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# Virulence profile and genetic variability of *Staphylococcus aureus* isolated from artisanal cheese

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## ABSTRACT

The objectives of this study were to characterize *Staphylococcus aureus* isolated from artisanal and industrialized Minas frescal cheeses, to determine their antimicrobial susceptibility profile as well as the genetic similarity among the isolates. The isolates were also tested for staphylococcal enterotoxin genes and other virulence factors. Fifty-six artisanal raw milk cheeses sold at street fairs and 10 industrialized cheeses commercialized in supermarkets of Goiânia, Goiás, were analyzed. *Staphylococcus aureus* was confirmed in 19 samples (33.9%) of artisanal cheese by detection of *femA* gene, in which 29 isolates were obtained. These isolates were submitted to the antimicrobial susceptibility test and classified into 9 different profiles (A–I). Thirteen isolates (44.8) were resistant to penicillin and 3 (10.3) to tetracycline, with 2 (7.4) resistant to both. The multiplex PCR technique was performed to detect virulence genes that code for the production of hemolysins (Hla and Hlb), toxic shock syndrome toxin (TSST-1), exfoliative toxins (ETa and ETb), and staphylococcal enterotoxins [SE; SEA–SEE, SEG–SEJ, SEM–SEO]. All the isolates amplified for the *hla* gene and 14 (48.3%) for the *hlb* gene. The *seh* gene was the most frequently detected (n = 11, 37.9%), followed by *seo* gene (n = 3; 10.3%). In one isolate (3.4%), 4 enterotoxins genes were detected, and in another, 6 (3.4%) were detected. The comparison performed by pulsed-field gel electrophoresis of the 29 isolates revealed 18 genotypic profiles, which were grouped into 5 clusters. The genotyping found high genetic similarity among the isolates. Identical isolates were obtained from different samples and one sample showed more than one genetically different isolate. The high prevalence of *S. aureus* in the Minas Frescal cheese samples, as well as the detection of toxin encoding genes identified in this study, warns of the necessity to reduce the contamination levels in this type of cheese through monitoring and controlling the production and trade of the product.
**Key words:** *Staphylococcus aureus*, cheese, antimicrobial resistance, staphylococcal enterotoxin

## INTRODUCTION

*Staphylococcus aureus* is one of the most important human and animal pathogens. It has a broad set of virulence mechanisms associated with infectivity, production of toxins, and antimicrobial resistance (Argudin et al., 2010).

Some strains of *S. aureus* can produce toxins that are members of pyrogenic toxin superantigen family, such as the staphylococcal toxic shock syndrome toxin (**TSST-1**), which is responsible for generating an immune hyper-response in the host and has already been detected in *S. aureus* isolated from foods (Cha et al., 2007). These bacteria can produce exfoliative toxins, which promote the cleavage of the skin extract, causing severe cutaneous syndromes (Zschöck et al., 2005). Most strains secrete enzymes and cytotoxins that include coagulase and 4 hemolysins (α, β, gamma, and delta). Alpha and β hemolysins (**Hla** and **Hlb**) are dermonecrotic and neurotoxic, turn off the immune system by direct cytotoxic effect, and degrade soft tissues (Vandenesch et al., 2012).

Among the toxins produced by *S. aureus*, staphylococcal enterotoxins are a concern, because when produced in foods staphylococcal enterotoxins can cause food poisoning, which is one of the most prevalent foodborne diseases around the world (ECDC, 2015). These toxins present emetic activity and are called classic staphylococcal enterotoxins (**SE**; SEA–SEE), which occur more frequently in cases and outbreaks of food poisoning, along with other more recently identified (SEG, SEH, SEI, SER, and SET (Wallin-Carlquist et al., 2010; Xu and McCormick, 2012). In addition to these, other enterotoxins called staphylococcal enterotoxin-like proteins (**SE*l***) include the SE*l* J, K, L, M, N, O, P, Q, S, U, and V. Although the SE*l* toxins are homologous and structurally similar to the staphylococcal enterotoxins,

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emesis activity has not yet been confirmed in humans (Xu and McCormick, 2012).

