

# Bovine mastitis caused by rapid-growth environmental mycobacteria



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## Abstract

Rapid-growth mycobacteria were isolated from two cases of cow mastitis with similar clinical appearance and within a narrow time frame. Mycobacteria were isolated on blood esculine agar. The isolated mycobacteria were Gram stained, Ziehl-Nielsen stained and tested for growth at 25°C, 37°C and 42°C, iron uptake, growth on Löwenstein-Jensen (LJ) agar with and without 5% NaCl, arylsulphatase (3 days), tween 80 hydrolysis, tellurite reduction, nitrate reductase and niacin synthesis. Molecular identification was performed using the Mycobacteria GenoType CM and AS tests (Hain Diagnostika, Nehren, Germany). One isolate was additionally sequenced for the *hsp65*, *rpoB*, 16S rRNA gene sequence and *transcribed spacer sequence* (ITS) DNA. Susceptibility testing of isolates was performed on the Sensititre Rapmycol plate (TREK Diagnostic Systems Ltd.) for trimethoprim/sulfamethoxazole, linezolid, ciprofloxacin, imipenem, moxifloxacin, cefepime, ceftazidime, amoxicillin/clavulanic acid, amikacin, ceftazidime, doxycycline, minocycline, tigecycline, tobramycin and clarithromycin. Gram-positive acid-resistant

rods were observed in stained smears. Both strains grew at 25°C, 37°C and 42°C on LJ medium, and on LJ medium containing 5% NaCl. The conventional biochemical tests for iron uptake, arylsulphatase (3 days), Tween 80 hydrolysis, tellurite reduction and nitrate reductase were positive, while the niacin test was negative. Both isolates were identified by the GenoType Mycobacterium CM as *Mycobacterium fortuitum* II/ *Mycobacterium mageritense*, while application of the GenoType Mycobacterium AS kit identified both isolates as belonging to the species *Mycobacterium smegmatis*. Analysis of the isolate sequences (strain DS) for 16S ribosomal RNA confirmed a 100% identical result with *Mycobacterium smegmatis* strain INHR2. According to the CLSI criteria, both strains were sensitive to sulfamethoxazole/trimethoprim, linezolid, doxycycline, amikacin and tobramycin. The strains differed in their sensitivity to ceftazidime, and both strains were resistant to clarithromycin. There was a strong difference between the isolates in sensitivity toward ceftazidime and tigecycline.

**Key words:** *mycobacterium*; mastitis; cow

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## Introduction

Mastitis in dairy cows is considered a cause of greatest losses in dairy production, and it most commonly is caused by bacteria (Cvetnić et al., 2016; Turk et al., 2017; Benić et al., 2018; Mimoune et al., 2021; Lamari et al., 2021). The contemporary literature lists over 135 different causative agents of mastitis (Watts, 1988). Some udder pathogens are important as potential causes of infections in humans or food-borne infections and intoxications (Juhász-Kaszanyitzky et al., 2007; Scallan et al., 2011; Burović, 2020; Saidi et al., 2021; Cvetnić et al., 2021; Knežević et al., 2021).

Mycobacteria are gram-positive aerobic bacteria belonging to the family *Mycobacteriaceae*. In terms of the time needed for growth on nutritive agar, they are differentiated into slow and rapid-growth species. Slow-growth species include the members of the *M. tuberculosis* complex and other slow growing species. Colonies of slow-growth species on solid agar appear after seven days or longer. Rapid-growth species appear on agar in less than seven days (Han et al., 2007). According to the Runyon classification, rapid-growth species are included in category 4. They are often isolated in microbiology laboratories, and are associated with numerous infections in humans (Esteban et al., 2007). The clinically most significant, unpigmented, rapid-growth mycobacteria are divided into three groups: *M. fortuitum* group, *M. chelonae/abscessus* group and *M. smegmatis* group (Brown-Elliott and Wallace, 2002).

