Staphylococcus aureus produces exoproteins that contribute to its ability to colonize the mammary gland such as hemolysins, coagulase, slime, and protein A. This study characterized phenotypically and genotypically these virulence factors in 50 Staph. aureus isolates. These isolates were obtained from milk samples from subclinical mastitis cases identified in 15 dairy cattle farms located in the state of Rio de Janeiro, Brazil. All of the confirmed Staph. aureus samples were PCR positive for the coa gene, which displayed 3 different size polymorphisms. The amplification of the spaA X region yielded a single amplicon for each isolate with the prevalent amplicon sized 315 bp. The Staph. aureus isolates were 24 and 16% positive for the hla and hlb genes, respectively, and 22 and 20% positive for the icaA and icaD genes, respectively. Amplification of the agr gene RNAIII was positive in 74% of the strains. Twenty-seven different profiles were identified among the samples, indicating a great diversity of Staph. aureus involved in the etiology of mastitis cases in the analyzed region. These findings are valuable to the comprehension of the distribution of the profiles of Staph. aureus strains isolated from subclinical mastitis cases in the state of Rio de Janeiro.

Key words: Staphylococcus aureus, virulence factor, bovine mastitis

Short Communication

Staphylococcus aureus is a ubiquitous pathogen that causes a variety of infections in humans and animals. It is recognized as a major pathogen causing subclinical IMI in dairy cows, which may lead to severe economic losses worldwide (Godden et al., 2002). In Brazil, Staph. aureus isolates are commonly recovered from bovine mastitic milk samples (Coelho et al., 2009). The molecular process responsible for the disease manifestation are poorly understood but presumed to be caused partially by differences in gene content and by allelic variations between Staph. aureus strains. Nevertheless, little information is available about the genetic diversity of Staph. aureus isolated from cows with mastitis. This bacterium produces a range of virulence factors that contribute to its ability to colonize the mammary gland, including hemolysins, slime, protein A, and coagulase.

The α- and β-hemolysins are the most important factors in the pathogenesis of IMI. The β-toxin is an Mg2+-dependent sphingomyelinase C, which degrades sphingomyelin in the outer phospholipid layer of the membrane (Linehan et al., 2003).

The adherence and fixation of Staph. aureus on biological surfaces represent the fundamental steps in the development of infections. The production of slime mediates adhesion to implanted surfaces acting as a cementing matrix, making bacteria less accessible to the host’s defense system (Coelho et al., 2009). Slime production is controlled by the ica operon (icaADBC) and the co-expression of the icaA and icaD genes leads to a significant increase in such production (Arciola et al., 2001).

Staphylococcal protein A is a membrane-bound exoprotein characterized and well known for its ability to bind to the fragment crystallizable (Fc) region of immunoglobulins of most mammalian species. This protein is encoded by the spaA gene with a polymorphic (X) and a conserved region. The polymorphic X region consists of a variable number of repeated 24 pairs of bases located in the coding region for the cellular wall C-terminal extremity (Koreen et al., 2004).

The coagulase protein has the ability to turn fibrinogen into fibrin threads by a mechanism different from natural clotting. Coagulase has also been shown to be a virulence factor in IMI. This protein is encoded by the coa gene, which possesses a conserved and a repeated polymorphic region that can be used to measure relatedness between Staph. aureus isolates. This region consists of repeated short sequences of 81 bp that are variable in number and sequence, and a fixed sequence of 330 bp (Reinoso, 2004).
The accessory gene regulator (agr) operon (agrA, agrB, agrC, agrD, and hld) is recognized as a quorum-sensing gene cluster that upregulates production of secreted virulence factors such as α- and β-hemolysins, proteases, DNase, and sphingomyelinase. This same cluster also downregulates the production of cell-associated virulence factors in a cell density-dependent manner in Staph. aureus (Lyon et al., 2000). The agr locus comprises 2 divergent transcriptional units under the control of the promoters P2 (RNA II) and P3 (RNA III). The P3 transcript, an RNA III molecule, mediates the upregulation of secreted virulence factors as well as the downregulation of surface proteins (Novick, 2000).

These virulence factors have significant importance through the different stages of mastitis pathogenesis; hence, the aim of the present study was to characterize these factors phenotypically and genotypically in Staph. aureus strains isolated from subclinical mastitis cases in the state of Rio de Janeiro, Brazil. The continuous addition of information about this bacterium represents a valuable source for understanding the genetic diversity of Staph. aureus and can contribute to new strategies toward decreasing the spread of infection.