*Staphylococcus aureus* was the second most common pathogen responsible for foodborne diseases in Brazil between 2000 and 2014. Milk and its derivatives were among the foods most frequently involved in outbreaks of foodborne diseases (ANVISA, 2015).

Minas frescal cheese is one of the most consumed cheeses in Brazil and presents high susceptibility to microbiological and proteolytic changes due to its high moisture content and handling (Chalita et al., 2009). According to Brazilian law, this type of cheese must be produced in dairy industry with pasteurized milk. However, this food is also manufactured from raw milk and marketed in the informal sector, without regulation and sanitary supervision. For these reasons, it is frequently involved in the transmission of pathogenic bacteria and staphylococcal food poisoning outbreaks (Dorigon, 2010).

Additionally, bacteria isolated from foods of animal origin may show resistance to several antimicrobial agents used to treat diseases, which may influence the effectiveness for treatment (Delsol et al., 2010; Araújo et al., 2011).

The aims of the study were to (1) evaluate the prevalence of *S. aureus* in artisanal and industrialized Minas frescal cheese sold in the municipality of Goiânia, Brazil; (2) determine the antimicrobial susceptibility profile of the isolates; (3) molecularly characterize the isolates for the presence of virulence genes and genetic similarity by macrorestriction analysis using pulsed-field gel electrophoresis (**PFGE**).

## MATERIALS AND METHODS

### Cheese Sampling

In this study, 56 artisanal and 10 industrialized samples of Minas frescal cheese were purchased from retail sale sites along the market streets and from supermarkets, respectively, in the municipality of Goiânia, Goiás, Brazil, from June to August 2012. All samples were immediately transported to the laboratory in a refrigerated box (4 to 8°C) and kept at 4°C until analysis.

### Isolation of Coagulase-Positive Staphylococci

To enumerate coagulase-positive staphylococci (**CPS**), the samples were processed by surface plating on Baird-Parker agar with egg yolk tellurite emulsion and incubated at 37°C for 48 h under aerobic conditions. Up to 5 typical or atypical (or both) presumptive colonies were selected and tested using standard microbiological procedures such as Gram staining, catalase, and thermostable nuclease detection. The coagulase production was performed by tube test using rabbit serum (Bennett and Lancette, 2001). A pure culture of each isolate was kept frozen at −80°C for further analysis.

### Antimicrobial Susceptibility Test–Disk Diffusion Method

All CPS isolates were subjected to an antimicrobial susceptibility test by the disc diffusion method on Müeller Hinton agar (CLSI, 2015). Penicillin (10 UI), cefoxitin (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), rifampin (5 μg), ciprofloxacin (5 μg), tetracycline (30 μg), erythromycin (15 μg), quinupristin/dalfopristin (15 μg), and clindamycin (2 μg) were used as antimicrobial agents. *Staphylococcus aureus* ATCC 25923 was used as a reference strain for antimicrobial susceptibility testing.

### DNA Extraction

The DNA extraction procedure was performed according to Aires-de-Sousa et al. (2007). The pure culture was thawed and streaked onto Tryptone Soya Agar culture and incubated at 35°C for 18 to 24 h. Three to 4 colonies were suspended in 50 μL of TE 1× buffer (10 m*M* Tris, 1 m*M* EDTA, pH 8.0) with 1 μL of lysostaphin (10 mg/mL) and incubated at 37°C. After 30 min, cell suspensions were placed in a boiling-water bath at 95°C for 15 min. Then, 150 μL of $H_2O$ Milli-Q was added and the solution was centrifuged (13,000 × *g*) for 5 min at 4°C. The resulting supernatants were used as DNA templates in the PCR reactions.

### Identification of Staphylococcus aureus

The CPS isolates were screened by PCR for the presence of *femA* gene, which is specific for *S. aureus*, according to Mehrotra et al. (2000). The primers used for gene amplification were *femA*-F: 5′AAAAAAGCACATAACAAGCG 3′ and *femA*-R: 5′GATAAAGAAGAAACCAGCAG 3′, to obtain a 132-bp amplicon.