To date, the *Mycobacterium fortuitum* group has been isolated from water, water supply systems and various types of soil (Franco et al., 2013). This group includes three taxa: *M. fortuitum*, *M. peregrinum* and one unnamed taxon. It is most often a member of the non-tuberculosis mycobacteria isolated from the mammary glands of dairy cows. Until the 1970s, it was considered a saprophyte; however,

its pathogenicity for humans has since been demonstrated many times (Hand and Sanford, 1970; Dreisin et al., 1976; Nolan et al., 1991; Rotman et al., 1993). The *M. fortuitum* group causes localised post-traumatic infections, infections from catheter use, and infections of surgical wounds, particularly following mammoplastic and heart operations (Brown-Elliott and Wallace, 2002). *M. mageritense* is included in an unnamed complex that includes several other species (*M. houstonense*, *M. septicum* and *M. bonickei*). All have been associated with human disease. The species was first described in 1997 (Domenech et al., 1997) when it was isolated from human sputum. It is assessed that 75% of infections caused by this complex are associated with infections of the skin, soft tissues or bone (Brown-Elliott and Wallace, 2002).

The *M. chelonae/abscessus* complex includes a number of species, including *M. chelonae*, *M. abscessus* and *M. immunogenum*. Infections in humans manifest as chronic lung disorders, localised cellulitis, osteomyelitis and arthritis (Lee et al., 2015).

The *M. smegmatis* group includes multiple members. This group differs from the *M. fortuitum* and *M. chelonae/abscessus* groups due to the lack of sensitivity to new macrolids. It was first isolated and described in 1885. Until recently, members of the group were considered saprophytes without any clinical significance. The first described case in humans caused by a member of this group was reported in 1986, when it was isolated from a patient with lung disease. Following this, this group was associated with cases of cellulitis, localised abscesses and osteomyelitis following traumatic injuries. Additionally, sporadic cases of complications have been reported following surgical procedures, such as catheterisation, pacemaker implants and

plastic surgery procedures (Wallace et al., 1988, 1989; Xu et al., 2013).

The aim of this research was to identify strains of mycobacteria isolated from two bovine mastitis cases and to examine their susceptibility to selected antimicrobials.

## Material and methods

Two samples of dairy gland secretions were delivered to the laboratory at one-month intervals. Samples originated from geographically remote farms without any mutual contact. In both cases, the medical history records coincided. Samples were taken from Simmental breed dairy cows, ages 6 and 7 years, at about 5 months gravidity. Cows were kept indoors and milked mechanically. The infected cows did not display any clinical signs. In both cows, the infected udder was predominantly harder than the remainder of the gland, with an increased somatic cell count in milk. The sample from the infected udder was delivered to the diagnostic laboratory for testing.

### Bacteriological testing

Milk samples were inoculated on esculin-blood agar and incubated at a temperature of 37°C. The growth of microbial cultures was examined at 24-hour intervals. Cultures were Gram-stained and Ziehl-Nielsen stained. Both isolates were typed biochemically and using molecular methods. The prescribed procedure of NTM species in Croatia is conducted using the determination of growth properties and biochemical properties of the strain (growth at 25, 37 and 42°C, ability to produce pigment, semi-quantitative catalase test, Tween 80 hydrolysis, arylsulfatase test (3 and 14 days), thermostable catalysis (pH 7, 68°C), pyrazine amidase (4 and 7 days), urease, nitrate reduction and colony morphology). Subcultivation was performed using the BACTEC

MGIT culture system (Becton Dickinson, Maryland, USA) and by inoculation on Löwenstein-Jensen agar in duplicate.

### Molecular identification

#### DNA isolation

Mycobacterial isolates were prepared on solid nutritive agar. Bacterial culture was suspended in 1 mL distilled water. Bacterial DNA was extracted by boiling at 100°C for 15 minutes. Samples were centrifuged at 13400 g during 2 minutes, and the obtained supernatant was used for further analysis.

#### Species identification

Identification was performed using the Mycobacteria GenoType CM and AS tests (Hain Diagnostika, Nehren, Germany). The system is intended for the identification of the *M. tuberculosis* complex and for the 40 most common NTM species cultivated from the species.

#### Geno-typing

The mycobacteria test was performed according to manufacturer's recommendations (Hain Germany). The procedure included replication of the PCR product, hybridisation of the PCR product and detection of bound PCR products. For more precise identification of the mycobacterial species for the strain DS (Dugo Selo), additional comparisons were made of the sequences *hsp65*, *rpoB*, 16S rRNA gene and *transcribed spacer sequence* (ITS) DNA, using gene fragments with the forward and reverse primers:

Tb11 (5'-ACC AAC GAT GGT GTG TCC-3') and

Tb12 (5'-CTT GTC GAA CCG CAT ACC CT-3' (Telenti et al., 1993; Adékambi and Drancourt, 2004)

Myc-F  
(5'-GGCAAGGTCACCCCGAAGGG-3')  
and

Myc-R  
(5'-AGCGGCTGCTGGGTGATCATC-3');  
16MycF (59-CGTGCTTAACA-

CATGCAAGTCCG-39) and 16MycR (59-GTGAGATTTACGAACA-ACGC-39) (Devulder et al., 2005), Ec16S.1390b (5'-TTG TAC ACA CCG CCC GTC A-3') and Mb23S.44n (5'-TCT CGA TGC CAA GGC ATC CAC C-3').

