Veterinarski Glasnik 2022, 76 (1), 85-90
UDC: 636.2.09:618.19-002
579.862.085.2
https://doi.org/10.2298/VETGL210929003S

Case Report

MUCOID BOVINE MASTITIS ISOLATES OF *STREPTOCOCCUS UBERIS* – DIFFICULTIES IN IDENTIFICATION

Oliver STEVANOVIĆ[1], Dubravka MILANOV[2], Milan ĐILAS[3], Nevenka ALEKSIĆ[4]*

[1]Dr Vaso Butozan Veterinary Institute of the Republic of Srpska, Banja Luka, Bosnia and Herzegovina
[2]Novi Sad Scientific Veterinary Institute, Novi Sad, Serbia
[3]Institute of Public Health of Vojvodina, Novi Sad, Serbia
[4]Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia

Received 29 September 2021; Accepted 30 December 2021
Published online: 17 February 2022

How to cite: Oliver Stevanović, Dubravka Milanov, Milan Đilas, Nevenka Aleksić. Mucoid bovine mastitis isolates of *Streptococcus uberis* – difficulties in identification. *Veterinarski Glasnik*, 2022. 76 (1): 85-90. https://doi.org/10.2298/VETGL210929003S

Abstract

Highly mucoid *Streptococcus uberis* were isolated on blood agar from five milk samples originating from two dairy cow farms. All the isolates were CAMP test and esculin hydrolysis negative. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) following prior extraction with 70% formic acid was used for identification to the species level. Given that it is plausible that such strains could be isolated in the future from dairy cows by laboratories in Serbia and the Balkans, we consider this case report to be a useful instruction for veterinary bacteriology laboratories.

Key words: cow, MALDI-TOF MS, mastitis, mucoid *Streptococcus uberis*

CASE PRESENTATION

Samples. In March 2021, five cow's milk samples originating from two farms in the Semberija region (The Republic of Srpska, Bosnia and Herzegovina) were submitted for analysis to the Laboratory for Infectious Disease Diagnostics in Bijeljina, at the Dr Vaso Butozan Veterinary Institute. All milk samples differed noticeably in color and consistency from normal milk. According to anamnestic data, the diseased animals

*Corresponding author – e-mail: nenaaleksic@vet.bg.ac.rs

suffered from chronic clinical recurrent mastitis, and their long-lasting intramammary and intramuscular treatment with ß-lactam antibiotics was unsuccessful.

Isolation and identification. The milk samples were inoculated onto Columbia blood agar base (CM0331, Oxoid, Basingstoke UK) supplemented with 5% sheep blood, MacConkey agar (CM0007, Oxoid, Basingstoke, UK) and Sabouraud dextrose agar (BK 025HA, Biokar Diagnostics). After 24 h of incubation at 37°C in aerobic conditions, smooth, partially merged, non-transparent and non-hemolytic colonies, with a diameter about 2 mm were detected on blood agar (Figures 1 A and B). MacConkey and Sabouraud dextrose agar remained without microbial growth. Subpassaging of isolates with an inoculation loop was difficult owing to the rubbery, tough and elastic consistency of the colonies. The inoculation with a smear loop to nutrient agar, to test for the presence of catalase and oxidase, was unsuccessful: the growth was not visible even after 48 h of incubation at 37°C. Thus, passaging was done through tryptone soya broth (TSB) and thioglycolate medium. After 24 h of incubation at 37°C, all isolates produced diffuse opacity in the TSB, and their growth in thioglycolate was noticeable in the zone of aerobiosis.

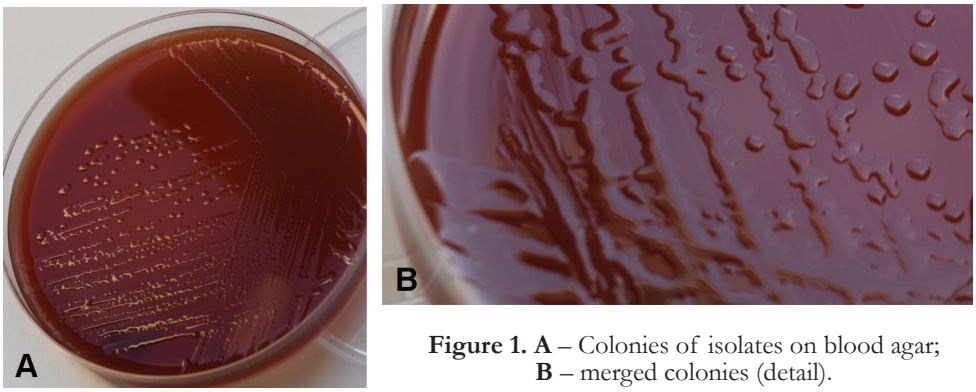

Figure 1. A – Colonies of isolates on blood agar; **B** – merged colonies (detail).

