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Verification of molecular characterization of coagulase positive *Staphylococcus* from bovine mastitis with matrix-assisted laser desorption ionization, time-offlight mass spectrometry (MALDI-TOF MS) mass spectrometry

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Besides *Staphylococcus aureus*, other coagulase-positive *Staphylococcus* (CPS) species such as *Staphylococcus hyicus* and *Staphylococcus intermedius* are implicated in bovine mastitis etiology. These species are often misdiagnosed as *S. aureus*. Also, some atypical *S. aureus* isolates can test negative for coagulase production and consequently be misdiagnosed as coagulase-negative *Staphylococcus* (CNS). Several currently available methods for the identification of *Staphylococcus* spp., including molecular techniques, are widely used worldwide. Recently, matrix-assisted laser desorption ionization, time-offlight mass spectrometry (MALDI-TOF MS) has been attracting attention for its fast and precise identification of several microorganisms at the species level. The present work evaluated the efficiency of a protocol for *S. aureus* characterization using PCR and M-PCR procedures. MALDI-TOF was considered the gold standard test to evaluate the sensitivity and specificity of the proposed identification protocol. Seventy-two *Staphylococcus* spp., isolates were evaluated. All samples were submitted to PCR for *coa*, *nuc* and 23S rDNA. Out of 33 isolates, genotypically characterized as *S. aureus* and confirmed by MALDI-TOF MS, 2 (6.1%) tested negative for coagulase production. Three isolates were identified as *S. hyicus* (2) and *S. intermedius* (1) by MALDI-TOF MS. The proposed molecular identification schedule achieved 100% sensitivity and specificity as compared to MALDI-TOF MS.

Key words: Bovine mastitis, coagulase-positive *Staphylococcus*, matrix-assisted laser desorption ionization, time-offlight mass spectrometry (MALDI-TOF MS), molecular identification.

INTRODUCTION

Bovine mastitis is an inflammatory disease usually caused by bacterial and mycotic pathogens (Capurro, 2009). It is recognized as a major disease affecting milk production and consequently dairy enterprises. Among the infectious

agents implicated in the etiology of mastitis, *Staphylococcus* spp. are usually the most frequent bacteria (Taponen and Pyörälä, 2009).

According to the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net/staphylococcus.html), the genus *Staphylococcus* comprises 49 species and 26 subspecies, separated into two distinct groups based on their ability to produce coagulase. The coagulase-negative *Staphylococcus* (CNS) was long regarded as non pathogenic species assembled in an undistinguishable group. Today, their importance in animal infections is becoming clear and there are several reports implicating CNS in bovine mastitis.

Eight coagulase-positive *Staphylococcus* species have been reported: *Staphylococcus aureus*, *Staphylococcus intermedius*, *Staphylococcus delphini*, *Staphylococcus pseudintermedius*, *Staphylococcus schleiferi* subsp. *coagulans*, *Staphylococcus hyicus*, *Staphylococcus lutrae* and *Staphylococcus agnetis* (Freney et al., 1999; Devriese et al., 2005; Sasaki et al., 2010; Taponen et al., 2012). *S. aureus* is the most frequent species isolated from bovine mastitis samples. *S. intermedius* and *S. hyicus* are rarely identified and the other CPS seems to be misidentified as *S. aureus* (Capurro, 2009).

The failures in the identification protocol are mostly related to phenotypic procedures, since distinguishing between species is a difficult task. The use of molecular markers has greatly improved species differentiation and allows the elucidation of the taxonomy of *Staphylococcus* spp., (Lange et al., 2011). Description of new species (Foster et al., 1997; Devriese et al., 2005) and reclassification of known ones have happened as a consequence of new methods and techniques (Sasaki et al., 2007; Blaiotta et al., 2010).

Molecular identification methods are keys to achieving phenotypic identification spaces as gene specific markers are being recognized. Nucleic acid-based detection approaches offer rapid and sensitive methods that are easily reproducible. Several identification schedules considering the amplification of *nuc*, *coa* and 23S rDNA genes have been previously reported for *S. aureus* (Hookey et al., 1998; Straub et al., 1999; Ciftci et al., 2009). Sasaki et al. (2010) developed a *multiplex* PCR (M-PCR) of *nuc* gene which encodes for thermonuclease in different *Staphylococcus* species.