Sequences were analysed against public gene sequence repositories at the GenBank National Centre for Biotechnology Information. The sequence comparison with the reference strains from GenBank was performed using the algorithm BLAST.

### Antimicrobial Susceptibility testing

Susceptibility testing of isolates was made by Sensititre Rapmycoi plate (TREK Diagnostic Systems Ltd.), in accordance with the Sensititre protocol. The plate was dosed with antimicrobial agents at appropriate dilutions: trimethoprim/sulfamethoxazole 0.25/4.75-8/152, linezolid 1-32, ciprofloxacin 0.12-4, imipenem 2-64, moxifloxacin 0.25-8, cefepime 1-32, cefoxitin 4-128, amoxicillin/clavulanic acid 2/1-64/32, amikacin 1-64, ceftriaxone 4-64, doxycycline 0.12-16, minocycline 1-8, tigecycline 0.015-4, tobramycin 1-16, clarythromycin 0.06-16.

The mycobacterium culture was emulsified in demineralized water (Api Suspension Medium, 5 mL, bioMérieux SA) and adjusted to a 0.5 McFarland (Densimat, bioMérieux). A total of 50 µL of suspension was transferred into the cation tube of the adjusted Mueller-Hinton broth with TES (Thermo Scientific, Remel Inc.). Then 100 µL of suspension was transferred to each well. Plates were incubated aerobically at 30°C in a non-CO<sub>2</sub> incubator for 96 hours. *Staphylococcus aureus* ATCC 29213 was used for quality control. The result was read manually by visual reading of growth with a mirror viewer and interpreted according to CLSI M24-A2 (2).

## Results

### Bacteriological testing

The growth of pure cultures of bacterial colonies was observed after 72 hours of incubation on esculin-blood agar. The strains are designated as DS (Dugo Selo) and BJ (Bjelovar) based on the place of origin. In gram-stained culture smears, gram-positive rods were visible. Smears were stained with Ziehl-Nielsen stain, following which acid-resistant rods were observed.

### Biochemical properties

**Table 1.** Biochemical/culture properties of the isolates DS and BJ

Test/property	DS	BJ
Growth at 25°C	positive	positive
Growth at 37°C	positive	positive
Growth at 42°C	positive	positive
Iron uptake	positive	positive
Growing on LJ with 5% NaCl	positive	positive
Arylsulphatase (3 days)	positive	positive
Tween 80 hydrolysis	positive	positive
Tellurite reduction	positive	positive
Nitrate reductase	positive	positive
Niacin	negative	negative

The cultures grew at 25°C, 37°C and 42°C on LJ medium, and on LJ medium containing 5% NaCl. The conventional biochemical tests for iron uptake, arylsulphatase (3 days), Tween 80 hydrolysis, tellurite reduction and nitrate reductase were positive, while niacin was negative. (Table 1).

Both isolates (BJ and DS) were identified by the GenoType Mycobacterium CM as *Mycobacterium fortuitum* II/ *Mycobacterium mageritense*,

**Table 2.** CLSI interpretative criteria and observed MIC break-points of two strains

Agent	CLSI criteria			Observed	
	S	I	R	Strain BJ	Strain DS
Sulfametoxazole/trimethoprim	≤ 2/38	-	≥ 4/76	≥ 0.254/4.75	≥ 0.254/4.75
Linezolid	≤ 8	16	≥ 32	≥ 2	≥ 2
Ciprofloxacin	≤ 1	2	4	≥ 0.5	≥ 0.5
Imipenem	≤ 4	8-16	≥ 32	≥ 4	≥ 4
Moxifloxacin	≤ 1	2	≥ 4	≥ 0.25	≥ 0.25
Cefepime	-	-	-	r	r
Cefoxitim	≤ 16	32-64	128	≥ 32	≥ 16
Amoxicillin with clavulanic acid	-	-	-	≥ 32/16	≥ 32/16
Amikacin	≤ 16	32	≥ 64	≥ 1	≥ 1
Ceftriaxone	-	-	-	r	r
Doxycycline	≤ 1	2-4	≥ 8	≥ 0.12	≥ 0.12
Minocycline	-	-	-	≥ 1	≥ 1
Tigecycline	-	-	-	≥ 0.12	≥ 0.03
Tobramycin	≤ 2	4	≥ 8	≥ 1	≥ 1
Clarithromycin	≤ 2	4	≥ 8	r	≥ 8

and application of the GenoType Mycobacterium AS kit identified both isolates as belonging to the species *Mycobacterium smegmatis*.

