Case Report

Bovine pyogranulomatous mastitis caused by *Mycobacterium goodii*

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Introduction: *Mycobacterium goodii* is a rapidly growing non-tuberculous mycobacterium that has recently been associated with severe infections in animals and humans. The ecological niche of *M. goodii* remains unclear, and cases in large animals remain either undiagnosed or misdiagnosed.

Case presentation: We present a case of a 5-year-old Holstein cow showing mastitis signs of pronounced glandular hardening that did not respond to antibiotic therapy. During the milk bacteriological culture, we observed Gram-positive and acid-fast rods with an unusual profile in the milk diagnostic routine. Biochemical tests were performed and the results showed a bacterium belonging to the group *Mycobacterium smegmatis*. Antimicrobial susceptibility testing was performed, and the result for tobramycin indicated the presence of *M. goodii*. In order to confirm its identity, 16S rRNA gene sequencing and phylogenetic analysis were performed, showing 100 % nucleotide similarity with *M. goodii*. Histological analyses of a biopsy specimen obtained from the affected mammary quarter showed evidence of pyogranulomatous and diffuse mastitis, both suggestive of bacterial intracellular infection.

Conclusion: To the best of our knowledge, this is the first confirmed case of mycobacterial mastitis caused by *M. goodii* infection in cows, identified through isolation of the bacteria and 16S rRNA gene sequencing. Although the role of this agent in bovine mastitis remains unclear, we highlight its potential source for humans and the implications for the dairy industry.

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Introduction

Mycobacteria are aerobic, non-motile, Gram-positive rods with a cell wall rich in mycolic acids and mycosides (Brown-Elliott & Wallace, 2002). Rapidly growing mycobacteria (RGM) are ubiquitous in the environment, can be isolated from soil and water, and need up to 7 days to grow on solid medium (Brown-Elliott & Wallace, 2002). RGM comprise the following groups: *Mycobacterium chelonae-abscessus*, *Mycobacterium fortuitum* and *Mycobacterium smegmatis*

(Brown-Elliott & Wallace, 2002). *Mycobacterium goodii* was recently identified as a rapidly growing non-tuberculous mycobacterial species of the *M. smegmatis* group (Friedman & Sexton, 2001). This group may be divided with approximately 90 % accuracy based on their susceptibility to tobramycin (10 µg): *M. smegmatis sensu stricto* is susceptible to tobramycin (agar disk diffusion zone >30 mm), *M. goodii* has intermediate susceptibility (agar disk diffusion zone 11–30 mm) and *Mycobacterium wolinskyi* is resistant (agar disk diffusion zone ≤11 mm) (Brown *et al.*, 1999).

The diagnosis of RGM infections may be quite difficult by conventional methods (Lemarie, 1999). Although several phenotypic characteristics can help identify *M. goodii*, they do not have a strong discriminating power for

Abbreviations: Abbreviation: RGM, rapidly growing mycobacteria.

The GenBank/EMBL/DDBJ accession number for the sequence of *M. goodii* strain SB 314/96 is JF338856.

differentiating this pathogen from other members of the *M. smegmatis* group (Brown *et al.*, 1999). *M. goodii* was previously associated with sporadic cases of cellulitis, osteomyelitis, infected pacemaker sites, lipoid pneumonia (Brown-Elliott & Wallace, 2002) and bursitis in humans (Friedman & Sexton, 2001). Therefore, *M. goodii* is considered a pathogen with zoonotic potential (Krimer *et al.*, 2010).

The ecological niche of *M. goodii* remains unclear, although *M. goodii* has been acknowledged as a spotted hyena pathogen (van Helden *et al.*, 2008) and has been found in dogs with a concurrent infection caused by hyperadrenocorticism (Bryden *et al.*, 2004). Cases in large animals remain undiagnosed or misdiagnosed. Non-tuberculosis mycobacterial bovine mastitis is uncommon, but it has been proved that the *M. smegmatis* group species may cause clinical mastitis in sheep and dairy cows (Thomson *et al.*, 1988). Here, we describe an unusual case of bovine pyogranulomatous mastitis caused by *M. goodii*.

