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Case Report

MOLECULAR IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY OF *MYCOLICIBACTERIUM SMEGMATIS* ASSOCIATED WITH BOVINE MASTITIS

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Abstract

Bovine mastitis presents a significant health and economic issue in dairy farming, typically caused by common bacterial pathogens, but atypical organisms such as non-tuberculous mycobacteria (NTM) may occasionally be involved. Among rapidly growing mycobacteria (RGM), *Mycolicibacterium smegmatis* is usually considered an environmental saprophyte, although sporadic mastitis cases have been described. In this article, a rare case of bovine mastitis in a Simmental cow is presented. The animal showed no systemic clinical signs and failed to respond to intramammary antibiotic therapy. Milk culture revealed acid-fast rods with features consistent with RGM, but MALDI-TOF MS did not provide conclusive identification. Final confirmation was obtained by 16S rRNA gene sequencing, showing 99.73% similarity to *M. smegmatis*. Antimicrobial susceptibility testing showed resistance to cefoxitin, clarithromycin, and trimethoprim-sulfamethoxazole, while the isolate was sensitive to aminoglycosides, fluoroquinolones, tetracyclines, carbapenems, and linezolid. Rifampicin and azithromycin exhibited high minimum inhibitory concentration (MIC) values consistent with known reduced

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susceptibility. This case illustrates the diagnostic difficulties associated with atypical mycobacteria, particularly the limitations of conventional phenotypic methods and the importance of molecular approaches. It also underlines the need to consider RGM in cases of mastitis that do not respond to conventional therapy and adds to the limited body of evidence documenting their role in bovine pathology.

Key Words: bovine mastitis, *Mycolicibacterium smegmatis*, non-tuberculous mycobacteria, rapidly growing mycobacteria, 16S rRNA sequencing

CASE PRESENTATION

Mastitis in dairy cows is one of the most significant diseases in these animals, causing substantial economic losses through reduced milk yield, discarded milk, veterinary expenses, and early culling of affected animals (Benić et al., 2018., Cvetnić et al., 2022). Despite intensive control measures, mastitis continues to challenge dairy production worldwide, and atypical pathogens occasionally emerge, complicating both diagnosis and treatment (Gulaydin et al., 2023; Tomanić et al., 2023).

Among the less common agents are non-tuberculous mycobacteria (NTM), a diverse category of acid-fast organisms widely distributed in the environment (Bercovier and Vincent, 2001). Although NTM are increasingly recognized in veterinary medicine, their role in bovine pathology remains less defined compared with classical slow-growing mycobacteria and common mastitis pathogens (Supré, 2019). Among the NTM, rapidly growing mycobacteria (RGM), including members of the *M. fortuitum*, *M. chelonae-abscessus*, and *M. smegmatis* complexes, are generally regarded as environmental saprophytes, yet have been implicated in sporadic cases of bovine mastitis and other opportunistic infections (Bercovier and Vincent, 2001; Ghielmetti et al., 2017). Formerly designated as *Mycobacterium smegmatis*, this species has been reclassified as *Mycolicibacterium smegmatis* following phylogenomic and comparative genomic analyses that demonstrated the polyphyly of the genus *Mycobacterium* (Gupta et al., 2018; LPSN, 2025).

Although *M. smegmatis* is generally considered nonpathogenic or opportunistic, it has been associated with disease in both humans and animals (Siqueira et al., 2016; Reil et al., 2023; Crowley et al., 2024). In veterinary medicine, beyond bovine mastitis, reports include cutaneous infections such as canine dermatitis and panniculitis (Barletta and Steffen, 2013), systemic disease in dogs (Grooters et al., 1995), pyogranulomatous panniculitis in cats (Malik et al., 1994), infections in wild boars (Zanetti et al., 2018), and chronic granulomatous lesions in captive deer (Channakeshava et al., 2006). Experimental studies have also confirmed its ability to induce mastitis in bovine mammary glands (Richardson, 1970). Reports of mastitis in cows caused by *M. smegmatis* have been described in many countries, often linked to recurrent pyogranulomatous inflammation and poor response to conventional antibiotic therapy (Richardson, 1970; Siqueira et al., 2016; Ghielmetti et al., 2017; Supré et al., 2019). Environmental sources such as contaminated bedding material or repeated intramammary treatments