The PCR reaction was performed in a reaction mixture with a final volume of 50 μL containing 10 μL of 5× reaction buffer [20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl], 1.5 m*M* $MgCl_2$, 100 μ*M* of each dNTP, 0.2 mM of *femA* primers, 2.5 U of *Taq* polymerase, and 20 ng of template DNA. The volume of this mixture was adjusted to 50 μL with sterile deionized water. As positive controls, PCR reactions were carried out in paral-

lel and contained template DNA extracted from the standard strains of *S. aureus* ATCC 25923. The PCR was performed under the following conditions: initial denaturation at 94°C for 5 min, subsequently followed by 35 cycles at 94°C for 2 min, 57°C for 2 min, and 72°C for 1 min, with a final extension of 7 min at 72°C. The amplified products were detected by electrophoresis on 1.5% gel agarose containing ethidium bromide (0.5 μg/mL). The electrophoresis was carried out at 100 V for 1 h. The PCR products were visually examined and photographed under UV illumination.

### Detection of Virulence Genes

The multiplex PCR technique was performed on all *S. aureus* isolates to detect virulence genes that code for the production of hemolysins (Hla and Hlb), TSST-1, exfoliative toxins (ETa and ETb), and enterotoxins (SEA–SEE, SEG–SEJ, SEM–SEO), through 4 sequential reactions, according to Jarraud et al. (2002) and Holtfreter et al. (2007) with modifications. All the primer pairs used in this study and the multiplex reactions are listed in Table 1.

The PCR reactions were performed in a total volume of 25 μL containing reaction buffer (5×; 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, pH 8.0), 5.0 m*M* $MgCl_2$, 100 m*M* of dNTP, 150 to 400 n*M* of each primer, 1.0 U of Taq DNA polymerase, and 10 to 20 ng of DNA template.

The PCR samples were subjected to amplification using a thermocycler (Axygen, Tewksbury, MA) according to the following program: initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, 72°C for 50 s, and a final extension at 72°C for 10 min. Amplicons were separated by 1.5% agarose gel electrophoresis in 1× Tris-borate-EDTA (**TBE**) at 100 V for 90 min and viewed under Gel Doc XR UV transilluminator (Bio-Rad Laboratories, Hercules, CA). The gel images were analyzed with the QuantityOne software (Bio-Rad Laboratories) after ethidium bromide (0.5 μg/mL) staining.

**Table 1**. Primers used in multiplex PCR reactions for virulence gene detection[1]

| Primer[2] | Sequence (5′–3′) | bp | Reaction |
|---|---|---|---|
| TSST F | TTCACTATTTGTAAAAGTGTCAGACCCACT | 180 | I |
| TSST R | TACTAATGAATTTTTTTATCGTAAGCCCTT | | |
| SEC F | GTAAAGTTACAGGTGGCAAAACTTG | 297 | |
| SEC R | CATATCATACCAAAAAGTATTGCCGT | | |
| SEB F | ATTCTATTAAGGACACTAAGTTAGGGA | 404 | |
| SEB R | ATCCCGTTTCATAAGGCGAGT | | |
| SEI F | CTCAAGGTGATATTGGTGTAGG | 576 | |
| SEI R | AAAAAACTTACAGGCAGTCCATCTC | | |
| SEO F | AGTTTGTGTAAGAAGTCAAGTGTAGA | 180 | II |
| SEO R | ATCTTTAAATTCAGCAGATATTCCATCTAAC | | |
| SEJ F | TAACCTCAGACATATATACTTCTTAACG | 300 | |
| SEJ R | AGTATCATAAAGTTGATTGTTTTCATGCAG | | |
| SEE F | CAAAGAAATGCTTTAAGCAATCTTAGGC | 482 | |
| SEE R | CACCTTACCGCCAAAGCTG | | |
| ETB F | CAGATAAAGAGCTTTATACACACATTAC | 612 | |
| ETB R | AGTGAACTTATCTTTCTATTGAAAAACACTC | | |
| ETA F | ACTGTAGGAGCTAGTGCATTTGT | 190 | III |
| ETA R | TGGTACTTTTGTCTATCTTTTTCATCAAC | | |
| SEM F | CTATTAATCTTTGGGTTAATGGAGAAC | 326 | |
| SEM R | TTCAGTTTCGACAGTTTTGTTGTCAT | | |
| SED F | GAATTAAGTAGTACCGCGCTAAATAATATG | 492 | |
| SED R | GCTGTATTTTTCCTCCGCTAAATAATATG | | |
| SEG F | AATTATGTGAATGCTCAACCCGATC | 642 | |
| SEG R | AAACTTATATGGAACSSSSGGTACTAGTTC | | |
| HLA F | CTGATTACTATCCAAGAAATTCGATTG | 209 | IV |
| HLA R | CTTTCCAGCCTACTTTTTTATCAGT | | |
| HLB F | GTGCACTTACTGACAATAGTGC | 309 | |
| HLB R | GTTGATGAGTAGCTACCTTCAGT | | |
| SEH F | CAATCACATCATATGCGAAAGCAG | 376 | |
| SEH R | CATCTACCCAAACATTAGCACC | | |
| SEA F | GAAAAAAGTCTGAATTGCAGGGAACA | 560 | |
| SEA R | CAAATAAATCGTAATTAACCGAAGGTTC | | |
| SEN F | ATGAGATTGTTCTACATAGCTGCAAT | 680 | |
| SEN R | AACTCTGCTCCCACTGAAC | | |