Case report

A 5-year-old Holstein cow belonging to a herd of 67 milking cows, originally located in the south of Brazil, showed mastitis signs of pronounced glandular hardening. The herd characteristics included a semi-intensive dairy system and the majority of the cows belonged to the Holstein breed. All animals were vaccinated against foot-and-mouth disease and brucellosis. Tuberculosis was routinely controlled, and veterinarians and technical staff of a cooperative provided assistance to the farmer. The cow underwent several intramammary (aminoglycoside and β -lactam) and parenteral (tetracyclines) antibiotic therapies with treatment protocols for 2 weeks. Due to the lack of adequate response, the owner requested milk bacteriological testing.

Investigations

Milk samples from all quarters, collected twice with a 15-day interval between collections, were submitted to a standard milk culture (blood agar and MacConkey plates with aerobic incubation at 37 °C), and routine biochemical tests were performed (MacFaddin, 2000). Antimicrobial susceptibility testing was determined using a disc diffusion method (Acar, 1980), according to Clinical and Laboratory Standards Institute standards (CLSI, 2013). The antimicrobials tested were: oxacillin (1 µg), ampicillin (10 µg), penicillin (10 IU), cefoperazone (75 µg), cephalotin (30 µg), tobramycin (10 µg), gentamicin (10 µg), neomycin (30 µg), enrofloxacin (5 µg), norfloxacin (10 µg), lincomycin (2 µg), nitrofurantoin (300 µg), tiamulin (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), tetracycline (30 µg) and rifampin (5 µg). Standard control strains of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) as recommended by the CLSI were used for monitoring the accuracy of susceptibility tests.

A biopsy sample of the affected mammary quarter was fixed in 10 % buffered formalin, paraffin embedded,

sectioned at 4 µm and stained with haematoxylin and eosin and Ziehl–Neelsen/Fite (EasyPath) for acid-fast rods (Berg, 1953) in order to verify tissue damage.

The DNA from an *M. goodii* strain SB 314/96 isolate was extracted by lysis with cetyltrimethylammonium bromide (CTAB), according to Sambrook & Russell (2001). Molecular diagnosis was performed by analysis of the partial sequencing of triplicate DNA product of approximately 1500 bp amplified from the 16S rRNA gene using universal primers (Fredricks & Relman, 1998). The PCR product was sequenced with ACT Gene Análises Moleculares LTDA (Centro de Biotecnologia) using an automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequences were submitted to a consensus analysis based on chromatogram reliability obtained with the Staden Package Gap 4 program (Staden *et al.*, 2000) and alignment by National Center for Biotechnology Information BLAST with DNASIS software (v.2.5; Hitachi Software Engineering Co.). In order to confirm the identification of the isolate as *M. goodii*, we performed a phylogenetic analysis based on the 16S rRNA gene using the neighbour-joining method. In order to calculate the evolutionary distances and the pairwise deletion of gaps, the *p*-distance was implemented with MEGA v.5.2.2 software.

Diagnosis

After 3 days of incubation, a slow and sparse growth of lightly pigmented, shiny and smooth colonies ranging from 1 to 2 mm was observed on the blood agar plate, and

Fig. 1. Bovine mammary gland showing pyogranulomatous mastitis. The lesion was characterised by a central area of necrosis with large numbers of neutrophils, mostly degenerated (*), surrounded by macrophages, epithelioid cells (arrows), Langhans giant cells (arrowhead) and fewer lymphocytes and plasma cells (x). Fibroblasts (○) were present in the peripheral zone. Haematoxylin and eosin stain; magnification, $\times 40$.

