Case Report

Isolation of Brucella from a White’s tree frog (Litoria caerulea)

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Introduction: Brucellosis is a zoonotic disease that has a significant economic, social and public health impact in many parts of the world. The causative agents are members of the genus Brucella currently comprising 11 species and with an expanding known host range in recent years.

Case presentation: One of a pair of White’s tree frogs (Litoria caerulea) developed skin lesions from which a pure growth of a haemolytic organism was obtained. The isolate was identified as Brucella melitensis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, although the colony morphology was inconsistent with this identification. Applying the classical biotyping approach used to subdivide members of the genus Brucella, the isolate did not correspond to any known Brucella sp. However, PCR targeting of genes specific for members of the genus Brucella was strongly positive and 16S rRNA gene sequencing revealed a close relationship with extant Brucella spp. In order to place the isolate more accurately, a multilocus sequencing approach was applied, which confirmed that the isolate represented a novel member of the emerging ‘atypical’ Brucella group, which includes isolates from human disease, from rodents and, more recently, reported isolations from frogs in Germany.

Conclusion: This case represents the first report of isolation of a Brucella sp. from frogs outside Germany and suggests that these isolates may be widespread. Whilst there is no evidence to date that these isolates represent a zoonotic threat, the association of other ‘atypical’ Brucella sp. with human disease suggests that appropriate measures should be taken to avoid unnecessary contact with potentially infected amphibians until the zoonotic potential of this group is better understood.

Keywords: Brucella, brucellosis, frog, MALDI-ToF.

Introduction

Brucellosis is a zoonotic disease with a significant global impact. Of the 11 recognized species, Brucella melitensis, Brucella abortus and Brucella suis, causative agents of small ruminant, bovine and swine brucellosis, respectively, are considered the most significant animal and human pathogens. In recent years, the host range of the genus has increased, notably with new species identified from rodents, marine mammals and baboons (Whatmore et al., 2014). Here, we add to this picture describing a case of isolation of Brucella sp. from skin lesions on a frog housed in a tropical animal collection in the UK.

Case report

One of a pair of White’s tree frogs (Litoria caerulea) that had been used as handling exhibits in a tropical animal collection presented with lesions, appearing first as swellings on the frog’s lower back but progressing rather quickly from small swellings to quite obvious, fluid-filled lesions (Fig. 1). The lesions were incised, curedt and drained, and a swab sent to a commercial laboratory for identification. The lesions appeared to subside after treatment with enrofloxacin, but the main lesion reappeared shortly after...
treatment and required a second drainage before resolution. The second animal remained symptomless throughout.

Bacteriological analysis

The swab was initially received at IDEXX Laboratories for characterization and inoculated onto blood agar with 5 % sheep blood (Oxoid). After overnight aerobic incubation at 36 °C, a pure growth of a haemolytic organism was observed. Following on-target extraction using formic acid, the isolate was examined using a Bruker Microflex LT system (Bruker Daltonics) using the standard and security-related (SR) databases. Using the standard database, no reliable identification was returned (score <1.7), but using the SR database the MALDI Biotyper real-time classification (RTC) software returned a secure identification of B. melitensis with a score of 2.316, with scores ≥2.3 being considered secure to the species level. The identification was repeated three times to verify the original findings, and each time the score was >2.3. The isolate was not morphologically consistent with B. melitensis and was therefore referred to the national and international Brucella reference laboratory at the Animal and Plant Health Agency (APHA) for further characterization.