Recently, matrix-assisted laser desorptionionization, time off light mass spectrometry (MALDI-TOF MS) has been attracting attention for its fast and precise identification of several microorganisms at the species level, even in mixed cultures (Bizzini and Greub, 2010; Bannoehr and Guardabassi, 2012). Mass spectrometry (MS) is a technique based on the analysis of ionized molecules in a gaseous phase. Decristophoris et al. (2011) reported high specificity (95%) and sensitivity (100%) in the identification of species of the SIG group, the *S. intermedius* reclassification proposed by Devriese et al. (2005), that comprises *S. intermedius,* the new species *S. pseudintermedius* and *S. delphini*. Böhme et al. (2012) also reported its use for *S. aureus* identification.

In the present study, we proposed a molecular schedule based on PCR amplification of the *nuc*, 23S rDNA and *coa* genes in coagulase-positive *Staphylococcus* isolated from dairy farms. The results obtained were compared with those yielded by MALDI-TOF MS, considered the gold standard technique due to its reliability and speed.

MATERIALS AND METHODS

Sampling

The 72 *Staphylococcus* spp. isolates evaluated in this study were obtained from samples of mastitic cow's milk and dairy workers' hands, obtained from dairy farms in the state of Rio de Janeiro, Brazil.

The samples were first inoculated on blood agar (blood agar base enriched with 5% sheep blood) and incubated at 35°C (± 2°C) for 24 h. Then, the isolates were submitted to routine microbiological diagnostics, including inoculation in selective medium for analysis of cultural properties and catalase and coagulase production. The coagulase-positive samples were evaluated for maltose and $_D$-mannitol fermentation, acetoin production and nitrate reduction (Winn et al., 2006). Coagulase-negative isolates were stored in 45% glycerol added to Brain Heart Infusion (BHI) broth for complementary analysis. To its identification, a modified scheme based on Cunha et al., (2004) was used, comprising the following tests: fermentation of the sugars xylose, arabinose, sucrose, trehalose, maltose, mannitol, lactose, xylitol, ribose, fructose and mannose; production of hemolysin; presence of urease; and resistance to novobiocin 5 mcg.

Molecular and proteomic analysis

After phenotypic identification, all strains including CNSs, were submitted to polymerase chain reaction for 16S rRNA to confirm the presence of *Staphylococcus* spp. (Zhang et al., 2004). PCR for *coa* (Hookey et al., 1998), *nuc* (Ciftci et al., 2009) and 23S rDNA (Straub et al., 1999) genes were performed to characterize *S. aureus* (Table 1). *S. aureus* standard strain ATCC29213 was used as control.

Multiplex PCR (M-PCR) for *nuc* gene was performed according to Sasaki et al. (2010) to characterize coagulase-positive *Staphylococcus* species (Table 1). Strains ATCC 29213 *S. aureus* and ATCC 29663 *S. intermedius* and two strains from UFRJ culture collection, the *S. hyicus* 5368 and *S. schleiferi* 3975 were used as quality controls.

Furthermore, all 72 isolates were evaluated by the MALDI-TOF MS. To perform this procedure, the samples were inoculated in BHI agar at 37°C for 24 h. Each culture was transferred to a microplate

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Table 1. Primers and cycles employed to identify *S. aureus* and other CPS species.

Gene (fragment)	Species	Primer Sequence (5'-3')	Cycling*
16S rRNA(756 pb)	*Staphylococcus* spp.	AAC TCT GTT ATT AGG GAA GAA CA CCA CCT TCC TCC GGT TTG TCA CC	1
23S *rDNA*(1250 pb)	*S. aureus*	ACG GAG TTA CAA AGG ACG AC AGC TCA GCC TTA ACG AGT AC	1
coa (Variável)	*S. aureus*	ATA GAG ATG CTG GTA CAG G GCT TCC GAT TGT TCG ATG C	2
nuc (279 pb)	*S. aureus*	GCG ATT GAT GGT GAT ACG GTT AGC CAA GCC TTG ACG AAC TAA AGC	3
nuc (359 pb)	*S. aureus*	TCG CTT GCT ATG ATT GTG G GCC AAT GTT CTA CCA TAG C	4
nuc (430 pb)	*S. intermedius*	CAT GTC ATA TTA TTG CGA ATG A AGG ACC ATC ACC ATT GAC ATA TTG AAA CC	4
nuc (526 pb)	*S. schleiferi* sub sp. *coagulans*	AAT GGC TAC AAT GAT AAT CAC TAA CAT ATC TGT CTT TCG GCG CG	4
nuc (661 pb)	*S. delphini* group A	TGA AGG CAT ATT GTA GAA CAA CGR TAC TTT TCG TTA GGT CG	4
nuc (1135 pb)	*S. delphini* group B	GGA AGR TTC GTT TTT CCT AGA C TAT GCG ATT CAA GAA CTG A	4
nuc (793 pb)	*S. hyicus*	CAT TAT ATG ATT TGA ACG TG GAA TCA ATA TCG TAA AGT TGC	4
nuc (926 pb)	*S. pseudintermedius*	TRG GCA GTA GGA TTC GTT AA CTT TTG TGC TYC MTT TTG G	4

