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Agreement of conventional microbiological and molecular identification of streptococci isolated from bovine milk

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ABSTRACT

Pathogenic streptococci are implicated in clinical and subclinical mastitis. Most laboratories identify streptococci on the basis of microbiological examination, but molecular diagnostic methods have become the gold standard of mastitis diagnosis in the last few years. Therefore, this study aims to determine the agreement of microbiological and molecular identification of streptococci isolates from bovine milk. Milk samples were taken before the evening milking into sterile tubes. Samples were examined bacteriologically by inoculation on aesculin blood agar. Identification of grown colonies was carried out using internationally accepted methodology. Sequencing of the 16S rRNA gene was the reference method used to confirm *Streptococcus* sp. in all bacterial isolates. In the study, 54 strains of bacteria isolated from milk samples from the udder quarters of dairy cows with subclinical mastitis were examined using molecular methods. By conventional microbiological examination, the strains were identified to the species (*Strep. agalactiae, Strep. dysgalactiae and Strep. uberis*) or the genus level (*Streptococcus* spp.) without final identification of the species. On the basis of 16S rRNA analysis, 47 out of 54 examined streptococcal strains were found to belong to the genus *Streptococcus* sp. Among the streptococci identified, 6 isolates belonged to *Strep. agalactiae*, ¸8 isolates to *Strep. dysgalactiae*, 2 isolates to *Strep. canis* and 31 isolates belonged to *Strep. uberis*. Among the seven remaining isolates, three were identified as *Enterococcus faecalis* and four as *Lactococcus lactis*. Agreement between the identification procedures used was fair, with a Kappa index of 0.2181 (SE=0.0612; Z=3.56; p=0.0002).

Key words: streptococci; bovine mastitis; culture; molecular tools; 16S rRNA

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Introduction

Mastitis is a multifactorial disease that is usually the result of interaction between microorganisms, the host and the environment. Mastitis occurs frequently in dairy cows and causes significant economic losses, manifested in reduced production and low milk quality (MAĆEŠIĆ et al., 2016; BUROVIĆ, 2020). Mastitis is the inflammation of udder tissue that can be caused by various infectious agents. It is characterized by an increase in somatic cell count, and is usually accompanied by physical, chemical and/or microbial changes in the milk. Recent papers list about 150 known pathogens of mastitis, and this number is very likely to increase with the application of molecular identification methods (BENIĆ et al., 2018; SAIDI et al., 2021; SUKALIĆ et al., 2021). Mastitis pathogens are usually bacteria, but molds, yeasts and protothecae can also cause mastitis (EL-SAYED et al., 2017). An important group of bacteria associated with intramammary infections in cattle are *Streptococcus* spp. (*Strep.*) and other Gram-positive, catalase-negative cocci (HALASA et al., 2007; REAMY et al., 2013; BUROVIĆ, 2020). Pathogenic streptococci are implicated in subclinical and clinical mastitis, and are usually observed in high yielding dairy herds (WERNER et al., 2014; WALD et al., 2017). *Strep. uberis*, *Strep. dysgalactiae* and *Strep. agalactiae* are prominent pathogens involved in bovine mastitis. In the case of *Strep. uberis*, a high prevalence has been reported throughout the world (REAMY et al., 2013). Mastitis caused by *Strep. agalactiae* has been rarely observed in Croatia in recent decades, probably because it can be controlled by improved milking management, and it has good sensitivity to antibiotics (MAĆEŠIĆ et al., 2012; CVETNIĆ et al., 2016).

