

# Accuracy of PCR targeting different markers for *Staphylococcus aureus* identification: a comparative study using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as the gold standard

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**Abstract.** *Staphylococcus aureus* is considered a major pathogen in veterinary and human medicine, and the emergence of multidrug-resistant strains, such as livestock-associated methicillin-resistant *S. aureus*, means that reliable, inexpensive, and fast methods are required to identify *S. aureus* obtained from animal sources. We tested the accuracy of a PCR targeting the genes *femA*, *nuc*, and *coa* in identifying *S. aureus* from animals. A total of 157 *Staphylococcus* spp. isolates were examined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry; 18 different *Staphylococcus* species were identified. Of 68 *S. aureus* isolates, the genes *femA*, *nuc*, and *coa* were found in 61, 53, and 32 isolates, respectively. Considering MALDI-TOF as the gold standard, the PCR assays targeting all 3 genes showed 100% specificity; the sensitivity values were 89.7, 77.9, and 47.0% for *femA*, *nuc*, and *coa*, respectively. Sensitivity was 100% when *femA* and *nuc* markers were targeted simultaneously. These results confirm PCR as an accurate method to identify *S. aureus* species from animal sources and strongly suggest the simultaneous use of primers targeting *femA* and *nuc* genes.

**Key words:** *coa*; *femA*; MALDI-TOF; molecular identification; *nuc*.

*Staphylococcus* is a ubiquitous microbial genus comprised of >40 different species colonizing humans and animals.<sup>4</sup> Based on the capacity to produce the enzyme coagulase, these organisms are generally classified into 2 major groups: coagulase-positive (CoPS) and coagulase-negative (CoNS) staphylococci (<https://goo.gl/3nw55T>).

*Staphylococcus aureus* is a typical CoPS and has been frequently associated with nosocomial infections in humans.<sup>22</sup> Although reports have highlighted the increasing importance of CoNS species as opportunistic pathogens, *S. aureus* remains a major disease-causing agent in veterinary medicine.<sup>9</sup> Furthermore, the increasing role of animals as sources of methicillin-resistant *S. aureus* (MRSA), potentially pathogenic to humans,<sup>23</sup> reinforces the importance of accurately identifying *S. aureus*.

The isolation of *Staphylococcus* spp. normally does not represent a challenge for microbiologists, and the subsequent identification of *S. aureus* is traditionally performed by means of Gram staining, microscopy, and catalase and coagulase tests. Although this is a standard procedure in routine veterinary testing,<sup>18,24</sup> confirmatory tests are still needed to exclude CoPS strains other than *S. aureus*, especially in situations where species identification is required. In such cases, phenotypic tests such as carbohydrate fermentation patterns

are performed. However, strain variation related to the capacity to metabolize certain sugars has been identified as a cause of misidentification.<sup>24,26</sup> Moreover, the identification of *Staphylococcus* isolates using fermentation-based reactions is expensive, laborious, and time-consuming.<sup>26</sup>

Among the many microbial identification systems available, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has been accepted as a reference identification system in clinical microbiology that provides accurate and very rapid results at a low cost per sample.<sup>3</sup> However, the high cost of the equipment<sup>12,21</sup> has

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**Table 1.** Primers and cycling conditions used in the identification of *Staphylococcus aureus*.

Gene	Amplicon size (bp)	Oligonucleotide primer sequences (5'–3')	Cycling conditions*	Reference
<i>femA</i>	132	F: AAAAAAGCACATAACAAGCG R: GATAAAGAAGAAACCAGCAG	2	16
<i>nuc</i>	279	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAATAAAGC	2	5
<i>coa</i>	Variable	F: ATAGAGATGCTGGTACAGG R: GCTTCCGATTGTTTCGATGC	1	10

\* Condition 1 = 94°C for 4 min, then 94°C for 1 min, 60°C for 1 min, 72°C for 1 min for 30 cycles, and 72°C for 5 min. Condition 2 = 94°C for 5 min, then 94°C for 40 s, 58°C for 40 s, 72°C for 1 min for 10 cycles, then 94°C for 1 min, 50°C for 1 min, 72°C for 2 min for 25 cycles, and 72°C for 10 min.

been a factor, limiting the use of this technology in veterinary diagnostic laboratories.

Alternatively, PCR has been extensively used and recommended as a reliable approach in the identification of *Staphylococcus* species.<sup>6,10,17,19,20</sup> Using MALDI-TOF as the gold standard for identification of *S. aureus* from animal sources, we therefore investigated the accuracy of PCR using 3 different targets: the thermonuclease gene (*nuc*), the aminoacyl transferase FemA gene (*femA*), and the coagulase gene (*coa*).

