Madagascar and was indistinguishable by means of its recA sequence.

Genome sequencing of strain 10RB9215 and annotation

One African bull frog strain (10RB9215) isolated from a granulomatous / purulent skin lesion was selected for whole genome sequencing and compared with available genomes of classical Brucella species in order to shed light on the evolutionary background of amphibian brucellae. De novo assembly of the PacBio reads resulted in a high quality genome, consisting of two closed contigs (one for each chromosome) with a total size of 3,562813 bp and an average coverage of 120 x. The sizes of the chromosomes were 2.256.786 bp and 1.306.027 bp, respectively. The GC content was 57.2%. Annotation using RAST (http://rast.nmpdr.org/) revealed a total of 3279 coding sequences (CDS) and 67 RNAs.

Core-genome based phylogenetic reconstruction

The genome-based phylogenetic reconstruction of strain 10RB9215 and other known Brucella species and their biovars revealed two major clusters (Fig 2), formed by the classical Brucella species together with B. microti and B. papionis and the distant brucellae, consisting of frog isolate 10RB9215, B. inopinata BO1, B. vulpis F60, strain BO2, and the Australian rodent isolates 83–13 and NF 2653. Strain 10RB9215 formed a separate lineage and was most closely related to B. inopinata and the B. inopinata related isolate BO2.

Genes present in 10RB9215 but absent from classical Brucella species

Singleton analysis of strain 10RB9215 resulted in a total of 338 genes (10.3% of the entire coding sequences) absent from all classical Brucella species (S1 Table). Of the 338 singletons, 277 CDS had a BLAST hit against the reference database. Most of the hits with average similarities of 85–100% were detected with other atypical brucellae (B. inopinata and strain BO2) and Ochrobactrum, the closest genetic relative within the family Brucellaceae, a genus largely consisting of environmental bacteria occasionally infecting humans. Other hits were specific for a wide range of soil living or facultative pathogenic Alphaproteobacteria, like Rhizobium, Agrobacterium, Mesorhizobium, Paracoccus and Bartonella. The majority of genes with a BLAST hit
(27.7%) encoded for additional phages and prophages, followed by genes for metabolism/ABC transporters (18.6%), carbohydrate metabolism (10.8%), and iron acquisition (5.4%). Several novel transposases, integrases and a DNA-modification system were identified. A list of all singletons together with the coding sequences and possible function is provided as supplementary material (S1 Table).

Many of the additional genes were organized in clusters (S1 Table), either flanked by mobile genetic elements or t-RNAs in combination with bacteriophages (not shown), suggesting extensive horizontal gene transfer. One of these clusters, encoding an ectoine uptake system is located on a 12.7 kb fragment on chromosome 1 (peg 1652–1661). The cluster, which is absent from all classical Brucella species consists of nine genes and shows high sequence similarity (av. 94%), to the ectoine system found in Ochrobactrum (S1 Table).

Fig 1. Phylogenetic tree from maximum likelihood analysis of the recA gene alignment of Brucella isolates from exotic frogs including classical and ‘atypical’ Brucella species. The tree was calculated with 100 bootstrap repetitions. Ochrobactrum served as outgroup. Bar: 0.08 substitutions per site. Accession numbers are given in brackets.

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Fig 2. Core-genome-based phylogenetic neighbor-joining tree with 200 repetitions. Bar: 0.002 substitutions per site. Isolate 10RB9215 is indicated in bold letters. Accession numbers are given in brackets.

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Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is a natural compound found in a wide range of Gram-negative and Gram-positive bacteria. It acts as an osmolyte and confers resistance towards salt and temperature [18]. Only recently a similar ectoine system has been described in an atypical Brucella isolate from a Pac-Man frog (Ceratophrys ornata). In the latter case ectoine could be used as a sole carbon source.