have been suggested as potential risk factors for introducing these organisms into the udder (Siqueira et al., 2016; Ghielmetti et al., 2017). Reports of bovine mastitis caused by atypical mycobacteria are rare, and each confirmed case contributes valuable insight into the epidemiology, diagnostic challenges, and clinical management of mycobacterial infections. In this article, we report a rare case of bovine mastitis caused by *M. smegmatis*, confirmed through 16S rRNA gene sequencing, underscoring the importance of recognizing atypical pathogens in dairy medicine and highlighting the role of molecular methods in definitive diagnosis.

On a dairy farm in Bijeljina (Bosnia and Herzegovina), a localized case of mastitis with swelling and redness was observed in an adult Simmental cow. The animal had no change in body condition and did not display systemic clinical signs. The affected quarter presented no gross abnormalities, and no additional cases were detected within the herd. The cow received intramammary antibiotic therapy with amoxicillin and gentamicin, but no clinical improvement was achieved, after which the animal developed chronic mastitis. Therefore, milk was sampled from the affected quarter and was submitted for bacteriological examination.

An aseptically collected milk sample was cultured on Columbia blood agar (Becton Dickinson, New Jersey, USA), MacConkey agar (Becton Dickinson, New Jersey, USA), and Sabouraud dextrose agar (HiMedia, Mumbai, India), and incubated under aerobic conditions at 37 °C. Conventional biochemical tests were subsequently performed. Following incubation of 48 hours, smooth, shiny and circular colonies with light pigmentation, approximately 1 mm in diameter were observed on blood agar (Figure 1).

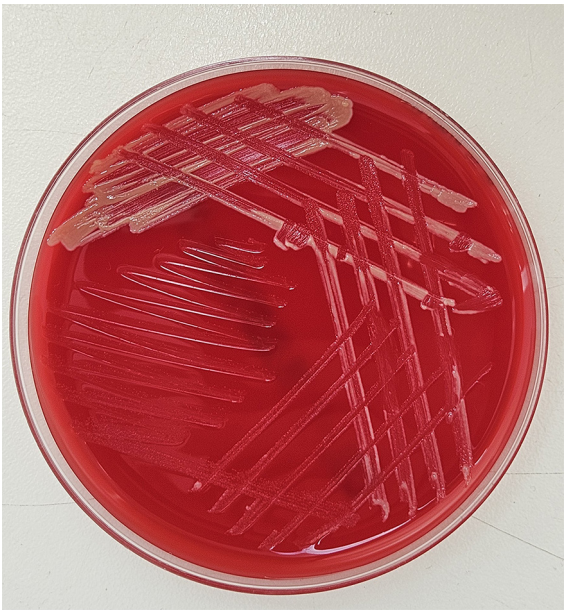

Figure 1. Colonies of *Mycolicibacterium smegmatis* on blood agar (48 h incubation)

No growth was detected on MacConkey or Sabouraud dextrose agar. Morphological and biochemical characteristics were examined, and growth on Löwenstein-Jensen slants was confirmed. Gram and Ziehl-Neelsen staining showed Gram-positive and acid-fast rods (Figure 2).