A total of 157 *Staphylococcus* spp. isolates, including CoPS ( $n = 74$ ) and CoNS ( $n = 83$ ), were randomly selected from the microbiologic bank of the Laboratory for Food Safety at the College of Agricultural Sciences of the Federal University of Paraíba (Brazil). The isolates originated from cows ( $n = 5$ ), goats ( $n = 100$ ), horses ( $n = 13$ ), pigs ( $n = 38$ ), and a bird ( $n = 1$ ), and were cultured by means of conventional microbiologic procedures using mannitol salt agar and Columbia blood agar base supplemented with 5% sheep blood. Presumptive isolates were subjected to staining, and tests for catalase, oxidase, and coagulase, according to reference procedures.<sup>18</sup>

A MALDI-TOF system (Vitek MS, bioMérieux, Mercy l'Etoile, France) was used as the gold standard in our investigation. The identification was performed at the Department of Clinical and Toxicological Sciences, University of São Paulo (Brazil). Briefly, isolates were streaked onto trypticase soy agar (TSA) for 24 h at 37°C and 3–5 colonies transferred to 1 of the 48 wells of the standard steel plate. The samples were covered by the matrix solution of  $\alpha$ -cyano-4-hydroxycinnamic acid and dried at room temperature. The prepared plates were placed into the equipment, and the mass spectrum was analyzed and compared with the on-board database.

For PCR analysis, DNA was extracted by a crude lysate method,<sup>2</sup> with some modifications. Briefly, 3–5 colonies were resuspended in 100  $\mu$ L of purified water, frozen for 10 min, and immediately boiled at 100°C for 10 min. Afterwards, samples were centrifuged (12,902  $\times$  g, 4°C, 3 min), and the supernatant (~85  $\mu$ L) was carefully pipetted and transferred to a new DNase-free tube and stored at 4°C until use (within 1 wk). The master mix was prepared in a 25- $\mu$ L volume using 1 U of *Taq* DNA polymerase (LGC Biotechnology, Middlesex,

England), 2 mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTPs (Thermo Fisher Scientific, Waltham, MA), 10 pmol of each oligonucleotide primer, and 2  $\mu$ L of DNA template. The oligonucleotide primer sequences targeting the *S. aureus*-specific genes *femA*, *nuc*, and *coa*, and the respective cycling conditions used for *S. aureus* identification are shown in Table 1. The sequences were analyzed using Primer Blast (<https://go.gl/2DLYDn>), and homology with *S. aureus* only was confirmed.

The PCR assays targeting the genes *femA* and *nuc* were performed simultaneously in a duplex PCR assay. A reference *S. aureus* strain (USA 400) harboring the 3 genes, kindly provided by the Infectious Diseases Molecular Epidemiology Laboratory of The Ohio State University, was used as control in all PCR assays. Amplification products were electrophoresed in 2% agarose gel, stained (GelRed, Biotium, Hayward, CA), visualized under ultraviolet light, and documented (Gel Logic 212 PRO v.5.0, Carestream Health, Rochester, NY).

The correlation among the PCR assays targeting the genes *femA*, *nuc*, and *coa* was determined by the Cohen kappa index of concordance using interpretation criteria (Supplementary Table 1) that have been established previously.<sup>13</sup> The sensitivity and specificity values for *S. aureus* identification by PCR were calculated based on presence of *femA*, *nuc*, and *coa*, and using MALDI-TOF as the reference standard.

Of the 74 CoPS isolates, 68 were identified as *S. aureus* by MALDI-TOF. The other 6 CoPS were identified as *S. hyicus* (2), *S. intermedius* (2), and *S. pseudintermedius* (2). Among the 83 CoNS isolates, the following species were identified: *S. caprae* (21), *S. chromogenes* (13), *S. sciuri* (12), *S. epidermidis* (8), *S. simulans* (7), *S. cohnii* subsp. *cohnii* (6), *S. warneri* (5), *S. pasteurii* (2), *S. xylosus* (2), *S. saprophyticus* (2), *S. capitis* (2), *S. lentus* (1), *S. lugdunensis* (1), and *S. haemolyticus* (1). Detailed information about the isolates investigated in our study is shown in Supplementary Table 2.