Analysis of the isolate sequences (strain DS) for 16S ribosomal RNA confirmed a 100% identical result with *Mycobacterium smegmatis* strain INHR2, complete genome, Sequence ID: CP009496.1 and a high similarity of 99% for the ITS sequence with *Mycobacterium smegmatis* strain INHR2, complete genome, Sequence ID: CP009496.1, of the rpoB gene with *Mycobacterium smegmatis* genome strain NCTC8159, GenBank: LN831039.1 and of the hsp65 gene for *M. smegmatis* strain RGTB229 65 kDa heat shock protein (hsp65) gene, partial cds, Sequence ID: HM454229.1.

According to the CLSI criteria for the interpretation of sensitivity of rapid-growth mycobacteria, both

strains are sensitive to sulfametoxazole/trimethoprim, linezolid, doxycycline, amikacin and tobramycin. The strains differ in their sensitivity to cefoxitim. (Table 2).

## Discussion

The pathogenicity of *M. fortuitum* and *M. smegmatis* was proven following trial infections in fish (Talaat et al., 1999) and in cats (Brown et al., 1999). Infections in cats most often manifest as panniculitis, and arise from injuries during fights with other cats (Malik et al., 2000; Gunn-Moore et al., 2013).

Though literature reports associate mastitis in dairy cows with mycobacteria, there are few described cases and published reports. Richardson (1970) described multiple cases of mastitis in dairy cows caused by *M. smegmatis* and untypable mycobacteria, by associating

the infections with the application of medicine to the udder. Wetzstein and Greenfield (1992) described the appearance of mycobacterial mastitis in cows in British Columbia, Canada, and stated that of the 18 isolates, 17 were identified as *M. fortuitum*, and one as *M. chelonae*. Schultze and Brasso (1987) described and identified 20 isolates of mycobacteria isolated from the milk of cows with mastitis, and all isolates were identified as *M. smegmatis*. Thomson et al. (1988) described the appearance of acute clinical mastitis caused by *M. smegmatis* after antibiotic therapy of cows. Mycobacteria were also isolated from milk samples collected from collecting stations (Franco et al., 2013).

Both isolates of the mycobacteria described in this study originated from the mammary glands of dairy cows containing an increased number of somatic cells and displaying an altered consistency of the tissue in the affected udder quarter. Since the medical history of the infected cows did not list prior cases of mastitis, it is not possible to determine whether mycobacteria were the primary cause of the mastitis, or whether the infection arose following the improper application of medicine. The fact that one of the first signs of infection was a change in consistency of mammary gland tissue indicates that the primary cause of the mastitis could be mycobacteria.

The results of genetic identification of both isolates in this study were similar. The isolates were identified using the GenoType Mycobacteria CM test as *Mycobacterium fortuitum* II/ *Mycobacterium mageritense* and with the GenoType Mycobacteria AS kit as the species *M. smegmatis*. Since there is a possibility of overlap between individual species in the identification of species using the GenoType Mycobacteria test, and due to the description of new species in

recent years, gene fragments were also analysed to obtain precise identification. The results of testing on gene fragments indicated the highest overlap of the analysed isolates with the species *M. smegmatis*, which was further supported by the established insensitivity to macrolids (clarithromycin). The fundamental biochemical properties of the isolated mycobacteria are similar to the properties of rapid-growth mycobacteria described elsewhere (Nasr Esfahani et al., 2012).

There are very few reports in the literature on the sensitivity of mycobacteria isolated from animals. Infections caused by members of the *Mycobacterium tuberculosis* complex are notified to competent authorities and the affected animals are removed from further breeding. Infections by rapid-growth mycobacteria are not subject to mandatory notification, and mammary gland infections in cows caused by mycobacteria are generally not treated. Despite this, sensitivity data towards antimicrobial compounds isolated from cows are useful to allow for the better knowledge of bacterial resistance dynamics, and the possible role of mycobacteria in the transmission of resistance genes. The *M. smegmatis* and *M. fortuitum* third biovariant is generally insensitive to macrolide therapy, including clarithromycin which has a dominant role in antimicrobial therapy for RGM infections (Brown-Elliott and Wallace, 2002).