Good veterinary practice to prevent antimicrobial resistance requires identification of the pathogen causing mastitis to ensure the best choice of antibiotic (EL-SAYED et al., 2017). Despite many diagnostic methods available today, routine mastitis diagnosis is still mostly based on microbiological examination (MBE) of properly collected udder quarter samples (CVETNIĆ et al., 2016). However, classification of mastitis-causing pathogens into environmental and contagious pathogens has begun to be modified by recent data delivered by the application of modern molecular epidemiological tools, which has counteracted the old classification. The data show that within the same species, some isolates can be classified as contagious and others as environmental, some as extremely pathogenic, and others as less pathogenic, some causing severe clinical mastitis, and others mild subclinical mastitis. This in turn provides a huge bonus point for molecular genotyping-based assays compared to the conventional bacteriological tools (ZADOKS et al., 2011; MAHMMOD et al., 2015; EL-SAYED et al., 2017). Most milk hygiene laboratories identify streptococci on the basis of serological and biochemical tests (REINOSO et al. 2010; CVETNIĆ et al., 2016), but molecular diagnostic methods have become the gold standard for mastitis diagnosis in the last few years (EL-SAYED et al., 2017). This study aims to determine the agreement of microbiological and molecular identification of streptococci isolates from bovine milk.

Materials and methods

Sampling and Bacteriology. Samples for the study were taken before the evening milking. After washing and drying, the teat ends were disinfected with cotton swabs soaked in 70% ethanol. The first few streams were discarded. Approximately 10 mL of milk from each udder quarter was taken into sterile tubes. Samples were transported to the laboratory on ice and stored at 4 °C until laboratory examination, which was performed within 12 hours from sampling. MBE was carried out according to the method recommended by NMC (2017). Primary isolation was done by inoculating the samples on nutrient agar (Merck, Germany) containing 5% ovine blood and 0.1% aesculin, and incubating at 37 °C. The inoculated plates were checked at 24-hour intervals. The colonies grown were evaluated according to their appearance on the agar, checked for catalase and oxidase production, aesculin hydrolysis, haemolysis, stained according to Gramm (Merck, Germany), and further subcultured on differential or selective media. Colonies were subcultured on kanamycin

aesculin azid (KAA) agar to differentiate between streptococci and enterococci. Additionally, the Christie Atkins Munch-Petersen (CAMP) assay was performed to identify *Strept. agalactiae* among presumptive streptococci. After identification, the strains were stored as cryocultures in glycerol broth at a temperature of - 20 °C. The frozen strains were revitalized by repeated inoculations on aesculin blood agar. During storage, the vitality of the strains was examined by a random selection procedure.

Molecular methods. DNA extraction for further analysis was performed using the commercial kit QIAcube DNA Mini Kit (QIAGEN, Hilden, Germany) and an automated instrument. Sequencing of the 16S rRNA gene was the reference method to confirm *Streptococcus* sp. in all bacterial isolates. We performed DNA extraction and amplified fragments of bacterial 16S rRNA genes by PCR with primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) according to EDWARDS et al. (1989). The cycling conditions for 16S rRNA gene amplification were as follows: 95°C for 15 min, followed by 30 cycles 94°C for 30 s, annealing at 59°C for 60 s, elongation at 72°C for 60 s, a final elongation at 72°C for 10 minutes, and the reaction was completed at 4°C. PCR products were analysed using QIAxcel capillary electrophoresis (QIAGEN, Hilden, Germany) with fragment sizes ranging in size from 100 to 2500 bp. The amplification products obtained were sequenced by the Sanger method in Macrogen Europe (Amsterdam, Netherlands). The sequences obtained were compared with the sequences in the internationally available databases NCBI (National Centre for Biotechnology Information) and RDP (Ribosomal database Project), using BioNumeric 7.6.3 software.

Statistical Analysis. The results obtained with different methods were compared with the Kappa test using the Stata 13.1 (Stat Corp., USA) computer program.

Results

In the study, 54 strains of bacteria isolated from milk samples from the udder quarters of dairy cows with subclinical mastitis were examined using molecular methods. By conventional microbiological examination, the strains were identified to the species (*Strep. agalactiae, Strep. dysgalactiae i Strep. uberis*) or to the genus level (*Streptococcus* spp.) without final identification of the species. On the basis of 16S rRNA analysis, 47 out of the 54 examined streptococcal strains belonged to the genus *Streptococcus sp.* Among the streptococci identified, six isolates belonged to *Strep. agalactiae*, eight isolates to *Strep. dysgalactiae*, two isolates to *Strep. Canis,* and 31 isolates belonged to *Strep. uberis*. Among the seven remaining isolates, three were identified as *Enterococcus faecalis* and four as *Lactococcus lactis*.