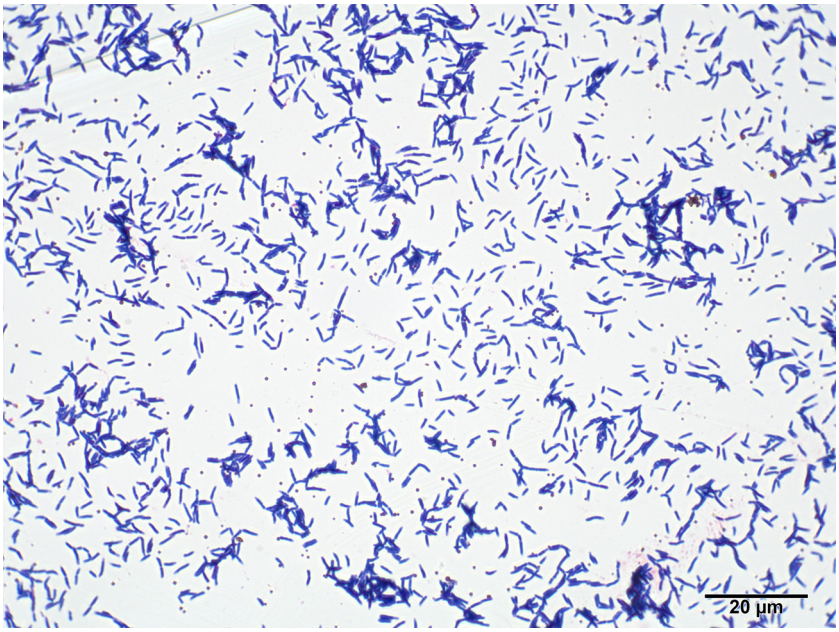

Figure 2. Microscopic preparation of *M. smegmatis* (Gram stain)

The isolate fermented different compounds (glucose, sucrose, mannitol), showed nitrate reduction and gave a positive catalase reaction, while tests for maltose, oxidase, and aesculin were negative. The *in vitro* profile was consistent with *M. smegmatis*, as previously reported (Quinn et al., 1994; Siqueira et al., 2016). However, species-level identification by MALDI-TOF MS was inconclusive, possibly due to insufficient protein extraction. MALDI-TOF MS was performed by the direct colony method using Microflex BioTyper spectrometer with FlexControl software ver. 3.4 (Bruker Daltonics, GmbH, Germany).

Molecular identification was performed by 16S rRNA gene sequencing. Nucleic acid extraction was performed with the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions for Gram-positive bacteria. DNA quality and concentration were assessed with a BioSpec-nano UV-Vis spectrophotometer, and the extracts were stored at −20 °C until analysis. Sequencing was carried out by Macrogen Europe (Amsterdam, Netherlands) using universal primers 785F (5' GGATTAGATACCCTGGTA 3') and 907R (5' CCGTCAATTCMTTTRAGTTT 3'). Subsequent BLAST analysis showed 99.73% similarity, with all of the top ten hits belonging to the species *M. smegmatis*. Phylogenetic

analysis was performed in MEGA11 (Tamura et al., 2021) using the Neighbor-joining method with the Kimura 2-parameter model and 1,000 bootstrap replications (Figure 3). The obtained 1459 bp sequence has been deposited in GenBank under the accession number PV992554.