*1. 94°C 5 min (94ºC 1 min, 55°C 1 min, 72°C 1 min) x 30 and 72ºC 10 min; 2. 94°C 4 min (94ºC 1 min, 60°C 1 min, 72°C 1 min) x 30 and 72ºC 5 min; 3. 94°C 5 min (94°C 45 s, 68°C 45 sec and 72°C 90 s) x 30 and 72°C 10 min; 4. 95°C 2 min (95°C 30 sec, 56°C 35 sec and 72°C 1 min) x 30 and 72°C 2 min.

(96 MSP, Bruker® - Billerica, USA). Each bacterial sediment was covered by a lysis solution (70% formic acid; Sigma-Aldrich®). Additionally, a 1-µL aliquot of matrix solution (alpha-ciano-4-hidroxi-cinamic acid diluted in 50% acetonitrile and 2.5% trifluoracetic acid, Sigma-Aldrich®) was added to each sediment. The spectra of each sample were generated in a mass spectrometer (MALDI-TOF LT Microflex, Bruker®) equipped with a 337 nm nitrogen laser in a linear path, controlled by the FlexControl 3.3 (Bruker®) program. The spectra were collected in a mass range between 2,000-20,000 m/s, and then were analyzed by the MALDI Biotyper 2.0 (Bruker®) program, using the standard configuration for bacteria identification, by which the spectrum of the sample is compared with the references in the database. The results vary on a 0-3 scale, where the highest value means a more precise match and reliable identification (Table 2). In this study, we accepted values for matching greater than or equal to 2.

The percentage of sensitivity, specificity and positive and negative predictive values for the employed molecular methods were measured considering MALDI-TOF MS proteomic analysis as the gold standard technique in this study.

RESULTS

Out of a total of 72 *Staphylococcus* spp. isolates evaluated in this study, 52.8% (38/72) tested negative for the phenotypic coagulase production test, so they were initially considered to be coagulase-negative *Staphylococcus*. Phenotypic identification of the 47.2% (34/72) of isolates that tested positive for coagulase production demonstrated that 79.4% (27/34) were *S. aureus*. Seven coagulase-positive isolates (20.6%) from the 34 could not be phenotypically identified.

PCR amplification of the 16S rRNA gene (756 pb) tested positive in all 72 isolates, corroborating the *Staphylococcus* spp., phenotypic identification. Additionally, PCRs for *coa*, nuc and 23S rDNA genes were carried out for all 72 isolates to characterize *S. aureus*. The decision to evaluate even the phenotypic coagulase-negative strains was due to the report of the detection of atypical coagulase-negative *S. aureus* strains misdiagnosed as CNSs (Akineden et al., 2011). The *coa* gene was detected in 41.7% (30/72) isolates, yielding variable size amplicons. Each *nuc* (279 pb) and 23S rDNA (1250 pb) gene was detected in 37.5% (27/72) of the isolates. Strains were characterized as *S. aureus* when positive for the amplification of at least one of these specific genes, consisting of 45.8% (33/72) of the samples. Interestingly, 6.1% (2/33) tested negative for phenotypic coagulase production. Also, none of the studied genes were detected in 4.2% (3/72) of the coagulase-positive isolates. These isolates were submitted to M-PCR for *nuc* genes of *S. intermedius*,

Table 2. Comparison of the phenotype, genotype and proteomic identification of 36 coagulase-positive *Staphylococcus* isolates analyzed by the MALDI-TOF MS technique and their respective scores.