[1]Source: Jarraud et al., 2002.

[2]TSST = toxic shock syndrome toxin; ET = exfoliative toxin; HLA = hemolysin A; HLB = hemolysin B; SE = staphylococcal enterotoxin. F = forward; R = reverse.

### Molecular Subtyping of S. aureus Isolates

Strains identified as *S. aureus* by PCR were submitted to PFGE using 20 U of *Sma*I restriction enzyme according to Chung et al. (2000). Electrophoretic run was performed in 0.5× TBE buffer (90 m*M* Tris, 90 m*M* boric acid, and 2 m*M* EDTA) using CHEF DRII System (Bio-Rad Laboratories, Hercules, CA), under the following conditions: 6 V/cm for 22 h, pulse times from 5 to 35 s at 14°C. *Staphylococcus aureus* strain NCTC 8325 was used as the reference standard.

After being run, the gels were stained with ethidium bromide (0.5 μg/mL) and viewed with a UV transilluminator. Gel images were captured by Molecular ImagerGelDoc XR (Bio-Rad) and analyzed with the BioNumerics program (version 5.1; AppliedMaths, Ghent, Belgium). Clustering was performed by unweighted pair group method using arithmetic averages. Similarity of strains was estimated using the Dice correlation coefficient with 0.7% optimization and 1.0% tolerance setting. For clustering of strains, a cut-off of 80% was applied (Carriço et al., 2005). Identical PFGE profiles (100% of similarity) were defined as a pulsotype. Gels were also analyzed by visual examination of the banding patterns to differentiate between the undistinguishable closely related strains, which were possibility related or unrelated (Tenover et al., 1995).

## RESULTS AND DISCUSSION

### Coagulase-Positive Staphylococci

From 10 industrialized Minas frescal cheese samples analyzed, 2 (20.0%) presented CPS counts higher than the limits established as maximum legal limit ($1.0 \times 10^3$ cfu/g) by Brazilian legislation (Brasil, 2001). The artisanal Minas frescal cheese was found to be potentially hazardous to public health because levels of contamination ranged from $1.2 \times 10^3$ to $1.5 \times 10^7$ cfu/g in 19 (33.9%) of the evaluated samples. Eight (14.3%) of them were higher than $10^5$ cfu/g, presenting a high risk of food poisoning (Balaban and Rasooly, 2000).

The presence of CPS in cheeses made from pasteurized milk may indicate a failure in pasteurization or recontamination after pasteurization due to insufficient hygienic and sanitary practices (O'Brien et al., 2009). High counts of CPS in raw milk cheese are expected because that milk can be contaminated directly from the production environment, infected animal, or food handler (Spanu et al., 2014). Variable results have been observed by other authors in samples obtained in Brazil such as 21.0% in Bahia and 31.2% in Pernambuco (Evêncio-Luz et al., 2012) and 54.2% in Goiás (André et al., 2008).

### S. aureus Identification

Of the 19 samples of artisanal and 2 of industrialized cheeses that were positive for CPS, 29 isolates were identified as *S. aureus* by phenotypic characterization and *femA* detection. All of these isolates were recovered from artisanal cheeses.