The culture obtained by bacterial multiplication in thioglycolate broth was spread onto nutrient agar and two blood agar plates that were simultaneously incubated at 37°C in aerobic and anaerobic conditions (GasPak EZ Becton Dickinson and Company, USA). Very poor growth of minute, dusty colonies was detected on nutrient agar after 48 h of incubation. On blood agar, the isolates grew well in both aerobic and anaerobic conditions. Those colonies were less mucoidal and merged to a lesser extent, but were as tough as in the prime culture. Catalase and oxidase tests were negative. On Gram-stained microscope slides, Gram-positive cocci, solitary and in short chains, were detected. All isolates were negative in the Christie-Atkins-Munch-Petersen (CAMP) test. Based on the mucoid colonies, microscopic appearance and negative catalase and CAMP test, *Streptococcus uberis* was suspected. Esculin broth was inoculated with colonies from the blood agar and the suspension of subcultures in TSB. In both cases, the reaction was negative.

Given that esculin hydrolysis is one of the fundamental criteria in the phenotypic characterization of bovine mastitis-causing *S. uberis* isolates, species identification was not possible this basis. Thus, the isolates were further processed at the Institute of Public Health of Vojvodina in Novi Sad, where they were identified with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Milanov et al., 2018).The isolates were prepared using the standard Bruker's direct transfer sample preparation procedure for MALDI-TOF MS. A single bacterial colony was spotted directly onto a MALDI target plate (Bruker Daltonics, Germany), allowed to dry and immediately overlaid with 1.0 µL of matrix solution (Bruker Matrix HCCA; α-cyano-4-hydroxycinnamic acid). Two isolates were subjected to MALDI TOF, but the identification failed: the identification score was 1.33, indicating unreliable recognition, and *Eggerthella lenta* was identified. As many isolates cannot be correctly identified directly and may need some type of extraction (van Horn et al., 2003), 70% formic acid was applied before MALDI TOF analysis, and *Streptococcus uberis* was identified (identification score 2.22, Figure 2).

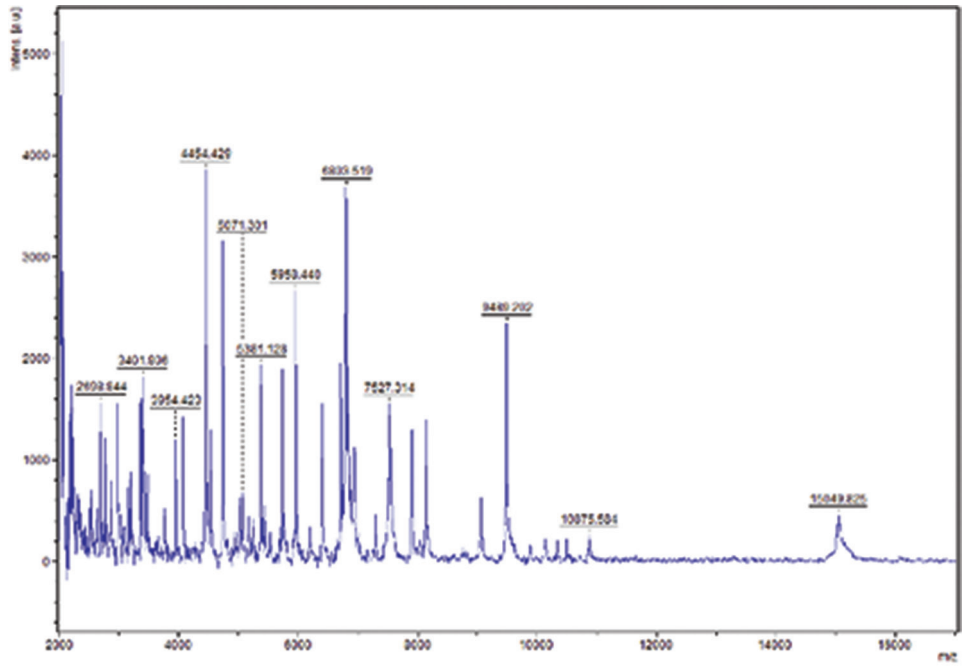

Figure 2. Spectrum of *Streptococcus uberis* isolate generated by MALDI-TOF Bruker flexControl software.