Twenty-five dairy cattle farms located in 6 different towns, comprising an important milk production region of the state of Rio de Janeiro, Brazil, were selected to be in this study due to its medium size comprising an average of 1,500 L of milk daily, mechanical milking system, and veterinary management. A total of 450 animals were evaluated for subclinical mastitis through the California Mastitis Test (CMT). Out of this total number, 280 cows presented positive results after the CMT, among which 57 cows were evaluated through 228 milk samples, representing 20% of the 280 investigated CMT-positive cases. Staphylococcus spp. were detected in 150 of these milk samples and Staph. aureus was detected in 50 of them (34%). Each Staph. aureus was isolated from a single cow, meaning that 50 of the above-mentioned 57 cows were Staph. aureus positive. After bacteriological and genotypic analyses, Staphylococcus intermedius (27%, n = 40), Staphylococcus hyicus (20%, n = 31), and Staphylococcus schleiferi coagulans (19%, n = 29) were also detected.

Milk samples were aseptically collected and transported to the laboratory in coolers with ice (4–8°C) for the bacteriological analyses. These samples were incubated at 37°C for 6 h and plated in duplicate in sheep blood agar (Oxoid Brasil Ltda, São Paulo–SP, Brazil), with one sample being incubated in aerobic conditions for 24 h and the duplicate sample being incubated in anaerobic conditions for 24 to 48 h at 37°C. The colonies were identified according to routine microbiological diagnostics, including cultural properties, catalase, coagulase, detection of hemolysis, maltose fermentation, acetoin production, and nitrate reduction (Koneman et al., 2005).

The Staph. aureus production of slime was detected using the Christensen et al. (1985) methodology. The adherence of each isolate to the surface of microplates has been determined quantitatively by a method standardized in our laboratory. They were also cultured in Congo red agar (CRA) and the black colonies were considered as normal slime producer strains, whereas the red ones were considered as strains unable to produce slime (Arciola et al., 2001).

To detect the production of α- and β-hemolysins, Staph. aureus isolates were radially streaked in sheep blood agar for evaluation of the type of hemolysis (Demo, 1996). Hemolytic synergism was determined by the cultivation of isolates in sheep blood agar at 37°C for 24 h in the presence of β-toxin from Staph. aureus (Demo, 1996).

Staphylococcus aureus DNA was extracted according to the methodology described in Coelho et al. (2009). Polymerase chain reaction analysis of the coa, spa (region X), icaA, icaD, hla, hlb, agr (rRNAIII gene), and rDNA genes were carried out using the primers, amplification cycles, and references described in Table 1. The reactions were performed in a final volume of 20 μL, containing PCR buffer (10 mM Tris HCl, pH 9.0; 50 mM KCl, and 0.1% Triton X-100), 3.5 mM MgCl2, 250 μmol of each of the deoxynucleoside triphosphates, 3.0 μmol of each gene-specific primer, 2.5 U of Taq DNA Polymerase (Promega, Madison, W1), and 5 μL of template (Reinoso, 2004). The hla and hlb genes were amplified by a PCR multiplex modified from the method described in Adhikari et al. (2002). Isolates from our collection that were previously characterized were used as controls in the assays.

The Staph. aureus isolates confirmed by PCR amplification by the 23S rDNA (Reinoso, 2004) were phenol and genotypically characterized by the presence of virulence factors. Correlation between the phenotypic and genotypic results were determined by the χ² and Fisher tests (IC = 95%).