The presence of *S. aureus* in fresh raw milk cheeses is a frequent finding around the world, and the prevalence varies, for example, 2.2% (Vitale et al., 2015) and 35.0% (Bianchi et al., 2014) in Italy, 10.9% (Jamali et al., 2015) and 45.0% (Saadat et al., 2014) in Iran, 37.5% in Turkey (Gücükoğlu et al., 2012), 54.2% in Brazil (André et al., 2008), and 69.0% in Sweden (Rosengren et al., 2010). In Switzerland, between 1996 and 2006, 63.6% of the staphylococcal food poisoning outbreaks reported were associated with cheese consumption (Baumgartner, 2008).

The handmade process in Brazil does not involve the step of milk pasteurization, which is very important for the quality control and safety of this product. André et al. (2008) observed that the main source of contamination of the final product was the raw milk used in its production, compared with strains isolated from handlers in artisanal cheese produced in the Goiás State, Brazil.

### Antimicrobial Susceptibility Profile

The resistance patterns of *S. aureus* to the tested antimicrobial agents are presented in Table 2. The tests demonstrated the susceptibility of all *S. aureus* isolates to cefoxitin, trimethoprim-sulfamethoxazole, rifampin, and erythromycin. Twelve (41.4%) isolates were susceptible to all 9 antimicrobials tested, and 13 (44.8%) and 3 (10.3%) were resistant to penicillin and tetracycline, respectively. Intermediate susceptibility was observed in one isolate (3.4%) for ciprofloxacin and clindamycin and in 5 (17.2%) for quinupristin/dalfopristin.

In this study, 12 isolates (41.4%) were resistant to 1, and 2 isolates (6.9%) were resistant to 2 antimicrobial agents. The resistance to penicillin remains the most common, as observed in other studies (André et al., 2008; Spanu et al., 2014; Jamali et al., 2015). The prevalence of resistance to β-lactam antibiotics is frequent in *S. aureus* strains obtained from milk and related products as reported by Daka et al. (2012), Hu et al. (2013), and Xu et al. (2014).

Several mechanisms are involved in β-lactam resistance, such as production of penicillinases or alteration of the target protein (Spanu et al., 2014). We did not find any isolate resistant to cefoxitin, indicating that the high resistance to penicillin observed should be as result of β-lactamase production.

**Table 2**. Antimicrobial susceptibility profile of *Staphylococcus aureus* isolated from artisanal raw milk cheeses sold in Brazilian street fairs

| Sample isolates[1] | Susceptibility profile[2] | Phenotype |
|---|---|---|
| Q3.2, Q12.2, Q12.3, Q15.2, Q18.1, Q18.3, Q34.3, Q35.2, Q35.3, Q35.4, Q44, Q45 | SSSSSSSSS[3] | A |
| Q9.2, Q9.3, Q14, Q15.3, Q24.3, Q36, Q46, Q49, Q56 | RSSSSSSSS | B |
| Q34.2, Q35.1 | SSSSSSSIS | C |
| Q3.1 | SSSSSRSSS | D |
| Q17 | SSSSSSSSI | E |
| Q27 | RSSSSRSIS | F |
| Q24.2 | RSSSSSSIS | G |
| Q11 | RSSSISSIS | H |
| Q54 | RSSSSRSSS | I |

[1]Q = raw milk cheese.

[2]S = susceptible; R = resistant; I = intermediate.

[3]Sequence of antimicrobials tested: penicillin, cefoxitin, trimethoprim/sulfamethoxazole, rifampim, ciprofloxacin, tetracycline, erytromycin, quinupristin/dalfopristin, clindamycin.

Tetracycline resistance observed in this study (10.3%) was also found in Italy by Spanu et al. (2014) in strains isolated from cheese (10.6%). Tetracycline is used in intensive livestock production, which may explain the resistance observed in the literature in bacteria obtained from animal products (Schneider et al., 2009; Lee et al., 2014).

According to the patterns generated by the antimicrobial susceptibility tests, the 29 isolates were grouped into 9 distinct profiles, A to I (Table 2). Profile A grouped 13 isolates (44.8%), and profile B grouped 9 (31.0%).