Another feature shared among strain 10RB9215 and members of the atypical Brucella clade (B. inopinata, BO2 and the Pac-Man isolate B13-0095) is the presence of a L-Rhamnose utilization gene cluster, a deoxy-hexose sugar commonly found in nature. Like in the other members of atypical brucellae the L-Rhamnose gene cluster of isolate 10RB9215 is located in close proximity to a flagellum gene cluster (not shown). The entire virB operon (B1-B11) is found on chromosome 2 (peg 1036–1046) of strain 10RB9215. In classical Brucella species this operon is important for virulence and is essential for the intracellular survival and multiplication within macrophages.

Discussion

Until recently the genus Brucella was considered to represent a genetically homogeneous and clonal group of bacteria intricately associated with mammalian hosts. The data presented here illustrate a much more complex ecological situation with extant Brucella species representing specialized ecotypes with yet undetermined pathogenic potential for human and animal health. They form a background of an apparently genetically and geographically disparate population of ‘atypical’ Brucella. In contrast to known classical Brucella species, recently reported brucellae from exotic frogs are genetically highly diverse and might represent several new Brucella species. The presence of additional genetic material from various other soil-living bacteria suggests extensive horizontal gene transfer and indicates a different ecology compared to classical Brucella species which to date have been considered largely clonal [19]. It appears that the amphibian isolates may represent a link between free living soil saprophytes and the pathogenic Brucella with a preferred intracellular habitat, and further study of these organisms may help understanding the crucial steps in the evolution of virulence in the latter group. The close relationship of amphibian isolates with ‘atypical’ Brucella isolates, previously been associated with severe human disease, suggests that these organisms may themselves have pathogenic potential that merits investigation. Apparently, amphibian brucellae are capable of causing disease in different anuran species ranging from localized manifestations to generalized organ infections [7–12] (this study). This indicates that they are at least facultative pathogens of veterinary importance in cold-blooded vertebrates. Further, the world-wide distribution in frogs suggests that amphibians are not only occasionally infected by ‘atypical’ brucellae, but may represent a yet undiscovered and ecologically significant natural host for this group.

Supporting Information

S1 Table. Results of a BLAST analysis of singletons present in strain 10RB9215 but absent from classical Brucella species. A maximum of two BLAST hits are given. C1 = chromosome 1; c2 = chromosome 2.

(XLSX)