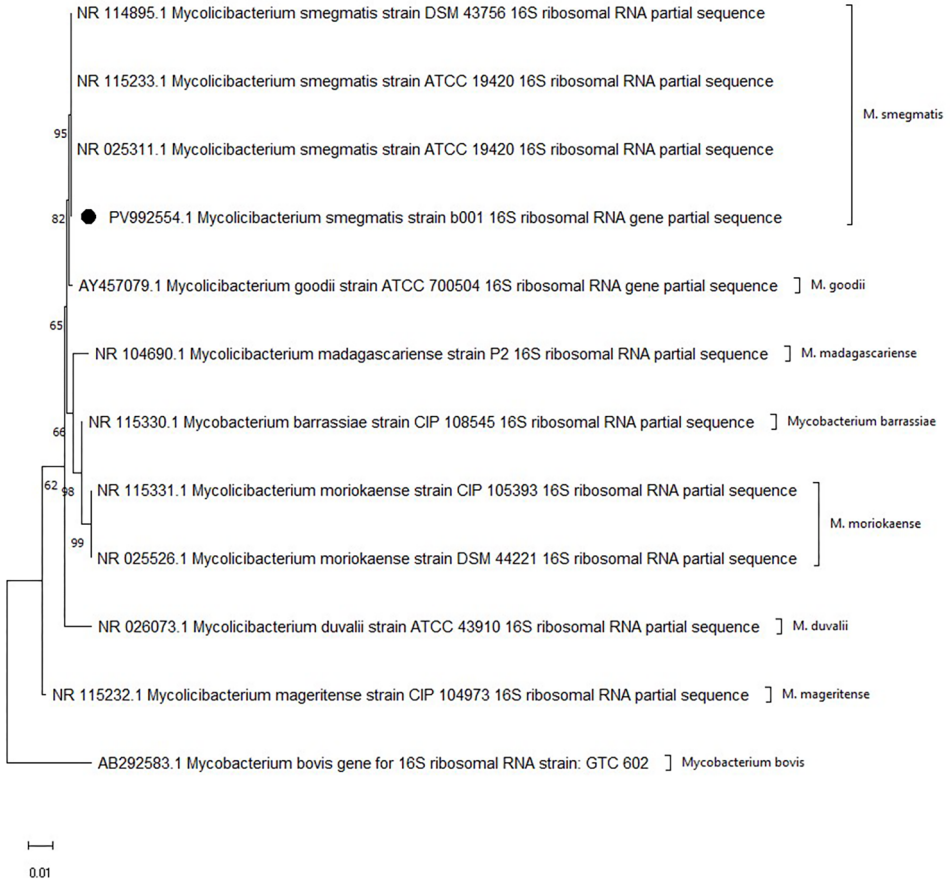

Figure 3. Phylogenetic tree of *Mycolicibacterium smegmatis*

As no veterinary standards exist for antimicrobial susceptibility testing of *M. smegmatis*, testing was performed according to CLSI guidelines for RGM in humans (M24 and supplement M24S) (CLSI M24, 2018; CLSI M24S, 2018). The broth microdilution method was used as recommended. Fourteen antibiotics were tested (Table 1). Each microdilution plate included a positive growth control and a sterility control. Standard quality control strains (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213) were used to verify the accuracy of antimicrobial panels across different antibiotic classes. Minimum inhibitory concentration (MIC) endpoints were read after incubation at 30 °C for 72 h, and for clarithromycin plates were read daily from day 3 through day 14 to assess potential inducible resistance (in this isolate, sufficient

growth was observed by day 5). Each test was performed in duplicate, and results were interpreted using CLSI breakpoints for RGM. The isolate was resistant to cefoxitin, clarithromycin, and trimethoprim–sulfamethoxazole (TMP-SMX), while it was susceptible to all other tested antibiotics.

Table 1. Antimicrobial susceptibility results for *Mycolicibacterium smegmatis*

Antibiotic	MIC[1] value (μg/mL)	Result[2]
Amikacin	4	S
Tobramycin	1	S
Cefoxitin	128	R
Ciprofloxacin	1	S
Moxifloxacin	0.5	S
Clarithromycin	8	R
Azithromycin*	16	Not interpreted
Rifampicin*	32	Not interpreted
Doxycycline	1	S
Minocycline	1	S
Imipenem	0.25	S
Meropenem	1	S
Linezolid	0.5	S
Trimethoprim/sulfamethoxazole	4/76	R

[1]**MIC** – Minimum Inhibitory Concentration
[2]**S** – susceptible; **R** – resistant
***According to CLSI M24 (3rd ed., 2018), no interpretive breakpoints are available for rifampicin or azithromycin in RGM. Rifampicin testing is not recommended, as *M. smegmatis* typically exhibits high MIC values (≥16-32 μg/mL) due to intrinsic resistance mediated by rifampicin ADP-ribosyltransferase activity. Likewise, azithromycin is not included in the CLSI panel, since clarithromycin is the recommended macrolide. Therefore, MIC values for these agents were reported without S/I/R categorization and are presented here for comparative purposes only.

Following a one-month course of therapy prescribed based on the MIC antibiotic testing, the cow showed clinical recovery. However, the swelling persisted until the time of publication of this report.