### Detection of Virulence Genes

*Staphylococcus aureus* groups are characterized by different virulence factors and by large variations in the presence of these virulence genes (Aydin et al., 2011). In this study, the presence of *tst*, *eta*, and *etb* genes was not found in the isolates. The *hla* gene was found in all isolates, and *hlb* gene was found in 14 (48.3%) of them (Table 3). Silva et al. (2012) also found 100.0% of hemolytic activity among *S. aureus* isolated from bovine mastitis and most of them presented co-production of toxins.

According to Lo et al. (2011), the cytolysin production, including hemolysins, determines the pathogenicity of several bacterial agents and is associated with the pathogenesis of diseases caused by *S. aureus*. The hemolysins can be produced by the most *S. aureus* isolates (Burnside et al., 2010).

The synergistic effect between α and β hemolysins is recognized as a determinant of the severity of *S. aureus* diseases, particularly bovine mastitis, with great ability of the pathogen to persist in the mammary gland, which may contaminate the milk and milk products (Aarestrup et al., 1999). The Hlb is characteristic of isolates obtained from bovine mammary gland; therefore, the findings suggest the probable source of cheeses contamination evaluated in this study.

From 29 *S. aureus* isolates, 13 (44.8%) presented genes coding for enterotoxins. The *seh* gene was the most frequently detected (n = 11, 37.9%), followed by the *seo* gene (n = 3; 10.3%), *seg*, *sem*, *sen* genes (n = 2; 6.9%), and *sec*, *sei* genes (n = 1; 3.4%). In one isolate (3.4%), 4 genes coding for SEG and SE*l* M, N, and O were detected. In another isolate (3.4%), 6 genes were present (staphylococcal enterotoxins C, G, I and SE*l* M, N, O). Both the isolates were obtained from the same artisanal raw milk cheese (Table 3).

The identification of new staphylococcal enterotoxins and SE*l* greatly increases the frequency of enterotoxigenic *S. aureus* isolates, which suggests an improvement on pathogenic potential of this bacteria (Bianchi et al., 2014). Among the staphylococcal enterotoxins, SEH has been responsible for staphylococcal food poi-

**Table 3**. Virulence genes detected in *Staphylococcus aureus* isolated from artisanal raw milk cheeses sold in Brazilian street fairs

| Sample isolates | Virulence genes |
|---|---|
| Q3.1, Q3.2, Q9.2, Q9.3, Q15.2, Q17, Q27, Q34.3, Q35.1, Q35.4, Q36, Q49 | *hla+hlb* |
| Q11, Q45 | *hla+hlb+seh* |
| Q12.3, Q14, Q15.3, Q18.1, Q34.2, Q35.2, Q35.3, Q56.2 | *hla+seh* |
| Q24.2 | *hla+seg+sem+sen+seo* |
| Q24.3 | *hla+sec+seg+sei+sem+sen+seo* |
| Q46 | *hla+seh+seo* |
| Q12.2, Q18.3, Q44, Q54 | *hla* |

soning involving milk products (Jørgensen et al., 2005; Ostyn et al., 2010). Ikeda et al. (2005) described an outbreak due to the consumption of reconstituted milk, which was contaminated with enterotoxigenic *S. aureus* producing SEA and SEH. Another outbreak was attributed to SEH produced by *S. aureus* in mashed potatoes made with raw milk (Jørgensen et al., 2005).

The SEH has been described as the most prevalent staphylococcal enterotoxin in bovine milk. Liu et al. (2014), who analyzed 116 isolates from bovine mastitis, detected the *seh* gene in 36.3% and *sec* gene in 3.4% isolates. This is similar to the results obtained in the present study, and reinforces the possible source of cheese contamination. Bianchi et al. (2014) evaluating milk products found genes *sea*, *sed*, and *sec* in 23.1, 47.0, and 12.9% of the isolates, respectively, as well as the genes *seg*, *seh*, *sei*, *sej*, *sep*, and *ser* in 33.3, 4.7, 34.5, 46.7, 1.17, and 52.5%, respectively.