On receipt at APHA, the isolate was labelled UK8/14 and subcultured onto serum dextrose agar and Farrell’s medium. The isolate grew well at 20, 30 and 37 °C with only marginally faster growth at the two higher temperatures, forming opaque colonies not initially conforming to the characteristic appearance of Brucella spp. On subculture, the colonies were concave, smooth with entire edges and did show the characteristic iridescence seen with classical Brucella spp. on Henry illumination. The isolate was examined by a slide agglutination test using unabsorbed Brucella antiserum and showed strong agglutination. However, this test is considered to lack specificity, and the gold standard for identification is a biotyping approach conventionally used to identify Brucella isolates to the species and biovar levels (Whatmore, 2009). By these approaches, the organism was excluded as a member of any of the classically described Brucella spp., failing to agglutinate with A, M or R antisera and failing to lyse with any of the standard Brucella typing phage (Tb, Wb, Bk2, Fi, R/C or Lz) at a routine test dilution. In other classical biotyping reactions, the isolate grew in the presence of thionin at 1/50 000 (w/v) but with an atypical uptake of dye, grew in the presence of fuchsin at 1/50 000 (w/v), produced H₂S, hydrolysed urea and did not require CO₂ for growth.

Molecular analysis

Given the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) result and two recent reports of the isolation of Brucella from frogs in a quarantine centre and from a pet shop in Germany (Eisenberg et al., 2012; Fischer et al., 2012), more extensive molecular analysis was undertaken to fully characterize the strain.

To determine whether the isolate belonged within the genus Brucella, a real-time PCR assay based on a genus-specific target present in the bscp31 gene (Probert et al., 2004) and the multiple-copy Brucella-specific insertion sequence IS711 (Matero et al., 2011) were performed. Four replicates were tested alongside duplicate Brucella positive and no-template controls. The cycle threshold (Cₚ) values of the replicates were all around 23.5 cycles for bscp31 and 18.5 for IS711, in agreement with the identification of this isolate as a member of the genus Brucella. No-template controls were negative up to 40 cycles. The isolate was further tested using Bruce-ladder, a multiplex PCR approach that can differentiate all known species of Brucella (López-Goñi et al., 2011). The isolate gave a profile of five bands distinct from those reported from extant Brucella spp. (Fig. 2a).

Analysis of the 16S rRNA gene sequence of UK8/14 showed over 99 % nucleotide identity to sequences of all type strains of Brucella with the best match to Brucella inopinata. Whilst most Brucella spp. have been reported to share identical 16S rRNA gene sequences (Gee et al., 2004), B. inopinata is the most variant to date, showing five nucleotide changes from other species type strains (Scholz et al., 2010). UK8/14 shared these changes but additionally had two 7 bp insertions not shared with other described Brucella spp.

In order to place the isolate within the existing genus of Brucella, a multilocus sequencing approach examining sequences at nine unlinked genetic loci was applied (Whatmore et al., 2007). All nine loci were amplified successfully by PCR, indicating a close relationship of the isolate to Brucella. Phylogenetic placement based on concatenated sequence data, excluding a single gene that does not amplify from B. inopinata, was performed, comparing the isolate with isolates representing the known genetic diversity of members of the genus Brucella (Fig. 2b). These included both ‘core’ Brucella spp., comprising all the classically described major pathogenic species, as well as
Fig. 2. Molecular characterization of UK8/14. (a) Comparison of the Bruce-ladder profile with members of existing Brucella spp. and vaccine strains. Lanes: 1, 1 kb ladder; 2, B. ceti 1/94; 3, B. melitensis 63/9; 4, B. melitensis Rev1; 5, B. ovis 63/290; 6, B. abortus 86/8/59; 7, B. abortus RB51; 8, B. abortus S19; 9, B. suis 1330; 10, Brucella canis RM6/66; 11, Brucella
the recently described ‘atypical’ Brucella spp. that include the recently described B. inopinata (Wattam et al., 2012). These isolates are genetically divergent from the ‘core’ or classical Brucella spp. but have been described as Brucella spp. based on a much closer relationship to the ‘core’ Brucella group than to the next closest phylogenetic neighbours (Ochrobactrum spp.).