DISCUSSION

Reports of bovine mastitis caused by RGM remain scarce, yet accumulating evidence demonstrates their potential role as opportunistic pathogens in dairy cattle. Early descriptions already linked *M. smegmatis* to clinical mastitis in cattle (Richardson, 1970;

Schultze and Brasso, 1987; Thomson et al., 1988), and subsequent studies confirmed its involvement in recurrent pyogranulomatous mastitis in Brazil and Belgium (Siqueira et al., 2016; Supré, 2019). Similar findings were reported in Switzerland, where *M. goodii* and *M. smegmatis* were recovered from mastitis cases after repeated unsuccessful antimicrobial treatments, underscoring the importance of considering RGM when conventional therapy fails (Ghielmetti et al., 2017). The diagnostic process in such cases is challenging. Phenotypic and biochemical features may suggest *M. smegmatis*, but definitive identification usually requires molecular methods such as 16S rRNA or *rpoB* sequencing (Ghielmetti et al., 2017; Siqueira et al., 2016). MALDI-TOF has been introduced as a rapid diagnostic tool, although its reliability for RGM identification still depends on updated databases (Supré, 2019). The present case highlights these diagnostic difficulties, since phenotypic suspicion alone was insufficient and molecular confirmation was required.

Identification in our study relied on partial 16S rRNA sequencing, which showed 99.73% identity to *M. smegmatis*. Because short 16S segments have limited resolving power for RGM, especially within the former *M. smegmatis* group that includes *M. smegmatis*, *M. goodii*, and *M. wolinskyi*, confirmation with alternative loci, such as rpoB or hsp65, is typically recommended (Brown et al., 1999; Ringuet et al., 1999; Brown-Elliott and Wallace, 2002; Adékambi et al., 2003; Jeong et al., 2012; Brown-Elliott and Philley, 2017). However, given that these tests were not available in our examination, we favored *M. smegmatis* based on the high 16S identity along with rapid growth, colony characteristics, and a biochemical pattern consistent with the *M. smegmatis* group, while MALDI-TOF did not yield conflicting results (Balada-Llasat et al., 2013; Machado et al., 2015; Rodríguez-Temporal et al., 2022). This approach, based on phenotype plus a single gene, is practical and aligns with accepted workflows when multilocus sequencing is not available (Brown-Elliott and Wallace, 2002; Griffith et al., 2007).

An additional complicating factor is the potential for cross-reactivity in serological assays used for bovine tuberculosis and paratuberculosis surveillance. Intramammary *M. smegmatis* infection in Belgian cows yielded false-positive results for *Mycobacterium avium* subsp. *paratuberculosis* ELISA tests, raising concerns about interference in disease control programs (Supré, 2019). This diagnostic overlap reinforces the need for molecular confirmation of atypical mycobacteria, especially in regions with ongoing tuberculosis and paratuberculosis eradication efforts. Because exposures to RGM can yield false-positive ELISAs, paratuberculosis serology in affected herds should be interpreted cautiously and, where relevant, confirmed by alternative methods (Osterstock et al., 2007).

Antimicrobial susceptibility patterns of *M. smegmatis* have been insufficiently studied, but a recent study confirmed the bacterium's resistance to macrolides, particularly clarithromycin (Reil et al., 2023), as well as cefoxitin, while susceptibility is usually confined to aminoglycosides, fluoroquinolones, carbapenems, tetracyclines, linezolid, and trimethoprim-sulfamethoxazole (Cvetnić et al., 2022; Crowley et al., 2024). Earlier reports also emphasized therapeutic failure in mastitis cases due to *M. smegmatis*

(Richardson, 1970; Siqueira et al., 2016). The strain in this study followed a similar pattern. Although CLSI M24 does not provide breakpoints for rifampicin and azithromycin in RGM, the high MIC values observed are consistent with the intrinsic resistance of *M. smegmatis* and explain why these agents are not considered therapeutic options.

From an epidemiological perspective, contamination from the environment is considered a potential source of infection. Bedding material, water, and repeated intramammary treatments have been suggested as risk factors facilitating the introduction of RGM into the mammary gland (Siqueira et al., 2016; Ghielmetti et al., 2017). The opportunistic nature of *M. smegmatis* aligns with its ubiquity in soil and water, which has also been emphasized in recent reports (Cvetnić et al., 2022).

In settings where additional diagnostics are not feasible, we advise immediate herd-level hygiene as follows: milk the affected quarter last and discard that milk; dedicate clusters/liners to the affected animal or use a validated high-level disinfection between animals; reassess bedding and water sources for moisture and organic load that favor RGM; avoid non-sterile fluids during intramammary procedures and use single-use teat cannulas; and document all intramammary treatments to trace potential iatrogenic introductions (National Mastitis Council, 2016; Ghielmetti et al., 2017; Honda et al., 2018). The zoonotic risk from *M. smegmatis* is generally low and standard BSL-2 practices are appropriate for laboratory work (PHAC, 2011).