Table 1. Distribution of pathogens based on culture and molecular confirmation of the species identity of isolates

Microbiology	Molecular probes						
	Enterococcus faecalis	*Lactococcus lactis*	*Streptococcus agalactiae*	*Streptococcus canis*	*Streptococcus dysgalactiae*	*Streptococcus uberis*	Total
Streptococcus agalactiae	0	0	2	0	0	0	2
Streptococcus dysgalactiae	0	0	4	2	4	1	11
Streptococcus spp.	2	2	0	0	1	12	17
Streptococcus uberis	1	2	0	0	3	18	24
Total	3	4	6	2	8	31	54

Agreement between the identification procedures used was fair, with a Kappa index of 0.2181 (SE=0.0612; Z=3.56; p=0.0002).

Discussion

In most diagnostic laboratories, several biochemical tests are used in routine diagnostics to identify bacteria. This approach is time-consuming, and can often lead to unsatisfactory results despite complex identification procedures, such as those used for streptococcal identification. Alternatives to these methods include miniature biochemical assays, such as API systems, more complex systems, such as the Vitek system, Matrix-Assisted Laser Desorption / Ionization Time from Flight Mass Spectrometry (MALDI-TOF MS) based on mass spectrometry, and more recently the analysis of genetic determinants, such as the detection of species-specific genetic markers, such as 16S rRNA (MCDONALD et al., 2005).

All the isolates used in our study were gram-positive cocci, catalase-negative, with morphology characteristic of streptococci. Their ability to hydrolyse aesculin was tested, as well as the presence of the CAMP factor. The ability to grow on a selective medium of KAA agar was tested, with the addition of bile salts to distinguish them from enterococci, and no isolate grew after incubation for 72 hours at 37 °C.

Of the 54 bacterial strains that we selected for study, 47 (87%) were shown to belong to the genus *Streptococcus*. Of the seven remaining strains, three belonged to the species *Enterococcus faecalis* and four to the species *Lactococcus lactis*. The highest degree of concordance of results was observed for the species *Strep. uberis*. Of the 24 strains identified microbiologically, 18 (75%) were confirmed by genetic methods. The remaining six were identified as *Strep. dysgalactiae* (3), *Lactococcus lactis* (2) and *Enterococcus faecalis* (1). Using MBE, genus identity was confirmed for 17 *Streptococcus* spp. isolates. Of 17 isolates identified as *Streptococcus* spp. on the basis of the culture, 12 (71%) were identified as *Strep. uberis, Enterococcus faecalis* (2), *Lactococcus lactis* (2) and as *Strep. dysgalactiae* (1) using molecular methods.

Our results are comparable to recently published results. WALD et al. (2017) obtained 336 isolates of *Strep. uberis* from quarter milk samples from dairy cows with intramammary infection, and classified the isolates into two clusters using biochemical characterization. The first cluster contained isolates with identical biochemical reactions to the reference strain (*Strep. uberis* ATTCC 700407), and the second cluster contained isolates derived from the reference strain in one trait or more. In the first cluster, identification of *Strep. uberis* by conventional biochemical methods achieved 96% accuracy, in the second cluster 64%, while the combination of both clusters had 91% accuracy. WALD et al. (2017) concluded that, in contrast to cluster 1, they observed high bacterial biodiversity and lack of accuracy in cluster 2 using conventional tests. For *Strep. uberis* isolates with divergent patterns, better accuracy can be achieved with simple additional tests. An inherent weakness of conventional microbiological methods seems to be that the expression of traits varies, depending on the *Strep. uberis* isolate (ODIERNO et al., 2006; KRÖMKER et al., 2014; DUARTE et al., 2015). The microbiological identification procedure may be misleading regarding the distinction between atypical *Strep. uberis* and other environmental streptococci species or lactic acid bacteria, especially *Lactococcus* spp. (FORTIN et al., 2003; WERNER et al., 2014).