Isolate UK8/14 clearly fell within the ‘atypical’ Brucella clade, which includes the B. inopinata type strain (De et al., 2008; Scholz et al., 2010) as well as a number of isolates yet to be formally described taxonomically. These include strain 83/13, a representative of a group of isolates from Australian rodents (Tiller et al., 2010a), strain BO2, described as a B. inopinata-like isolate from a human infection (Tiller et al., 2010b), and two additional isolates, 10RB9251 and 09RB8471. Interestingly, the latter isolates represent the first Brucella-like isolates from amphibians described recently in wild-caught African bullfrogs from Tanzania (Eisenberg et al., 2012). Isolate UK8/14 is thus clearly a member of the ‘atypical’ Brucella group most closely related to a strain isolated previously from frogs (09RB8471).

Conclusions
Whilst this isolate would be excluded as Brucella by conventional phenotyping approaches, molecular analysis confirmed that the isolate belongs to the rapidly expanding group of ‘atypical’ Brucella spp. Although the species-level MALDI-TOF MS identification turned out to be inaccurate, probably reflecting both the known homogeneity of the group and the poor representation of extant Brucella spp. in the commercial databases, MALDI-TOF MS was useful in identifying as a potential Brucella an isolate that would immediately be excluded by conventional phenotyping. In order to avoid ‘missing’ such isolates, diagnostic or reference laboratories should consider the use of 16S rRNA gene sequencing and/or the presence of IS711, considered specific for Brucella spp. (Whatmore & Gopaul, 2012), in their routine identification procedures. In addition, the reaction with unabsorbed Brucella antiserum may be a useful screen, despite the acknowledged lack of specificity, provided that confirmatory testing is then pursued. With regard to MALDI-TOF MS, it should be noted that, according to our findings and as reported elsewhere (Cunningham & Patel, 2013), only users with the SR database installed and enabled would be able to identify this isolate as a Brucella sp. Whilst MALDI-TOF MS might provide a useful initial screen, particularly as the strain would be excluded as a Brucella sp. by conventional biotyping, the identification as the highly pathogenic and zoonotic B. melitensis would have significant implications. Clearly, it is therefore valuable to use molecular approaches, such as multilocus sequence analysis, to help accurately place these isolates in context with extant Brucella strains.

One of the classical characteristics of Brucella is the ability to survive and replicate intracellularly. Whilst testing of this characteristic was outside the scope of this case report, it would be of interest to examine this property. Whilst some of the emerging atypical Brucella spp. appear to possess this capability (Jiménez de Bagués et al., 2014), others, such as BO2, which, like UK8/14, fails to react with either monospecific A or M antiseras, and has a novel O-polysaccharide biosynthetic pathway (Wattam et al., 2012), appear unable to replicate intracellularly (Wattam et al., 2014). LPS is considered the major virulence factor of classic Brucella spp. (Lapaque et al., 2005), and thus any modifications in its structure may impact host–pathogen interactions. Whole-genome sequencing currently in progress should provide a route to understanding the structure of UK8/14 LPS.

This is the first report outside Germany of the isolation of such strains from amphibians and both confirms the rapidly expanding host range of the genus and suggests that such isolates may be widely distributed. The lesions observed in this case appeared superficially similar to those reported in a previous case in another frog species (Leptopelis vermiculatus). Whilst there is no evidence to date to suggest that Brucella isolates associated with amphibians are pathogenic for humans, many members of the genus represent significant zoonotic pathogens (Godfroid et al., 2011). It is worth noting that the fact that strain UK8/14 was untypeable using the monospecific sera (A or M dominant) commonly used to classify Brucella spp. is suggestive of modifications in the LPS that may compromise the ability to detect any infection serologically, as tests are based largely on detection of antibodies against the O-polysaccharide of the LPS (Zygmun et al., 2012). Thus, it is possible that any human infections with such organisms would not be detected by routine serodiagnostic approaches. The association of other ‘atypical’ Brucella isolates (B. inopinata and B. inopinata-like organisms) with serious human infections (De et al., 2008; Tiller et al., 2010b) suggests that appropriate measures should be taken to avoid unnecessary contact with potentially infected amphibians until the zoonotic potential of this emerging group is better understood.
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References