no growth was observed on the MacConkey plate. Bacterial growth was found in two of the eight milk samples collected (the same kind of growth was found in both samplings). Gram and Ziehl–Neelsen staining revealed poorly staining, irregular, slender Gram-positive and acid-fast rods. Biochemical characterization of the isolate showed that it was catalase, urease, glucose, sucrose, mannitol and nitrate reduction positive and was negative for maltose, CAMP test, oxidase and aesculin. The isolate was considered to belong most probably to the *M. smegmatis* group due to its rapid growth, acid-fast staining and biochemical properties (Hartmans *et al.*, 2006). In the antimicrobial susceptibility testing, the isolate was sensitive to all antimicrobials tested and was intermediate to tobramycin (agar disk diffusion 22 mm), showing the pattern for *M. goodii*. This *in vitro* susceptibility pattern is similar to previously reported findings (Brown-Elliott & Wallace, 2002).

Histologically, there were multiple foci of severe and diffuse inflammatory infiltration. They were characterized by a central area of necrosis with large numbers of neutrophils, mostly degenerated, surrounded by macrophages, epithelioid cells and Langhans giant cells, and fewer lymphocytes and plasma cells. Fibroblasts were observed in the peripheral zone.

The glandular ducts were filled with a large number of neutrophils and necrotic epithelioid cells. These microscopic changes are characteristic of pyogranulomatous and diffuse mastitis (Fig. 1), suggestive of bacterial intracellular infection. Histological slides stained with Ziehl–Neelsen showed poorly positive acid-fast rods (Fig. S1 available in the online Supplementary Material).

Phenotypic characteristics can help identify *M. goodii*, although they do not have a strong discriminatory tool to differentiate *M. goodii* from other members of the *M. smegmatis* group. Thus, to assure the definitive identification of the isolate, we used a molecular approach. Here, although the 16S rRNA genes of *M. goodii* and *M. smegmatis sensu strictu* differed by only 3 bp, the phylogenetic analysis (Fig. 2) performed was a useful tool to differentiate these species. The consensus sequence showed 100 % nucleotide similarity with *M. goodii* sequences NR029341, Y12872 and AY457079 in GenBank.

Discussion

The various attempts at intramammary antibiotic treatment may have been the main reason for the introduction

Fig. 2. Phylogenetic analysis based on 16S rRNA gene sequences showing the homology between the 16S rRNA gene sequence of *M. goodii* SB 314/96 (with a label) and other 16S rRNA gene sequences of *Mycobacterium* species. The neighbour-joining method using the *p*-distance to calculate the evolutionary distances and the pairwise deletion of gaps was implemented with MEGA v.5.2.2 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to each branch. *Corynebacterium jeikeium* was used as the outgroup.

of *M. goodii* into the cow udder, as the majority of cases of infection by *M. goodii* are related to previous injuries (Ahmad & Khakoo, 2010). In fact, the circumstances reported here are very similar to cases of nocardial mastitis, which are characterized by being refractory to conventional therapy, especially after intramammary infusions for long periods (Ribeiro *et al.*, 2008). Our findings should be taken into consideration by veterinarians and laboratories during diagnosis, especially as *M. goodii* is an environmental RGM and domestic animals are a potential source for this non-tuberculous mycobacterial infection in humans (Krimer *et al.*, 2010). The current case report also emphasizes that milk contaminated with *M. goodii* may be a potential source for human infections, especially if drunk untreated. Another important factor is the lack of a disinfectant or antiseptic against *M. goodii* (Ahmad & Khakoo, 2010), which may contribute to its maintenance in the environment.

To the best of our knowledge, this is the first confirmed case of mycobacterial mastitis caused by *M. goodii* infection in cows, and was identified by isolation of the bacteria and 16S rRNA gene sequencing. Although the role of this agent in bovine mastitis remains unclear, we highlight its potential source for human infections and the implications for the dairy industry. We expect to clarify the role of *M. goodii* in bovine mastitis with further studies.

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