Several studies focused on the detection of genes coding classical staphylococcal enterotoxins (SEA–SEE). In those studies, the major prevalence was observed for the *sea* gene, although at low percentages such as 12.8% (Spanu et al., 2014), 5.0% (Saadat et al., 2014), 4.8% (Gücükoğlu et al., 2012), 2.2% (Vitale et al., 2015), and 1.6% (Ertas et al., 2010). Our study expanded the investigation for 7 more staphylococcal enterotoxins or SE*l* (G, H, I, J, M, N, O), and we observed that only *sec* gene among the classical staphylococcal enterotoxins was detected in 3.4% of the cheese samples. This fact demonstrates the importance of searching for other staphylococcal enterotoxins, especially in *S. aureus* obtained from food samples.

The presence of enterotoxins in milk products is considered a potential risk to public health. Several factors such as processing failures, inadequate refrigeration, improper hygienic practices, and contamination after processing are associated with *S. aureus* multiplication and enterotoxin production (Saadat et al., 2014). Therefore, the establishment and implementation of safety regulations is essential to prevent staphylococcal food poisoning.

### *Molecular Subtyping of S. aureus*

The comparison of the 29 *S. aureus* isolated during the study were performed by PFGE. The isolates were assigned to 18 pulsotypes and grouped into 5 clusters (each cluster grouped 2 or more isolates with $\geq$80% of similarity; Figure 1). Cluster 1 grouped 20 isolates obtained from 13 different raw milk cheese samples. The other 4 clusters grouped 2 isolates each, and one isolate was not related to any cluster. This shows high genetic similarity among the isolates. This similarity can be explained by a possible sale of products from the same supplier in different street fairs. André et al. (2008) and Tondo et al. (2000) found more diversity among isolates obtained from dairy products in the Middle West and Southern Brazil.

We observed that some samples (Q3, Q9, Q15, Q18, Q24, Q35) presented more than one genetically distinct isolate, and genetically identical isolates (100% similarity) were obtained from different samples. As demonstrated in Figure 1, one sample (Q35) contained 4 isolates that, although grouped in the same cluster, presented distinct pulsotypes, different susceptibility, or different virulence profile. This observation shows that during the cheese production chain, contamination could occur from several sources in the milk and cheese production environment, as well as from the animal or food handler (Spanu et al., 2014). This could be minimized if hygienic processing was intensified to prevent cross-contamination among the potential sources of food contamination.

All 11 *S. aureus* isolates harboring the *seh* gene, obtained from 10 different samples, were grouped in cluster 1, which demonstrates high similarity among these isolates, although they were isolated from samples produced in distinct places at different times. The genes coding for other staphylococcal enterotoxins (C, G, I, M, N, O), detected in 2 isolates obtained from the same sample, were grouped in cluster 3 with 92.7% similarity. It has been demonstrated that the *seh* gene is the most prevalent in *S. aureus* isolates from bovine mastitis (Liu et al., 2014). The present study found the *seh* gene in 37.9% of the *S. aureus* isolated from raw milk cheeses, which indicates the most probable source of the bacteria for the product was the bovine milk.

The association of distinct molecular typing techniques is helpful to improve the knowledge about the dynamics of infections caused by *S. aureus*. The combination of PFGE with PCR is an important tool for epidemiological diagnosis as well as to understand the patterns of pathogen transmission and sources of possible food contamination (Said et al., 2010).

## CONCLUSIONS

The presence of *S. aureus* above the limit established by law and at levels compatible with the production of enterotoxins in artisanal raw milk cheeses points to the potential risk to public health. Consumption of this product is still widespread in Brazil; therefore, an effective reduction of contamination levels could be achieved by improving sanitation and hygiene procedures. The consumption of artisanal raw milk cheese should be considered a potential risk of foodborne disease because genes coding for enterotoxin production were detected. The identification of enterotoxins, other than the classic