Taken together, this case adds to the limited evidence on atypical mycobacteria as causative agents of bovine mastitis. The lack of response to conventional therapy, the need for molecular tools to achieve a definitive diagnosis, and the resistance profile observed are in line with earlier descriptions and highlight the importance of vigilance. Because therapeutic options are often restricted and outcomes unsatisfactory, timely recognition of RGM remains essential to avoid ineffective treatments, reduce diagnostic errors, and improve mastitis control strategies.

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Authors' contributions

Conceptualization: I.P., D.K., A.R. Clinical case investigation and sample collection: O.S. Laboratory analyses: I.P., A.R., K.A. MALDI-TOF MS analysis and interpretation: D.M. Molecular analyses: I.P., A.R., V.G., O.S. Writing-original draft preparation: I.P. Writing-review and editing: A.R., D.K., O.S. Project supervision and administration: D.K. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethical statement

Informed consent was obtained from the owner of the animal prior to sample collection and publication of this case report. All diagnostic and therapeutic procedures were performed as part of routine veterinary practice and in accordance with national and institutional guidelines for animal welfare. No experimental procedures were conducted.

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MOLEKULARNA IDENTIFIKACIJA I ANTIMIKROBNA OSETLJIVOST SOJA *MYCOLICIBACTERIUM SMEGMATIS* IZOLOVANOG IZ KRAVE SA MASTITISOM: PRIKAZ SLUČAJA

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Kratak sadržaj

Mastitisi kod krava predstavljaju značajan zdravstveni i ekonomski problem u industriji mleka, a najčešće su izazvani uobičajenim bakterijskim patogenima. Međutim, u etiologiju mastitisa povremeno mogu biti uključeni i atipični mikroorganizmi, kao što su netuberkulozne mikobakterije (NTM). Među brzorastućim mikobakterijama, vrste *Mycolicibacterium smegmatis* se obično smatra saprofitom iz okoline, iako su sporadično opisani slučajevi mastitisa izazvanim ovim uzročnikom. U ovom radu prikazan je redak slučaj mastitisa kod odrasle simentalske krave. Životinja nije ispoljavala znake sistemskog oboljenja i nije reagovala na intramamarnu antibiotsku terapiju. Mikroskopskim pregledom izraslih kolonija otkriveni su acidoalkohorezistentni bacili sa karakteristikama koje odgovaraju brzo rastućim mikobakterijama, ali MALDI-TOF MS nije omogućio pouzdanu identifikaciju uzročnika. Konačna potvrda je dobijena sekvenciranjem *16S rRNA* gena, koje je pokazalo 99,73% sličnosti sa *M. smegmatis*. Ispitivanjem osetljivosti na antimikrobne lekove dokazana je rezistencija na cefoksitin, klaritromicin i trimetoprim–sulfametoksazol, dok je izolat pokazao osetljivost na aminoglikozide, fluorohinolone, tetracikline, karbapeneme i linezolid. Rifampicin i azitromicin su imali visoke vrednosti minimalne inhibitorne koncentracije, što je u skladu sa urođenom rezistencijom ove vrste na date antibiotike. Ovaj slučaj opisuje poteškoće u dijagnostici atipičnih mikobakterija, posebno u pogledu ograničenja konvencionalnih metoda i

značaj primene molekularnih metoda u dijagnostici retkih uzročnika mastitisa. Takođe, ovaj slučaj naglašava potrebu da se netipične mikobakterije uzmu u obzir u slučajevima mastitisa koji ne odgovara na konvencionalnu antimikrobnu terapiju i doprinosi skromnom broju podataka koji dokumentuju njihovu ulogu u patologiji goveda.

Ključne reči: mastitisi krava, *Mycolicibacterium smegmatis*, netuberkulozne mikobakterije, brzorastuće mikobakterije, 16S rRNA sekvenciranje