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SHORT COMMUNICATION

Comparison of Three Methods for Routine Detection of *Staphylococcus aureus* Isolated from Bovine Mastitis

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Summary

The present study aimed to compare three identification methods that are routinely used for the detection of *Staphylococcus aureus* as bovine mastitis agent. The evaluated methods were as followed: conventional biochemical method, commercial identification system BioLog (Gen III MicroPlate) and amplification of species-specific gene (*nuc*) by polymerase chain reaction (PCR). A total of 73 staphylococcal isolates were collected from 453 individual milk samples from dairy cows with subclinical and clinical mastitis from different farms in Bulgaria. This isolates were determined as 60 coagulase-positive, 3 catalase-negative and 10 coagulase-negative by conventional methods. BioLog system identified 72 isolates as *S. aureus* subsp. *aureus* and one coagulase-positive isolate as *S. schleiferi* subsp. *coagulans*. PCR amplification of *nuc* gene further confirmed *S. aureus* subsp. *aureus* isolates identified by the BioLog system. The primary identification of *S. aureus* on the basis of coagulase level led to erroneous determination of 14 (19.2%) of the isolates. Based on the findings, BioLog system and PCR appear to be more reliable detection systems for *S. aureus* from milk. In conclusion, the present study showed that a routine approach using a combination of phenotypic and molecular detection systems could improve *S. aureus* detection in milk.

Keywords: *Staphylococcus aureus, Bovine mastitis, Identification, BioLog, nuc gene, PCR*

Bovin Mastitislerde *Staphylococcus aureus*'un Rutin Tespitinde Üç Metodun Karşılaştırılması

Özet

Bu çalışma Bovin mastitis etkeni olarak *Staphylococcus aureus*'un belirlenmesinde rutin olarak kullanılan üç tespit yöntemini karşılaştırmayı amaçlamaktadır. Denenen yöntemler şunlardır; konvensiyonel biyokimyasal metot, ticari tespit sistem BioLog (Gen III MicroPlate) ve polimeraz zincir reaksiyonu (PZR) ile amplifiye edilmiş tür spesifik gen (*nuc*). Bulgaristan'daki farklı çiftliklerde subklinik ve klinik mastitisli sütçü ineklerden alınan 453 adet örnekten toplam 73 staphilococal izolat elde edildi. Konvensiyonel metotlarla bu izolatların 60'ı koagulaz pozitif, 3'ü katalaz negatif ve 10'u koagulaz negatif olarak belirlendi. BioLog sistemi ile 72 izolat *S. aureus* subsp. *aureus* olarak tespit edilirken bir adet koagulaz pozitif izolat *S. schleiferi* subsp. *coagulans* olarak belirlendi. PZR ile *nuc* geninin amplifikasyonu kullanılarak BioLog sistemi ile *S. aureus* subsp. *aureus* olarak tespit edilen izolatlar teyit edildi. Koagulaz seviyesine dayanarak *S. aureus*'un primer identifikasyonu 14 (%19,2) hatalı tespite neden oldu. Bu bulgulara dayanarak BioLog sistem ve PZR, *S. aureus*'un sütten tespitinde daha güvenilir yöntemler olarak belirlendi. Sonuç olarak, bu çalışma ile rutin olarak fenotipik ve moleküler tespit yöntemlerinin beraber kullanılmasının sütten *S. aureus*'un tespitinde başarıyı artıracağı ortaya konuldu.

Anahtar sözcükler: *Staphylococcus aureus, Bovin mastitis, Tespit,, BioLog, nuc geni, PZR*

INTRODUCTION

Bovine mastitis is among the leading issues that cause serious economic losses in dairy industry throughout the world, resulting in reduced milk production, reduction of the quality of milk through contamination and due to

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treatment-associated hazards [1]. Of all mastitis causative agents, *S. aureus* is most commonly isolated [2], while the coagulase-negative staphylococci (CoNS) are also common but not considered as significant mastitis pathogens [3]. Therefore, the identification of the staphylococci by their ability to produce coagulase is deemed the main criterion for differentiation of those with pathogenic potential from others. On the other hand, some coagulase-positive staphylococci (CoPS) other than *S. aureus* such as *S. intermedius* and *S. hyicus* could cause intramammary infections in cows [4]. In that respect, the differentiation of the staphylococcal mastitis causative agents is important not only for the therapeutic approach and the remedial schemes, but also for the ability of some of them to produce a wide range of virulent factors [5].

Several conventional phenotypic and molecular methods have been used as routine approaches for the identification of staphylococci. The conventional culture methods require isolation of the bacteria on suitable media and subsequent identification with numerous biochemical tests. Commercial identification systems recognizing *S. aureus* are available such as BD Phoenix [6], BBL Crystal and Api Staph system [7] and Vitek2 [8]. Over the last few years, molecular techniques have become more popular and are currently used as detecting systems of mastitis pathogens in many diagnostic laboratories [9].