It was not possible to prepare a 0.5 McFarland-turbidity suspension in physiological saline to test the susceptibility of the isolates to antibiotics. For this reason, the isolates were left to grow overnight in Müller Hinton broth and transferred and streaked to plates containing Müller Hinton agar with and without ovine blood. The isolates' susceptibility was tested to the following antibiotics (Bio-Rad, France): penicillin (6 µg), ampicillin

(10 μg), amoxicillin/clavulanic acid (20 μg + 10 μg), oxacillin 1 (μg), ceftriaxone (30 μg), trimethoprim/sulfamethoxazole (1.25 μg + 23.75 μg), lincomycin (15 μg), enrofloxacin (5 μg), tylosin (30 μg), erythromycin (15 μg), spiramycin (100 μg), tetracycline (30 μg) and florfenicol (30 μg) (EUCAST, 2021). The isolates grew on Müller Hinton agar both with and without ovine blood. All isolates were resistant to oxacillin, and two to tetracycline.

COMMENT

S. uberis is one of the most prevalent species of mastitis-causing pathogens (Bradley, 2002; Günther et al., 2016). Owing to its basic phenotypic traits: morphology, type of hemolysis, catalase activity, CAMP reaction and esculin hydrolysis (Quin et al., 2013), there have not been any difficulties in *S. uberis* identification in laboratory practice.

Our isolates formed large, merged, mucoid colonies. Such highly mucoid colonies of *S. uberis* have not been detected in our laboratories so far. Therefore, we underline the fact that the mucoid appearance of the colonies of some *S. uberis* isolates may be indicative of the species identity (Fortin et al., 2003). Previously, two selected mucoid strains of *S. uberis* isolated from milk obtained from cows with mastitis needed 11 and 16 serial transfers to produce non-mucoid variants, respectively (Misra and Marshall, 1971).

Esculin hydrolysis is an important differentiating characteristic of *S. uberis*, especially to distinguish CAMP-positive isolates of *S. uberis* from *Streptococcus agalactiae*. All strains of *S. uberis* are described as capable of hydrolyzing esculin (Khan et al 2003; Raemy et al., 2013; Kromker et al., 2014), although some reports indicate that approximately 83-89% of *S. uberis* isolates are esculin-positive (Lämmler, 1991; Odierno et al., 2006). In this research, all *S. uberis* isolates were esculin negative, unlike the mucoid strains isolated from cow's milk that were previously described (Misra and Marshall, 1971).

Due to significant differences of *S. uberis* in this research from its well-known characteristic*s*, we consider this case to be of interest to routine work in microbiological laboratories. There are scarce literature data on the isolation of mucoid *S. uberis* strains (Misra and Marshall, 1971). Our findings confirm that conventional phenotypic tests may be insufficient for reliable identification of atypical *S. uberis* isolates (Fortin et al., 2003; Odierno et al., 2006), which prescribes additional tests for identification as inevitable, but these may not be available in every laboratory. For the identification of mucoid strains of *S. uberis* by the MALDI TOF technique, an extraction procedure using 70% formic acid was necessary. MALDI-TOF MS is a rapid, reliable technique, which has the potential to replace traditional phenotypic methods of bacterial identification.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Financial support agreements no.451-03-9/2021-14/200031 and 4510368/202014/200143).

Authors' contributions

OS and DM completed the classical microbiological examinations and wrote the draft of the manuscript, MĐ did the MALDI-TOFF MS, and NA edited and critically reviewed the work in Serbian and English. All the authors have read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests pertinent to the present work.

REFERENCES

Bradley A. J. 2002. Bovine mastitis: An evolving disease. The Veterinary Journal, 164: 116-128.

EUCAST, 2021. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0, 2021. http://www.eucast.org

Fortin M., Messier S., Julie Pare J., Higgins R. 2003. Identification of catalase-negative, non-beta-hemolytic, Gram-positive cocci isolated from milk samples. Journal of Clinical Microbiology, 41:106-109. DOI: 10.1128/JCM.41.1.106–109.

