Acta Scientiarum. Biological Sciences
ISSN: 1679-9283
eduem@uem.br
Universidade Estadual de Maringá
Brasil

Márcio da Costa, Geraldo; Vilela Paiva, Luciano; Hilsdorf Piccoli, Roberta; Junqueira Figueiredo, Demétrio; Pádua Pereira, Ulisses de; Silva, Nivaldo da
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Acta Scientiarum. Biological Sciences, vol. 32, núm. 4, 2010, pp. 403-406
Universidade Estadual de Maringá
.png, Brasil

DOI: 10.4025/actascibiolsci.v32i4.6276

Evaluation of a simplified key for the identification of coagulase-positive *Staphylococcus* isolated from bovine mastitis

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ABSTRACT. Evaluation of a simplified key for the identification of coagulase-positive *Staphylococcus* isolated from bovine mastitis. Three hundred fourty four strains of coagulase-positive *Staphylococcus* (CPS), isolated from mastitis cases, underwent phenotypic and genotypic tests to evaluate the efficiency of a simplified key, based on phenotypic tests for the discrimination of these microorganisms. The tests consisted of amplification of the *femA* gene and hemolysis in blood agar, production of acetoin and fermentation of maltose, mannitol and trehalose. Strains that showed negative results in the amplification test of the *femA* gene or that were not identified as *Staphylococcus aureus* (*S. aureus)* by phenotypic tests were tested with the APISTAPH kit (Biomériux-France), for precise identification of species. Phenotypic tests revealed 338 strains (98.25%) as *S. aureus*, three strains (0.86%) as *Staphylococcus hyicus*, and three microorganisms (0.86%) as *Staphylococcus intermedius.* PCR demonstrated that 338 (98.25%) strains belonged to the *S. aureus* species, confirming the results for 336 strains from 338 identified, through a simplified phenotypic key. A high rate of correlation (98.83%) was verifeid between the results of genotypic and phenotypic tests for the identification of *S. aureus*, demonstrating the applicability of the proposed key, for the discrimination of this microorganism in CPS isolated from bovine mastitis.

Key words: mastitis pathogens, characterization, coagulase test, PCR, biochemical tests.

RESUMO. Avaliação de uma chave de identificação simplificada para *Staphylococcus* coagulase-positivos isolados de mastite bovina. Visando testar a eficiência de uma chave simplificada baseada em testes fenotípicos para a discriminação de *Staphylococcus* coagulase positivos (SCP) isolados de infecções intramamárias de bovinos, 344 amostras destes microrganismos foram submetidas a testes fenotípicos e genotípicos. Estes consistiram na amplificação do gene *femA,* na observação de hemólise em ágar sangue, produção de acetoína e fermentação de maltose, manitol e trealose. Amostras que apresentaram resultado negativo na amplificação do gene *femA* ou que foram identificadas com não *Staphylococcus aureus* (*S. aureus)* por meio dos testes fenotípicos foram submetidas ao kit APISTAPH (Biomériux-França) para identificação mais precisa. Os testes fenotípicos utilizados na chave simplificada permitiram identificar 338 amostras (98,25%) como *S. aureus*, três amostras (0,86%) como *Staphylococcus hyicus* e três (0,86%) como *Staphylococcus intermedius*. Por meio da reação em cadeia da polimerase (PCR) 338 (98,25%) amostras foram identificadas como *S. aureus,* ratificando os resultados para 336 das 338 amostras identificadas por meio da chave fenotípica simplificada. Observou-se elevada concordância (98,83%) entre os resultados dos testes genotípicos e fenotípicos para a identificação de *S. aureus*, demonstrando a aplicabilidade da chave de identificação proposta para a discriminação deste microrganismo entre SCP isolados de casos de mastite bovina.

Palavras-chave: patógenos da mastite, caracterização, teste de coagulase, PCR, testes bioquímicos.

Introduction

The *Staphylococcus* genus is vastly diversified, containing 39 species (HOLT et al., 1994), and it usually requires the use of laborious and expensive procedures for its correct discrimination. Among the species of the genus, *S. aureus* is prominent as one of the species most frequently associated with bovine intramammary infections (IMI) in all continents, and it is known as the isolate species that causes the greatest losses in dairy farming worldwide (ANNEMÜLLER et al., 1999; VASUDEVAN et al., 2003).