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References
1. De BK, Stauffer L, Koylass MS, Sharp SE, Gee JE, Helsel LO, et al. Novel Brucella strain (BO1) associated with a prosthetic breast implant infection. J Clin Microbiol. 2008; 46:43–49. doi: 10.1128/JCM.01494-07 PMID: 1797792
2. Tiller RV, Gee JE, Lonsway DR, Griibble S, Bell SC, Jennison AV, et al. Identification of an unusual Brucella strain (BO2) from a lung biopsy in a 52 year-old patient with chronic destructive pneumonia. BMC Microbiol. 2010; 10:23. doi: 10.1186/1471-2180-10-23 PMID: 20105296
3. Sohn AH, Probert WS, Glaser CA, Gupta N, Bollen AW, Wong JD, et al. Human neurobrucellosis with intracerebral granuloma caused by a marine mammal Brucella spp. Emerg Infect Dis. 2003; 9:485–488. doi: 10.3201/eid0904.020576 PMID: 1270223
4. Gee JE, De BK, Levett PN, Whitnry AM, Novak RT, Popovic T. Use of 16S rRNA gene sequencing for rapid confirmatory identification of Brucella isolates. J Clin Microbiol. 2004; 42:3649–3654. doi: 10.1128/JCM.42.8.3649-3654.2004 PMID: 15297511
5. Scholz HC, Nöckler K, Gölker C, Bahn P, Vergnaud G, Tomaso H, et al. Brucella inopinata sp. nov., isolated from a breast implant infection. Int J Syst Evol Microbiol. 2010; 60:801–808. doi: 10.1099/ijs.0.011148-0 PMID: 1966151
6. Scholz HC, Revilla-Fernández S, Al Dahouk S, Hammer JA, Zygmun MS, Cloeckaert A, et al. Brucella vulpis sp. nov., a novel Brucella species isolated from mandibular lymph nodes of red foxes (Vulpes vulpes) in Austria. Int J Syst Evol Microbiol. 2016; 66:2090–2098. doi: 10.1099/ijsem.0.000998 PMID: 26928956
7. Eisenberg T, Hamann HP, Kaim U, Schlez K, Seeger H, Schauerte N, et al. Isolation of potentially novel Brucella spp. from frogs. Appl Environ Microbiol. 2012; 78:3753–3755. doi: 10.1128/AEM.07509-11 PMID: 22407680
8. Fischer D, Lorenz N, Heuser W, Kämpfer P, Scholz HC, Lierz M. Abscesses associated with a Brucella inopinata-like bacterium in a big-eyed tree frog (Leptopelis vermiculatus). J Zoo Wildl Med. 2012; 43:625–628. doi: 10.1638/2011-0005R2.1 PMID: 23082529
9. Whatmore AM, Dale EJ, Stubberfield E, Muchowski J, Koylass M, Dawson C, et al. Isolation of Brucella from a White’s tree frog (Litoria caerulea). JMM Case Rep. 2015;
10. Soler-Lloréns PF, Quance CR, Lawhon SD, Stuber TP, Edwards JF, Ficht TA, Robbe-Austerman S, O’Callaghan D, Kierle A. A Brucella spp. isolate from a Pac-Man Frog (Ceratophrys ornata) Reveals Characteristics Departing from Classical Brucellae. Front Cell Infect Microbiol. 2016; 28:116.
11. Shilton CM, Brown GP, Benedict S, Shine R. Spinal arthropathy associated with *Ochrobactrum anthropi* in free-ranging cane toads (*Chaulius [Bufo] marinus*) in Australia. Vet Pathol. 2008; 45:85–94. doi: 10.1354/vp.45-1-85 PMID: 18192584

12. Mühldorfer K, Wibbelt G, Szentiks CA, Fischer D, Scholz HC, Zschöck M, Eisenberg T. The role of 'atypical' *Brucella* in amphibians: Are we facing novel emerging pathogens? J Appl Microbiol. 2016;

13. Scholz HC, Al Dahouk S, Tomaso H, Neubauer H, Witte A, Schloter M, et al. Genetic diversity and phylogenetic relationships of bacteria belonging to the *Ochrobactrum-Brucella* group by recA and 16S rRNA gene-based comparative sequence analysis. Syst Appl Microbiol. 2008; 31:1–16. doi: 10.1016/j.syapm.2007.10.004 PMID: 18222618

14. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013; 30:2725–2729. doi: 10.1093/molbev/mst197 PMID: 24132122

15. Blom J, Albaum SP, Doppmeier D, Pühler A, Vorhölter FJ, Zakrzewski M, et al. EDGAR: a software framework for the comparative analysis of prokaryotic genomes. BMC Bioinformatics. 2009; 10:154. doi: 10.1186/1471-2105-10-154 PMID: 19457249

16. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research. 2004; 32:1792. doi: 10.1093/nar/gkh340 PMID: 15034147

17. Felsenstein J. PHYLIP (Phylogeny Inference Package) version 3.6. 2005. Available: http://evolution.genetics.washington.edu/phylip.html.

18. Kuhlmann AU, Hoffmann T, Bursy J, Jebbar M, Bremer E. Ectoine and hydroxyectoine as protectants against osmotic and cold stress: uptake through the SigB-controlled betaine-choline-carnitine transporter-type carrier EctT from *Virgibacillus pantothenticus* J Bacteriol. 2011; 193:4699–4708 doi: 10.1128/JB.05270-11 PMID: 21764932

19. Whatmore AM, Perrett LL, MacMillan AP. Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. BMC Microbiol. 2007; 7:34. doi: 10.1186/1471-2180-7-34 PMID: 17448232