Slime production was detected in 80% (40/50) of the Staph. aureus strains by the microplate test. In addition, the CRA assay revealed that 24% (12/50) presented black colonies, characterizing positive strains; 34% (17/50) were considered almost black (an intermediate variant); and 42% (21/50) were red variants, characterizing nonproducer strains. The PCR for the icaA (1,315 bp) and icaD (381 bp) genes were positive in 7 (14%) and 6 (12%) isolates, respectively (Figure 1) and both genes were detected in 4 isolates (7.3%). The ica locus is a virulence marker for clinically significant staphylococcal isolates, which is required for the synthesis of the intracellular polysaccharide adhesin by staphylococci.
Table 1. Sequences of oligonucleotide primers with corresponding programs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’–3’)</th>
<th>Program1</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>23S rDNA</td>
<td>ACG GAG TTA CAA AGG ACG AC</td>
<td>1</td>
<td>Straub et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>AGC TCA GCC TTA AGC AGT AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGC TGT TGG TGT TCT TCC TC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>spaA (X region)</td>
<td>CAA GCA CCA AAA GAG GAA</td>
<td>2</td>
<td>Reinoso (2004)</td>
</tr>
<tr>
<td></td>
<td>AGC TCA GCC TTA AGC AGT AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGC TGT TGG TGT TCT TCC TC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coa</td>
<td>ATA GAG ATG CTG GTA CAG G</td>
<td>3</td>
<td>Karahan and Çetinkaya (2007)</td>
</tr>
<tr>
<td></td>
<td>GCT TCC GAT TGT TCG ATG C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>icaA</td>
<td>CCA TAC TAA CTA ACA AAT ATA C</td>
<td>4</td>
<td>Vasudevan et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>AAG ATA TAG CTA AAT AAT AAT C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>icaD</td>
<td>AAA CGT AAG AGA GGT GG</td>
<td>4</td>
<td>Vasudevan et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>GGC AAT ATG ATC AAG ATA C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hla</td>
<td>CTG ATT ACT ATG CAA AGA ATT CGA TTG</td>
<td>5</td>
<td>Adhikari et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>CTT TCC AGC CTA CTT TTT TAT CAG T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hlb</td>
<td>GTG CAC TTA GTA AAT GTA GTG C</td>
<td>5</td>
<td>Adhikari et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>GTT GAT GAG TAG CTA CCT TCA GT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAA TCG GTG ACT TAG TAA AAT G</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 = 37 × (94°C for 40 s, 64°C for 60 s, 72°C for 75 s); 2 = 30 × (94°C for 60 s, 60°C for 60 s, 72°C for 60 s); 3 = 30 × (94°C for 60 s, 58°C for 60 s, 72°C for 60 s); 4 = 30 × (92°C for 45 s, 49°C for 45 s, 72°C for 1 min); 5 = 94°C for 5 min, 30 × (94°C for 60 s, 50°C for 60 s, 72°C for 60 s), 72°C for 7 min; 6 = 94°C for 3 min, 30 × (94°C for 60 s, 55°C for 60 s, 72°C for 60 s) 72°C for 7 min.

Figure 1. Agarose (1.5%) gel electrophoresis of Staphylococcus aureus PCR products of virulence factor genes. M = molecular weight standard; RN = negative reaction; (a) 1 = icaD-positive isolate (381 bp), 2 = control reaction; (b) 3 = icaA-positive isolate (1,315 bp), 4 = control reaction; (c) 5 = hla-positive isolate (210 bp), 6 = positive isolate for both genes (hla and hlb), 7 = hlb-positive isolate (300 bp); (d) 8 = spaA-positive isolate (polymorphic region; 220 bp), 9 = spaA-positive isolate (250 bp), 10 = spaA-positive isolate (280 bp), 11 = spaA-positive isolate (300 bp); and 12 = spaA-positive isolate (315 bp).
playing a role in cell-to-cell interactions during the process of biofilm formation (Götz, 2002). However, in the present study, statistical analysis showed no correlation between the levels of phenotypic slime production detected by the microplate and CRA tests and the presence of those genes. This result indicates that other mechanisms of slime production may coexist in *Staph. aureus* and, apparently, the intensity of its production is more related to the phenotypic characteristic expression specific to each strain.

Twenty-six (52%) *Staph. aureus* were hemolytic and both α- and β-hemolysis were observed for 15 (30%) isolates. From the 24 (48%) non-hemolytic isolates, 3 were able to express hemolysis in the presence of a β-hemolytic strain. The interaction between α- and β-toxin increases the adherence to bovine mammary epithelial cells and the proliferation of *Staph. aureus*, thus contributing to the establishment of *Staphylococcus* strains in the mammary glands, as described previously by Bownik and Siwicki (2008). In this study, β-hemolysin is present in 69% (18/26) of the hemolytic isolates, suggesting that this hemolysin may have an important role in the pathogenesis of mastitis, such as increasing permeability with progressive loss of cell surface charge in mammalian mammary glands cells (Coelho et al., 2009). The Multiplex PCR for the hla and hlb genes showed positive results in 24% (12/50) and 16% (8/50) of the isolates, respectively. Additionally, 13% (3/23) of α-hemolytic and 5.5% (1/18) of β-hemolytic isolates presented the hla and hlb genes, respectively. A total of 81% (21/26) of the isolates showed some type of hemolysin and were negative for both genes, suggesting the involvement of other genes, possibly related to the expression of these toxins, which are not amplified with the primers used in the present work. In our results, 12% (3/24) of the non-hemolytic *Staph. aureus* were able to express hemolysins in the presence of a β-hemolytic strain. This phenomenon can

### Table 2. *Staphylococcus aureus* characterization considering the presence (+) or absence (–) of hla, hlb, icaA, icaD, and rnaIII genes and the polymorphisms of spaA and coa genes in different farms