Despite the fact that *S. aureus* is more relevant in the etiology of bovine mastitis in comparison to

other species of the genus (ROBERSON et al., 1996) and that its control requires the adoption of specific measures, the precise identification of this microorganism is not carried out by the majority of laboratories. This is due to the high costs of commercial kits used for the identification and the limitations associated to the use of molecular techniques, such as the high cost of equipment and supplies and the necessity of specialized labour. Therefore, the laboratories are only able to classify isolates into coagulase-positive (CPS) or coagulase-negative *Staphylococcus*, according to the results yielded by the tube coagulase test. In most veterinary laboratories, CPS isolates are empirically discriminated as *S. aureus.*

The CPS group includes *S. aureus*, *S. hyicus, S. intermedius, S. schleiferi* subsp. *schleiferi* and *S. delphini* (BES et al., 2000; KONEMAN et al., 2001), *Staphylococcus lutrae* (FOSTER et al., 1997) and *Staphylococus pseudointermedius* (DEVRIESE et al., 2005). *S. hyicus* and *S. intermedius* have been associated to IMI in bovines (BOTHA; BRAND, 1987; CAPURRO et al., 1999; ROBERSON et al., 1996); however, according to Roberson et al. (1996), about 95% of CPS samples that are isolated from IMI in bovines are represented by *S. aureus*.

Though *S. aureus* is the most frequently involved species in the etiology of bovine mastitis in comparison to other CPS, the non-discrimination of other species makes it difficult to measure the importance of each of them in the etiology of IMIs. In determined herds, such procedure may cause an over-dimensioning of the importance of *S. aureus*, or a sub-dimensioning of the importance of other CPS species. Therefore, the objective of this study is to test a simplified phenotypic identification key for the discrimination of CPS isolates in bovine mastitis cases.

Material and methods

For this study, 1,693 milk samples from bovines affected by mastitis were microbiologically analyzed. These samples came from 38 herds from the Minas Gerais State. The samples were aseptically collected into sterilised tubes, and then transported to the laboratory under refrigeration, being then incubated at 37°C/6-12 hours for enrichment. After this procedure, they were cultured on blood agar (Blood Agar Base, containing 10% of ovine blood) and incubated at 37°C/24-48 hours, after which they were evaluated for the presence of bacterial growth.

The sorting of microorganisms from the Staphylococcaceae family, belonging to the CPS group was performed through assumptive tests, according to Quinn et al. (1994). They were based on the macroscopic observation of colonies, verifying the presence of hemolysis, size and pigmentation; observation of microscopic morphology through Gram-stained smears, catalase test and tube coagulase test, employing leporine plasma.

Among the microorganisms identified as CPS, 344 were randomly and proportionally selected for the study within the herds. They were submitted to the simplified identification key, which consisted of hemolysis detection in ovine blood agar, trehalose, manitol and maltose fermentation, as well as acetoin production (VP), selected on the basis of the identification keys proposed by Holt et al. (1994). The interpretation of results was made according to Table 1.

Table 1. Simplified key for identification of coagulase positive *Staphylococcus*, adapted from Holt et al. (1994).

	S. aureus	*S. intermedius*	*S. hyicus*	*S. delphini*	*S. schleiferi* ssp. *coagulans*
Hemolysis	+	d	-	+	+
VP Test	+	-	-	-	+
Fermentation					
Mannitol	+	(d)	-	+	D
Maltose	+	(w)	-	nd	-
Trehalose	+	+	+	-	-

+ = > positive strains ≥ 90%; - = > negative strains ≥ 90% ; nd = > not determined; d = > 11-89% positive strains; w = > weak positive reaction; () delayed reaction; VP = > Voges Proskauer (acetoin production).

All the 344 samples selected for the study underwent PCR for the amplification of *femA*, aiming to identify *S. aureus* through its genotype. Samples with a negative result in this test or which were identified as non-*S. aureus* through the simplified identification key were tested by the APISTAPH kit (Biomériux-France), aiming a more precise identification.

The methodology described by Silva and Silva (2005) was used for the extraction of bacterial DNA, execution of the PCR reaction and electrophoresis. Primers obtained from specific *S. aureus* sequences and reported by Berger-Bachi et al. (1989) were used in the PCR. Fem1: AAA AAA GCA CAT AAC AAG CG and Fem2: GAT AAA GAA GAA ACC AGC AG. The amplification product of the *femA* gene must contain 132 base pairs.