Among the *S. aureus* isolates, 61 of 68 (90%) were positive for *femA* and 53 (78%) were positive for *nuc*. However, only 32 (47%) *S. aureus* isolates were positive for *coa*. No *Staphylococcus* species other than *S. aureus* (as identified by

**Table 2.** Genotypic patterns of 68 *Staphylococcus aureus* isolates based on detection of the *femA*, *nuc*, and *coa* genes.

Genotypic pattern	<i>femA</i>	<i>nuc</i>	<i>coa</i>	No. of isolates and relative frequency
1	+	+	+	30 (44)
2	+	+	-	16 (24)
3	+	-	-	14 (21)
4	-	+	-	6 (8.8)
5	+	-	+	1 (1.5)
6	-	+	+	1 (1.5)
Total				68 (100)

+ = positive; - = negative. Numbers in parentheses are percentages.

**Table 3.** Sensitivity and specificity values for *Staphylococcus aureus* identification by PCR based on the detection of the genes *femA*, *nuc*, and *coa* and using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as the reference method. The panel used for this study comprised 157 isolates including *S. aureus* ( $n = 68$ ) and coagulase-positive and -negative species other than *S. aureus* ( $n = 89$ ).

Targeted gene(s) in the PCR	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
<i>femA</i>	90	100	100	93
<i>nuc</i>	78	100	100	86
<i>coa</i>	47	100	100	71
<i>femA-nuc</i>	100	100	100	100
<i>femA-coa</i>	91	100	100	94
<i>nuc-coa</i>	79	100	100	86
<i>femA-nuc-coa</i>	100	100	100	100

MALDI-TOF) harbored any of the 3 genes, indicating PCR assays targeting those markers were highly specific (100%).

Based on the targeted genes, 6 different patterns were detected among the isolates (Table 2). The *nuc-femA-coa* combination was the most frequent (44%) pattern; *femA-coa* and *nuc-coa* patterns were each detected in just 1 (1.5%) isolate (Table 2). According to the Cohen kappa coefficient ( $p < 0.001$ ), a moderate (54.5%) agreement was observed between *femA-coa*; substantial agreements (64%, 62%, and 70%) were observed between *nuc-coa*, *nuc-femA-coa*, and *nuc-femA*, respectively (Supplementary Table 3). The PCR targeting *femA* and *nuc* genes had sensitivity values of 90% and 78%, respectively; the sensitivity of the PCR detecting *coa* was only 47%. When more than 1 marker was used, and a positive in either marker regarded as confirmed identification, the PCR targeting *femA* and *nuc* showed 100% sensitivity, but only 91.2% and 79.4% when targeting *femA* and *coa* and *nuc* and *coa*, respectively (Table 3).

The high frequency of *femA* in *S. aureus* isolates is in accordance with previous studies showing that the occurrence of this gene varies from 72.5% to 100% in *S. aureus*.<sup>11,15</sup> The gene *nuc* has been shown to be *S. aureus* specific<sup>20</sup> and has been reported to occur in all *S. aureus* isolates.<sup>5,14</sup> Although the frequency of *nuc* among *S. aureus* in our study was high, some isolates were not positive for the gene, suggesting that misidentification by PCR could be related to

deletions or mutations occurring in the gene, as previously reported.<sup>8</sup>

The low frequency of *coa* among *S. aureus* in our study was unexpected. Coagulase production is regulated by this gene in *S. aureus*.<sup>16</sup> The gene is associated with the capacity of the pathogen to coagulate fibrin and therefore plays a key role in the pathogenesis of the agent by conferring protection against host immune defense mechanisms and in bio-film production.<sup>26</sup> Using the same primers as in our study, a high frequency of *coa* gene in bovine *S. aureus* was reported in a 2014 study.<sup>6</sup> After excluding potential bias in the PCR assay by exhaustively testing different DNA extraction methods, master mix, and cycling conditions, we suggest that the low frequency of this gene might be associated with *coa* gene polymorphisms. Polymorphisms of the *coa* gene have been reported in *S. aureus* from different sources and animal species.<sup>1,7</sup>

The results of our investigation confirm the high specificity of PCR targeting *femA*, *nuc*, and *coa* and support the use of *femA* and *nuc* as a means to identify *S. aureus* species. These findings are of importance for clinical veterinary diagnostic laboratories, where PCR techniques have been extensively used, particularly with the continued reduction in the associated operating costs of the assay.<sup>25</sup> The precise identification of *S. aureus* is of key importance for the appropriate monitoring of strains resistant to antimicrobial

agents, especially in the context of the emergence of MRSA in livestock and companion animals that are potentially pathogenic to humans.

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### Declaration of conflicting interests

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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