<table>
<thead>
<tr>
<th>Profile</th>
<th>Farm (isolates)</th>
<th>icaA</th>
<th>icaD</th>
<th>hla</th>
<th>hlb</th>
<th>coa (repeats)</th>
<th>spaA (repeats)</th>
<th>rnaIII</th>
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<tr>
<td>1</td>
<td>A (n = 1)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>800 (9)</td>
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<td>+</td>
</tr>
<tr>
<td>2</td>
<td>A (n = 3)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>520 (6)</td>
<td>315 (13)</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>520 (6)</td>
<td>315 (13)</td>
<td>–</td>
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<tr>
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<td>C (n = 4)</td>
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<td>–</td>
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<td>+</td>
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<tr>
<td>5</td>
<td>E (n = 2)</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>520 (6)</td>
<td>220 (9)</td>
<td>–</td>
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<tr>
<td>6</td>
<td>H (n = 3)</td>
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<td>–</td>
<td>–</td>
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<td>520 (6)</td>
<td>220 (9)</td>
<td>+</td>
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<td>7</td>
<td>J (n = 1)</td>
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<td>–</td>
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<td>520 (6)</td>
<td>315 (13)</td>
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<td>8</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>23</td>
<td>X (n = 1)</td>
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<td>–</td>
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<td>520 (6)</td>
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<td>24</td>
<td>Y (n = 1)</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>520 (6)</td>
<td>300 (12)</td>
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<td>25</td>
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<td>26</td>
<td>A (n = 1)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>800 (9)</td>
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<td>+</td>
</tr>
<tr>
<td>27</td>
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<td>+</td>
<td>–</td>
<td>800 (9)</td>
<td>300 (12)</td>
<td>+</td>
</tr>
</tbody>
</table>
probably be explained by the production of a delta- 
hemolysin, whose expression depends on the presence of a β-hemolytic isolate (Ali-Vehmas et al., 2001).

The PCR of the coa gene displayed 3 different size 
polymorphisms with approximately 520 bp for 43 
(86%) strains, 800 bp for 5 (10%) strains, and 900 bp 
for 2 (4%) strains. According to Hookey et al. (1998) 
and based on these results, the calculated numbers of 
repeats were 2, 6, and 11 repeats, respectively. In 
a study performed by Lange et al. (1999) in southern 
Brazil, 7 different coa profiles were identified through 
PCR—2 of them accounted for more than 50% of the 
isolates—suggesting that, in some Brazilian regions, 
most cases of mastitis may be caused by similar Staph. 
aureus strains.

The amplification of the X region from the spaA 
gene yielded a single amplicon for each isolate, with 
the prevalent amplon size being 315 bp for 32 strains 
(64%), 300 bp for 6 strains (12%), 250 bp for 5 strains 
(10%), 220 bp for 5 strains (10%), and 280 bp for 2 
strains (4%). Fréhaz et al. (1996) affirmed that spaA 
gene lengths >260 bp tend to be more associated with 
epidemic than with sporadic strains.

A total of 37 (74%) of the investigated Staph. 
aureus isolates were positive for rnaIII. This gene, located in the agr locus, encodes the RNAIII molecule and 
regulates at least 15 genes encoding for potential viru-

lence factors. The Agr mutants are nonpathogenic, are 
related to decreased synthesis of extracellular toxins 
and enzymes, and are, at the same time, related to an 
increased synthesis of adhesion molecules, such as co-
agulase and protein A (Gilot and van Leeuwen, 2004).

Staphylococcus aureus was isolated from milk samples 
collected over 15 dairy cattle farms, located in 5 differ-
ent towns in the state of Rio de Janeiro. Twenty-seven 
profiles (Table 2) were established among the isolates, 
based on a remarkable variability detected in the studied 
genes and their polymorphic forms. The predominant 
profiles were 2, 3, and 7, with a frequency rate of 38, 
8, and 2%, respectively, showing the absence of icaA, 
icaD, hla, and hlb genes as their most significant char-
acteristic. The low percentage of studied genes among 
the isolates does not dismiss the important role played 
by these virulence factors; it emphasizes the need for 
detection of a more extensive range of virulence factors, 
as all of them were positive for the presence of the 
rnaIII gene. Other Staph. aureus isolates (50%) also 
yielded different profiles, confirming the variability of 
this bacterium in the studied region. These results help 
in the understanding of the distribution of infectious 
Staph. aureus strains in Brazil and contribute to the 
establishment of preventive strategies to decrease the 
spread of infection.

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Janeiro (FAPERJ; process E-26/171.366/2006).

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