The isolated strains in this study were not completely identical with regard to sensitivity towards antimicrobial compounds. According to the CLSI criteria for the interpretation of sensitivity of rapid growing mycobacteria, the BJ strain is moderately sensitive to cefoxitim, while the DS strain is sensitive to this antibiotic. Both strains are resistant to clarithromycin, even though the MIC differs. The difference between the

isolates in the sensitivity to tigecycline was evident, at 0.03 µg/mL (BJ) and 0.12 µg/mL (DS).

## Conclusion

Sporadic infections of the mammary glands caused by mycobacteria are evidence of the need for ongoing monitoring of mammary gland health. Both species described in this study may cause infection in humans, but also represent an infection model for other pathogenic species. Despite the fact that infections caused by mycobacteria are not treated in animals, there is an obvious resistance towards certain antimicrobial compounds. Since antibiotics are used to treat infections caused by other bacterial species, mycobacteria likely come into contact with antimicrobial compounds by activating their own resistance mechanisms. In all cases of mastitis in cows with unsuccessful antimicrobial treatment in the medical history or negative bacteriological tests for the usual bacterial causative agents, *Mycobacteriae* should be suspected as the possible causative agent.

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## Mastitis u krava prouzročen brzorastućim mikobakterijama iz okoliša

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Brzo-rastuće bakterije iz roda *Mycobacterium* izdvojene se iz dva klinički slična slučaja mastitisa krava u relativno kratkom vremenu. Mikobakterije su izdvojene na krvnom agaru s dodatkom eskulina, obojene po Grammu i Ziehl-Nielsenu te im je provjerena sposobnost rasta pri 25 °C, 37 °C i 42 °C, korištenje željeza, sposobnost rasta na Löwenstein-Jensen agaru bez i s dodatkom 5 % NaCl, tvorba arilsulfataze, sposobnost hidrolize tween 80, redukcije telurita i tvorbe nitrat reduktaze i niacina. Molekularna identifikacija izolata provedena je korištenjem testova *Mycobacteria* GenoType CM i AS. Odsječci gena *hsp65*, *rpoB*, 16S rRNK te tzv. (engl. *transcribed spacer sequence* (ITS)) jednog izolata su sekvencirani. Osjetljivost izolata provjerili smo pomoću SENSITITRE RAPMYCOL ploča (TREK Diagnostic Systems Ltd.) prema trimetoprim/sulfametoksazolu, linezolidu, ciprofloksacinu, imipenemu, moksifloksacinu, cefepimu, cefoksitinu, amoksicilinu s klavulanskom kiselinom, amikacinu, ceftriaksonu, doksiciklinu, minociklinu, tigeciklinu, tobramicinu i

klaritromicinu. Gram-pozitivne acido-rezistentne štapičaste bakterije uočili smo u obojenim razmascima. Oba soja rasla su pri temperaturama 25 °C, 37 °C i 42 °C te na podlozi LJ s i bez dodatka 5 % NaCl. U oba soja utvrdili smo sposobnost korištenja željeza, tvorbe arilsulfataze, hidrolize Tween 80, redukcije telurita i tvorbe reduktaze nitrata. Sojevi nisu tvorili niacin. Oba izolata su testom GenoType *Mycobacterium* CM identificirana kao *Mycobacterium fortuitum* II/ *Mycobacterium mageritense* dok su GenoType *Mycobacterium* AS testom identificirana kao *Mycobacterium smegmatis*. Sekvencijskom analizom odsječka gena 16S ribosomske RNK soja DS i usporedbom s javno dostupnim izolatima, utvrdili smo potpunu podudarnost sa sojem *Mycobacterium smegmatis* soj INHR2. Prema CLSI kriterijima, oba izolata su osjetljiva prema sulfametoksazolu s trimetoprimom, linezolidu, doksiciklinu, amikacinu i tobramicinu, a rezistentna prema klaritromicinu. Razlike između sojeva očitovale su se u osjetljivosti prema cefoksitimu i tigeciklinu.

**Ključne riječi:** mikobakterije, mastitis, krava