The present study aimed to compare three methods for routine identification of *S. aureus*, isolated from dairy cows, the first based upon the conventional identification and coagulase activity; the second using an identification system (BioLog) and the third - a molecular approach using PCR amplifying the species-specific gene, *nuc*, as a reference method.

MATERIAL and METHODS

Isolation and Primary Identification of S. aureus

A total of 453 individual milk samples were collected from cows with subclinical and clinical mastitis from different farms in Bulgaria. The indication for sampling from the subclinical mastitis animals was the positive result for increased somatic cell count after screening with Mastitis test NK (Biovet, Czech Republic). The milk sampling was done according to the guidelines of bacteriological examination. The samples (10 µL) were inoculated on trypticase soy agar (TSA, Fluka, India) supplemented with 5% defibrinated sheep blood (TSBA) and incubated at 37ºC for 24-48 h under aerobic conditions. The primary identification of staphylococcal isolates was made by Gram staining, catalase and oxidase activity, colony morphology and pigmentation, hemolysis, growth and mannitol fermentation on Chapman agar (NCIPD, Bulgaria). Further, the pathogenic potential of the isolates was tested by tube coagulase test with rabbit plasma (NCIPD, Bulgaria). A weak coagulase activity was recorded as positive reaction.

Identification of the Isolates with BioLog System

The suspicious for *S. aureus* isolates were checked with identification system BioLog Gen III microplates following precisely the manufacturer's instructions (Biolog, Hayward, USA). In brief, protocol A was used for identifying of *S. aureus*. The isolates were cultured on TSBA and the inocula were prepared in a special type of liquid medium, provided by the company until achieving the desired cell density determined by turbidimeter. The plates were filled with 100 mL of the readily prepared inocula and incubated at 33ºC for 24 h under aerobic conditions. After incubation, the plates were read by the computer system software OmniLog.

Extraction of DNA and PCR Analysis

Bacterial colonies were suspended in 100 µL bidistilled water and boiled for 10 min to extract DNA. After centrifugation at 12.000 g for 5 min at room temperature, the supernatant that contains the nucleic acids was used as DNA template for subsequent PCR analysis. DNA was also isolated by universal commercially available kit - prepGem (ZyGem, USA), according to the company's protocol. The concentration and purity of DNA extract was determined by DNA/RNA spectrophotometer Gene Quant 1300 at A260 and A280. The DNA extracts were then stored at - 20ºC until the beginning of the experiments.

The PCR methodology used in the study was described previously by Brakstad et al.[10] with an expected amplicon size of 270 bp. Positive and negative control strains were included for each PCR experiment.

RESULTS

From collected 453 milk samples, 73 staphylococcal strains were isolated. Gram-stained smears of the pure cultures exhibited clusters of Gram-positive cocci. Catalase test revealed three isolates with catalase-negative reaction. The hemolytic activity of isolates was different, variations were observed in the pigmentation of the colonies as well. Ten of the beta-hemolytic isolates gave negative results in coagulase tests in two independent trials. With exception of one coagulase-positive isolate, all others fermented mannitol of Chapman agar under aerobic conditions *(Table 1)*.

BioLog system determined catalase-negative and coagulase-negative isolates as *S. aureus* subsp. *aureus* with probability of 0.919 - 0.999. The typical coagulase-positive isolates were confirmed as *S. aureus* subsp. *aureus*. The coagulase-positive non-hemolytic isolate with white colonies and non-fermenting mannitol was identified as *S. schleiferi* subsp. *coagulans* with probability of 0.970. Three

Table 1. *Comparison of the methods for detection of S. aureus isolated from bovine mastitis*
Tablo 1. *Bovin mastitislerden S. aureus'un tespiti amacıyla kullanılan metotların karşılaştırılması*

Isolates n/%	Criteria for Primary Identification							BioLog ID	*nuc* gene
	Catalase /oxidase	Haemolysis			Pigment	Coagulase	Mannitol		
		Double	Beta	No					
3/4.1	-/-	+			creamy-white	+	+	*S. aureus*	+
10/13.7	+/-		+		greyish	-	+	*S. aureus*	+
32/43.8	+/-	+			yellow	+	+	*S. aureus*	+
6/8.2	+/-	+			greyish	+	+	*S. aureus*	+
14/19.2	+/-		+		greyish	+	+	*S. aureus*	+
7/9.6	+/-		+		yellow	+	+	*S. aureus*	+
1/1.4	+/-			-	white	+	-	*S. schleiferi coagulans*	-

Key: *+ = positive reaction; – = negative reaction*

independent trials were performed to confirm the species affiliation of the isolate.

The PCR protocol based on the *nuc* gene further confirmed *S. aureus* subsp. *aureus* isolates identified by BioLog system (*Table 1*). The two DNA isolation techniques were of similar quality as template for PCR.