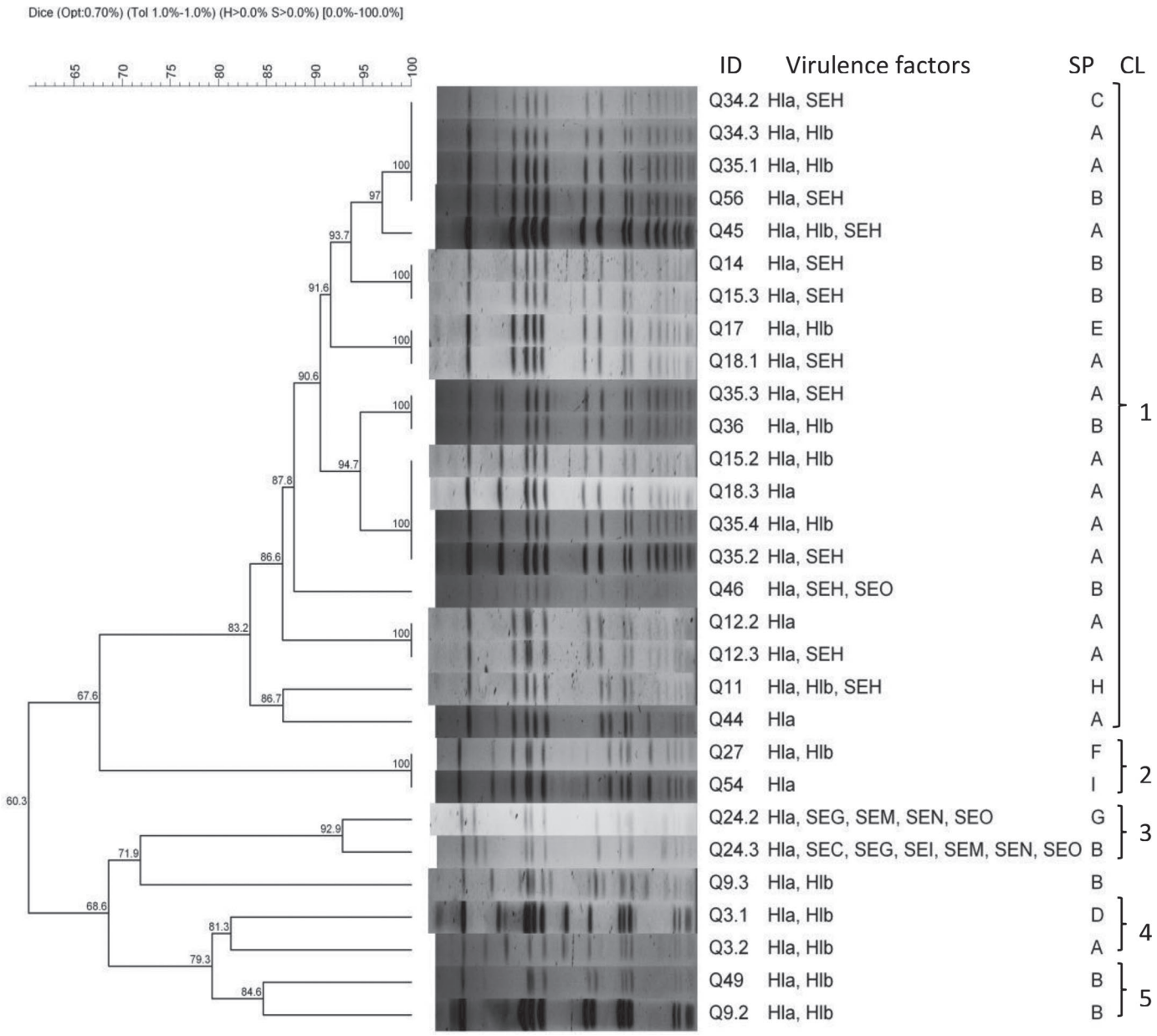


**Figure 1**. Clonal relationships, virulence, and susceptibility profile of *Staphylococcus aureus* isolated from raw milk cheese, established with *Sma*I pulsed-field gel electrophoresis analysis. ID = sample identification; SP = susceptibility profile; CL = cluster; Hla = hemolysin A; Hlb = hemolysin B; SE = staphylococcal enterotoxin.

ones, highlights the importance of expanding research to detect these virulence factors for food safety assurance. The combination of genotypic and phenotypic methods used helps to trace the origin of the strains. The virulence pattern observed in most of the isolates led us to conclude that the contamination of traditional and artisanal raw milk cheese with *S. aureus*, despite the handmade production, is probably of animal origin. Therefore, milk pasteurization, as recommended by the supervisory agencies, should solve most of the problem.

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