Isolate	Phenotype identification	PCR genes *coa*, *nuc*, 23S rRNA	M-PCR genes *nuc*	MALDI-TOF MS (score)
1	*S. aureus*	*S. aureus*	*S. intermedius*	*S. aureus*(2.354)
2	CPS	*S. aureus*	*S. aureus*	*S. aureus*(2.258)
3	CPS	*S. aureus*	*S. aureus*	*S. aureus*(2.380)
4	CNS	*S. aureus*	ND	*S. aureus*(2.329)
5	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.403)
6	CNS	*S. aureus*	ND	*S. aureus*(2.317)
7	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.459)
8	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.367)
9	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.408)
10	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.381)
11	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.441)
12	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.424)
13	S. aureus	*S. aureus*	ND	*S. aureus*(2.443)
14	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.351)
15	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.405)
16	CPS	*S. aureus*	ND	*S. aureus*(2.419)
17	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.371)
18	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.418)
19	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.426)
20	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.450)
21	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.455)
22	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.410)
23	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.461)
24	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.379)
25	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.428)
26	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.410)
27	CPS	*S. aureus*	ND	*S. aureus*(2.477)
28	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.442)
29	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.451)
30	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.397)
31	CPS	*S. aureus*	ND	*S. aureus*(2.425)
32	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.422)
33	*S. aureus*	*S. aureus*	ND	*S. aureus*(2.424)
34	CPS	Negative (CPS)	Negative	*S. hyicus*(2.157)
35	CPS	Negative (CPS)	Negative	*S. hyicus*(2.116)
36	*S. aureus*	Negative (CPS)	Nonspecific fragment	*S. intermedius*(2.178)

*CPS: Coagulase-positive *Staphylococcus*; CNS: coagulase-negative *Staphylococcus*; ND: not determined.

S. pseudintermedius, *S. schleiferi* subsp. *coagulans*, *S. delphini* group A and B, *S. hyicus* and *S. aureus* (Sasaki et al., 2010). Out of these three CPSs isolates evaluated, just one presented an atypical amplicon bigger than 1000 pb. The other two isolates and the *S. hyicus* 5368 standard strain could not be identified by this technique. MALDI-TOF MS confirmed the 33 isolates previously identified as *S. aureus* (45.8%), even the strain misidentified as *S. intermedius* by the M-PCR assay. Three isolates, previously identified as CPSs, were identified by MALDI-TOF MS as *S. hyicus* (2) and *S. intermedius*. The M-PCR assay for the *nuc* gene was not able to distinguish these strains. All 36 isolates previously identified as CNSs (45.8%) were confirmed by the MALDI-TOF MS proteomic analysis. *S. chromogenes* and *S. sciuri* were the prevalent species. The genotypic identification schedule based simultaneously on the detection of coa, *nuc* and 23S rDNA genes and showed correspondence of 100% with the MALDI-TOF MS technique.

Table 3. Percentages of sensitivity, specificity, positive predictive value and negative predictive value found for the proposed identification of *S. aureus*.

Genes	Values (%)			
	Sensitivity	Specificity	PPV	NPV
coa, *nuc* e 23S rDNA	100	100	100	100
coa	90.9	100	100	92.8
nuc	81.8	100	100	86.7
23S rDNA	81.8	100	100	86.7

*PPV: Positive predictive value; NPV: negative predictive value.

DISCUSSION

The phenotypic differentiation of CPS species is a difficult task due to the absence of specific biochemical markers. To overcome this problem, the use of molecular tools has become routine in human and veterinary microbiology diagnosis. Nonetheless, genotypic assays are relatively expensive, time consuming and most important may provide results that are difficult to analyze.

To evaluate susceptibility patterns, it is necessary to establish a reliable identification procedure of CPS species involved in several infections of distinct hosts. Parameters such as oxacillin minimum inhibitory concentration, antimicrobial susceptibility, incubation time and inhibition zones are specific to different *Staphylococcus* species (Sasaki et al., 2010).

In the present study, MALDI-TOF MS proteomic analysis was carried out to evaluate the sensitivity, specificity and positive and negative predictive values of a molecular identification schedule for *S. aureus* based on the *coa*, *nuc* and 23S rDNA genes. It proved to be an efficient tool for distinguishing *Staphylococcus* species. Also, it has high potential for routine automated analysis, allowing the identification of isolates from clinical sources on a large scale (CLSI) (2013). Nevertheless, although it proved to be a fast and easy method with high specificity and sensitivity, the equipment is very expensive and requires skilled staff, so it is not suitable for small laboratories.

The proposed genotypic identification schedule based on the *coa*, *nuc* and 23S rDNA genes achieved 100% sensitivity and specificity as compared to MALDI-TOF MS, the gold standard tool in this study (Table 3). So, this proposed identification schedule is reliable to characterize *S. aureus*, even the atypical coagulase-negative strains, and can be used in small research laboratories.

Despite the fact that it was reported as a 99.8% sensitive and a 100% specific method, the M-PCR technique, established by Sasaki et al. (2010) was not able to distinguish among the other CPS strains. In fact, although several molecular approaches have been suggested for the proper identification of CPS, since phenotypic methods are time consuming and unreliable for animal samples, this is still a goal to be achieved.

Conflict of interest

The author(s) have not declared any conflict of interests.

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