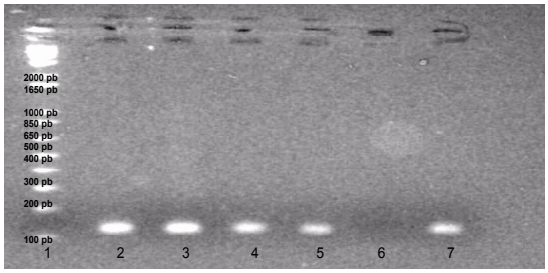
The results of phenotypic and genotypic identifications were compared by the Kappa (K) test, according to Siegel and Castellan (1988).

Results and discussion

Seven-hundred and ninety-six bacteria belonging to the Staphylococcaceae family were isolated, out of which 580 were identified as CPS. Among these, 344 samples were randomly and proportionally

chosen among the analyzed herds, and then submitted to phenotypic and genotypic tests, as previously described.

From the analyzed samples, the simplified phenotypic identification key allowed the identification of 338 samples (98.25%) as being *S. aureus*, three isolates (0.87%) as *S. hyicus*, and another three (0.87%) isolates as *S. intermedius*. As regards to the PCR, for the amplification of the *femA* gene (Figure 1), 338 samples (98.25%) showed a positive result, and the remaining six samples (1.75%), negative results.



1-DNA Ladder 1Kb plus, 2, 3, 4 and 5 *femA* positive strains; 6-negative control; 7- positive control (*Staphylococcus aureus* ATCC 25923). 2% agarose gel - 100V/60 min.

Figure 1. Amplification product of *Staphylococcus aureus femA* gene.

Among the 338 samples identified as *S. aureus* through the simplified key, 336 were identified through the PCR, resulting in a high correlation ($k = 0.98$; $p < 0.01$) between the results of phenotypic and genotypic tests for this species.

Included among the isolates with a negative PCR result (*femA*-negative), there were six samples that were previously identified by the simplified key as being *S hyicus* (three isolates), *S. intermedius* (one strain) and *S. aureus* (two isolates). These six PCR negative strains underwent identification using the APISTAPH kit, evidencing that the two isolates, previously and phenotypically, identified as *S. aureus* were indeed *S. aureus*, and the remaining ones were *S. hyicus*. From the three samples identified as *S. intermedius* by the phenotypic identification key, one was identified by PCR and by the APISTAPH kit as *S. hyicus* (PCR-negative for *femA* and APISTAPH-positive for *S. hyicus*), and the remaining ones as *S. aureus* (PCR-positive for *femA* and APISTAPH-positive for *S. aureus*). The three samples identified as *S. hyicus* by the simplified key showed a PCR-negative result, thus being confirmed as *S. hyicus* by the APISTAPH kit. Table 2 lists the relation of isolates whose identifications were discordants by use of phenotipic and genotypic tests and correct identification.

Table 2. Relation of *Staphylococcus* strains whose identifications were discordants by use of phenotipic and genotypic tests and correct identification by APISTAPH test.

Strains	Identification by Simplified key	Identification by Specific PCR (*fem*A)	Identification by APISTAPH
88	*S. intermedius*	*S. aureus*	*S. aureus*
118	*S. aureus*	non *S. aureus*	*S. aureus*
302	*S. intermedius*	*S. aureus*	*S. aureus*
325	*S. hyicus*	non *S. aureus*	*S. hyicus*
329	*S. hyicus*	non *S. aureus*	*S. hyicus*
345	*S. hyicus*	non *S. aureus*	*S. hyicus*
1195	*S. intermedius*	non *S. aureus*	*S. hyicus*
1340	*S. aureus*	non *S. aureus*	*S. aureus*

Though the three isolates identified as *S. hyicus* by the simplified key had their identification confirmed by the APISTAPH kit, the reduced number of isolates of this agent, and of *S. intermedius* in the studied sample did not allow a safe evaluation of the efficiency of the identification key proposed for the discrimination of these microorganisms.

We observed that *S. aureus* was the predominant species in the population studied; however, the epidemiological importance of different CPS species may vary among herds and different areas (BOTHA; BRAND, 1987; CAPURRO et al., 1999; ROBERSON et al., 1996), consequently requiring the judicious discrimination of them. Considering the importance of *S. aureus* in the etiology of bovine mastitis, and the potential risk represented to human health (ALTEKRUSE et al., 1998; BUYSER et al., 2001), its correct identification is indispensable to allow quantifying the relative importance in the epidemiology of IMIs in bovine herds, thus allowing, whenever necessary, the adoption or re-adaptation of specific control measures.

Conclusion

The results obtained evidenced the applicability of the identification key, proposed for the fast and accurate discrimination of *S. aureus* originated from bovine mastitis cases.

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Received on February 4, 2009.
Accepted on August 4, 2009.