The most prominent discrepancy in the results in our study was found in the species *Strep. agalactiae*. Two strains identified microbiologically were also confirmed by molecular analysis. However, four strains identified microbiologically as *Strep. dysgalactiae* were identified as *Strep. agalactiae* by molecular methods. For these four strains, the ability to form the CAMP phenomenon was not confirmed by microbiological tests. The CAMP test is used to identify the species *Strep. agalactiae,* and is highly specific for Lancefield group B, to which this species belongs. The test is based on the detection of a diffusible extracellular bacterial protein which, in synergy with a beta toxin of the species *Staphylococcus aureus*, causes complete lysis of sheep erythrocytes in the substrate.

The CAMP factor in *Strep. agalactiae* species encodes the cfb gene. Analogous genes are also present in other streptococcal species in which the CAMP phenomenon is expressed: cfu in *Strep. uberis* species (JIANG et al., 1996), cfa in *Strep.*

pyogenes (GASE et al., 1999) and cfg in *Strep. canis* (HASSAN et al., 2000), but they differ from each other by the cfb gene. Molecular tests confirmed the presence of the cfb gene in almost all members of the Lancefield group B streptococci (HASSAN et al., 2000; KE et al., 2000; ESTUNINGSIH et al., 2002). However, some members of Lancefield group B are phenotypically and, in some cases, genotypically CAMP negative (PODBIELSKI et al., 1994; VANDAMME et al., 1997; HASSAN et al., 2000; HASSAN et al., 2002). Concerning identification of *Strep. agalactiae* in routine tests, it was concluded that isolated strains of *Strep. dysgalactiae* should be subjected to additional biochemical tests or genetic analyses for more accurate identification. In addition, it would be advisable to analyse strains from the collection genetically that do not show the CAMP phenomenon, to see if they are carriers of the cfb gene. The microbial identification results from the same isolates collected from bovine milk samples were in high agreement to the genus and fair to the species level between the microbiological and molecular methods. The highest agreement was observed for the species *Strep. uberis* and the most prominent discrepancy in the results was found in the species *Strep. agalactiae*.

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SAŽETAK

Patogeni streptokoki povezuju se sa subkliničkim i kliničkim mastitisima. Većina laboratorija identifikaciju streptokoka temelji na mikrobiološkoj pretrazi, ali posljednjih godina molekularne metode postaju zlatni standard za dijagnostiku mastitisa. Cilj ovog istraživanja bio je odrediti podudarnost rezultata klasične mikrobiološke pretrage i identifikacije streptokoka molekularnim metodama. Uzorci mlijeka uzeti su u sterilne epruvete prije večernje mužnje. Mikrobiološka pretraga provedena je u skladu s opće prihvaćenim međunarodnim preporukama. Primarno izdvajanje i identifikacija streptokoka provedeni su korištenjem podloge eskulin krvni agar. Molekularna identifikacija vrste *Streptococcus* spp. učinjena je na osnovu 16S rRNA sekvencioniranja. U istraživanju su molekularnim tehnikama pretražena 54 soja bakterija izdvojena iz uzoraka mlijeka pojedinačnih četvrti vimena krava sa subkliničkim mastitisom. Klasičnom mikrobiološkom pretragom izdvojeni sojevi identificirani su do vrste (*S. agalactiae, S. dysgalactiae i S. uberis*) ili do roda (*Streptococcus* spp.) bez konačne identifikacije vrste. Metodom identifikacije na osnovi umnažanja gena 16S rRNA, od 54 pretražena bakterijska soja, za njih 47 je potvrđeno da pripadaju rodu *Streptococcus* spp. i to 6 *Streptococcus agalactiae*, 8 *Streptococcus dysgalactiae*, 2 *Streptococcus canis* i 31 *Streptococcus uberis* dok su 3 soja pripadnici vrste *Enterococcus faecalis*, a 4 soja pripadali su vrsti *Lactococcus lactis*. Podudarnost primijenjenih metoda za identifikaciju streptokoka je blaga, s Kappa indeksom od 0,2181 (SE=0,0612; Z=3,56; p=0,0002).

Ključne riječi: streptokoki; mastitis goveda; kultura; molekularne metode; 16S rRNA