Günther J., Czabanska A., Bauer I., Leigh J. A., Otto Holst O., HansMartin Seyfert H-M. 2016. *Streptococcus uberis* strains isolated from the bovine mammary gland evade immune recognition by mammary epithelial cells, but not of macrophages. Veterinary Research 47:13. DOI 10.1186/s13567-015-0287-8.

Khan I. U., Hassan A. A., Abdulmawjood A., Lämmler C., Wolter W., Zschöck M. 2003. Identification and epidemiological characterization of *Streptococcus uberis* isolated from bovine mastitis using conventional and molecular methods. Journal of Veterinary Science, 4:213–223. PMID: 14685025.

Kromker V., Reinecke F., Paduch J. H., Grabowski N. 2014. Bovine *Streptococcus uberis* intramammary infections and mastitis. Clinical Microbiology, 3:157. DOI:10.4172/2327-5073.1000157.

Lämmler C. 1991. Biochemical and serological properties of *Streptococcus uberis*. ZentralblattfürVeterinärmedizinReihe B, 38:737-742.DOI: 10.1111/j.1439 0450.1991. tb00937.x.

Milanov, D. S., Đilas, M. D., Babić, J. M., Prunić, B. Z., & Velhner, M. J. 2018. MALDI-TOF MS identification of citrobacter youngae isolated from food. Food and Feed Research, 45(2), 107-112. https://doi.org/10.5937/FFR1802107M

Misra B. and Marshall R. T. 1971. *Streptococcus uberis* of bovine mastitis origin: isolation, characterization, and serology of two mucoid strains. Journal of Dairy Science, 55:194-199. DOI: 10.3168/jds.S0022-0302(72)85458-4

Odierno L., Calvinho L., Traverssa P., Lasagno M., Bogni C., Reinoso E. 2006. Conventional identification of *Streptococcus uberis* isolated from bovine mastitis in Argentinean dairy herds. Journal of Dairy Science, 89:3886-3890. DOI: 10.3168/jds.S0022-0302(06)72431-6. PMID: 16960064.

Raemy A., Meylan M., Casati S., Gaia V., Berchtold B., Boss R, Wyder A., Graber H.U. 2013. Phenotypic and genotypic identification of streptococci and related bacteria isolated

from bovine intramammary infections. Acta Veterinaria Scandinavica, 55:53. http://www.actavetscand.com/content/55/1/53

Quin P. J., Markey B. K., Leonard F. C., FitzPatrick E. S., Fanning S., Hartigan P. J. 2013. The streptococci and related cocci, Section 2: Bacteriology, In: *Veterinary Microbiology and Microbial Disease*, 2nd Edition, Wiley-Blackwell, Hoboken, NJ, pp. 127-136.

Van Horn K., Woodward Z., Fernandez J. MALDI-TOF Identification of bacteria using 70% Formic acid on-plate direct extraction. Approached on 30 August 2021 at https://www.msacl.org/2013_posters/201302061445_69718_poster_Van%20Horn.pdf

MUKOIDNI IZOLATI *STREPTOCOCCUS UBERIS* IZ MLEKA KRAVA SA MASTITISOM– POTEŠKOĆE U IDENTIFIKACIJI

Oliver STEVANOVIĆ, Dubravka MILANOV, Milan ĐILAS, Nevenka ALEKSIĆ

Kratak sadržaj

Iz pet uzoraka mleka poreklom sa dve farme muznih krava, na krvnom agaru izolovane su izrazito mukoidne, žilave kolonije bakterija. Kolonije nisu rasle u primokulturi i supkulturi (presejavanjem sa krvnog agara) na hranljivom agaru. Svi izolati su dali negativne reakcije u CAMP testu i testu hidrolize eksulina. Identifikacija vrste primenom MALDI-TOFF masene spektrometrije bila je neuspešna bez prethodne ekstrakcije koja je urađena 70% mravljom kiselinom. U višegodišnjoj praksi u identifikaciji uzročnika mastitisa mlečnih krava, prvi put smo izolovali tako izrazito mukoidne sojeve *Strepococcus uberis*, pri čemu je pouzdana fenotipska identifikacija vrste bila onemogućena zbog negativne reakcije hidrolize eskulina. S obzirom je moguće da se ovi sojevi i ubuduće ustanove kod mlečnih krava u Srbiji i na Balkanu, prikaz slučaja smatramo korisnom praktičnom smernicom u radu veterinarskih bakterioloških laboratorija.

Ključne reči: krava, MALDI-TOF MS, mastitis, pogrešna identifikacija, mukoidni *Streptococcus uberis*