DISCUSSION

The primary conventional identification of CoPS is very important and suggests about *S. aureus*, however, prior to testing with rabbit plasma some tests as Gram staining, catalase and oxidase activity that are particularly valuable for the discrimination of the agent from streptococci and micrococci have to be done as routine tests [11]. An interesting and unique finding in this study was the three isolates with catalase-negative reaction which was preserved throughout subsequent subcultivations. The isolates showed double hemolysis, which gave us the clue and motivated us further to continue with the identification process. The review of the literature revealed reports for catalase-negative *S. aureus,* but they referred to isolates of human origin [7,12-14]. Piau et al.[8] determined a catalase-negative *S. aureus* strain with point mutations in the *katA* gene as a possible mechanism for the loss of catalase activity. To the best of our knowledge, this is the first report of catalase-negative *S. aureus* from bovine origin in particular from cases of subclinical mastitis, determined by the primary routine identification.

According to Bannerman [15] the detection of free coagulase in the plasma tube coagulase test is the gold standard for *S. aureus* identification. In this regard, another interesting finding in this study was the isolation of 10 coagulase-negative strains from subclinical mastitis with a wide range of beta hemolysis. Worldwide reports of coagulase-negative variants of *S. aureus* in bovine mastitis are still rare [16]. Akineden et al.[4] determined two *S. aureus* coagulase-negative strains, that would be interpreted as CoNS naturally. The very weak beta hemolysin activity of the isolates motivated the authors to further identify the strains on a molecular level, that confirmed the species affiliation of the isolates as *S. aureus*.

An important criterion for the presumptive primary identification of *S.aureus* is also the hemolytic activity of isolates in the initial cultures. In our study 41 (56.2%) of isolates showed double hemolysis (alpha + beta hemolysins), 31 (42.5%) beta and 1 (1.4%) was non-hemolytic. The isolates with double hemolysis coagulated rabbit plasma in the test tube by the 4th h, a quick (less than 4 h) coagulation was also observed in some beta hemolytic isolates (8/31) as well as in the non-hemolytic and the others required overnight incubation. In our study we did not find *S. aureus* isolates without hemolytic activity.

The staphylococcal isolates were subjected to further analysis and identification by the BioLog system. The atypical catalase-negative (n=3) and coagulase-negative (n=10) isolates were determined as *S. aureus* subsp. *aureus* with a very high probability (91.9-99.9%). Vitek 2 (bioMérieux, France) Gram-positive identification card identified the catalase-negative isolate as *S. aureus* with probability of 93 % [8]. To et al.[17] compared two identification systems for the identification of a catalase-negative *S. aureus* strain from a patient with endocarditis and pericarditis of the mitral valve. While Becton Dickinson Phoenix PID panel identified the strain as *S. aureus*, Vitek system Gram-positive identification test could not identify the bacterium. Our coagulase-positive isolate without hemolytic activity and mannitol fermenting ability was identified as *S. schleiferi* subsp. *coagulans*. *S. schleiferi* subsp. *coagulans* is considered pathogenic mainly in dogs as causative agent of otitis externa [11]. In our case the bacterium was isolated from clinical mastitis in pure culture. Working with BioLog Gen III does not require any preliminary tests, even Gram staining, which is important in selection of

other identification systems. BioLog system recognizes the biochemical profile of approximately 2500 bacterial species, including many important veterinary pathogens and those responsible for mastitis. In that respect, we do not agree with Ahmadi et al.[9] who affirmed that most commercial identification systems were not designed to determine the important veterinary pathogens.

S. aureus isolates identified with BioLog were confirmed by PCR amplification of the fragment of agent's species-specific *nuc* gene. The results of our research showed an excellent correlation between the BioLog system and PCR detection of *S. aureus* in bacterial colonies from bovine mastitis. A protocol for the identification of *S. aureus* in milk by PCR was proposed by Ahmadi et al.[9]. Real-time PCR-based commercial reagent kit is now available for investigation of bovine mastitis pathogens without conventional culturing [18]. Although the identification system BioLog and the PCR protocol for detection of *S. aureus* require a culture step, they proved to be very reliable methods, especially in the developing countries, where advanced molecular techniques are not yet applicable.

The comparison of the methods for routine detection of *S. aureus* showed that the conventional identification on coagulase activity level alone would result in false determination of 14/73 (19.2%) *S. aureus* isolates - 3 catalase-negative and 10 coagulase-negative, and also of one coagulase-positive identified as *S. schleiferi* subsp. *coagulans*. Special attention is required when working with atypical *S. aureus* strains in udder health laboratories, where the identification systems and PCR based methods are not currently used as diagnostic approaches. In conclusion, this study has shown that a routine approach using a combination of phenotypic and molecular detection systems could improve *S. aureus* detection in milk.

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