Molecular characterization and genetic diversity of *Staphylococcus aureus* isolates of dairy production farms in Rio de Janeiro, Brazil

Caracterização molecular e diversidade genética de *Staphylococcus aureus* isolados de fazendas leiteiras no Rio de Janeiro, Brasil

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**Abstract**

*Staphylococcus aureus* is an important pathogen involved in subclinical bovine mastitis, causing high economic losses for the dairy industry. The successful persistence of this pathogen in the host occurs due to a series of factors associated with colonization ability and the acquisition of virulence factors. This bacterial species carries genetic heterogeneity, and genetically diverse strains characterize the population. Analysis of the genetic variation is an important tool for epidemiological studies. For this study, *S. aureus* strains were randomly selected by molecular profiling. All strains were originated from the milk of cows of subclinical mastitis on farms in the State of Rio de Janeiro. Strains of *S. aureus* were profiled using virulence gene analysis profiles, agr and sps typing, Pulsed Field Gel Electrophoresis (PFGE), and Multilocus Sequence Typing (MLST). 47% (8/17) of the tested strains were positive for *ica* A gene; 82.3% (14/17) for *ica* D gene; 41% (7/17) for *fbn* A gene; 47% (8/17) positive for *fbn* B gene; 94% (16/17) for the *hl* A gene and 70.5% (12/17) for the *hl* B gene. These virulence results generated 11 different profiles. Most strains (58.8% - 10/17) were classified as type-II by *agr* system, spa typing identified seven different spa types. PFGE analysis found extensive genetic heterogeneity and no clones were observed. MLST analysis generated five different types of ST/CC. Considering the results observed at the present study, a high genetic variety of *S. aureus* strains associated of the presence of different virulence factors justified the absence of clonal strains at the properties evaluated. Besides, that, the definition of *S. aureus* clonal strain as well dynamic population in not fully understood since there are a limited number of studies in *S. aureus* associated with bovine mastitis.

**Keywords:** Bovine mastitis, virulence profile, molecular typing.

**Resumo**

*Staphylococcus aureus* é um importante patógeno envolvido na mastite subclínica bovina, causando altas perdas econômicas para a indústria de laticínios. A persistência bem-sucedida desse patógeno no hospedeiro ocorre devido a uma série de fatores associados à capacidade de colonização e aquisição de fatores de virulência. Esta espécie bacteriana possui heterogeneidade genética e a população é caracterizada por cepas geneticamente diversas. A análise da variação genética é uma ferramenta importante para estudos epidemiológicos. Para este estudo, cepas de *S. aureus* foram selecionadas aleatoriamente por perfil molecular. Todas as cepas foram originadas do leite de vacas de mastite subclínica em fazendas do Estado do Rio de Janeiro. As cepas de *S. aureus* foram caracterizadas usando perfis de análise de genes de virulência, tipagem *agr* e *spa*, eletroforese em gel de campo pulsado (PFGE) e tipagem de sequência de foco múltiplo (MLST). 47% (8/17) das cepas testadas foram positivas para o gene *ica* A; 82.3% (14/17) para o gene *ica* D; 41% (7/17) para o gene *fbn* A; 47% (8/17) positivo para...
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o gene *fbrB*, 94% (16/17) para o gene *hla* e 70.5% (12/17) para o gene *hlaB*. Esses resultados de virulência geraram 11 perfis diferentes. A maioria das cepas (58.8% - 10/17) foi classificada como o tipo II pelo sistema *agr*. A tipificação por *spa* identificou sete tipos diferentes. A análise por PFGE encontrou extensa heterogeneidade genética e nenhum clone foi observado. A análise do MLST gerou cinco tipos diferentes de ST / CC. Considerando os resultados observados no presente estudo, uma elevada variedade genética de cepas de *S. aureus* associada à presença de diferentes fatores de virulência justificou a ausência de cepas clonais nas propriedades avaliadas. Além disso, a definição de cepas clonais assim como a dinâmica populacional em *S. aureus* não é totalmente compreendida, pois há um número limitado de estudos em *S. aureus* associados à mastite bovina.

**Palavras-chave:** Mastite bovina, perfil de virulência, tipagem molecular.

**Introduction**

In Dairy cattle, various species of microorganisms are implicated in infectious mastitis, especially *Staphylococcus aureus*, which causes contagious mastitis that could be clinical or chronic (usually subclinical) whose infection and dissemination usually occur during milking (Bardiau et al., 2014).

Mastitis caused by *S. aureus* is a result of the production of several virulence factors (VFs), which may contribute in different ways to the pathogenesis of the organism. Pathogenic differences of *S. aureus* strains may be a result of the geographical distribution, host, tissue type, and number of combination of virulence genes may influence the pathogenic potential of *S. aureus* strains (Bar-Gal et al., 2015).

The ability of *S. aureus* to produce biofilm is considered as a critical virulence factor capable of influencing the pathogenesis of mastitis. Biofilm aids in the adhesion and colonization of the organism in the mammary gland epithelium. The association between biofilm in infections and drug resistance has led to a growing interest in the characterization of the genes involved in biofilm formation. A single locus has been detected in most isolates of *S. aureus* of mastitic origin, indicating its potential role as a virulence factor in the pathogenesis of mastitis in ruminants (Melchior et al., 2006).

Another VF that is associated not only with adhesion but also internalization by cells is fibronectin-binding proteins (FBN) A and B that are multifunctional microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that recognize fibronectin, elastin, and fibrinogen. FBN promotes the internalization of *S. aureus* in epithelial and endothelial cells that are generally not phagocytic, favors the spread of bacteria from the bloodstream to internal organs, and the evasion of the immune response and the action of antibiotics (Burke et al., 2010).

VFs involved in toxins production in *S. aureus* play a significant role in intramammary infections pathogenicity. α- and β-hemolysins are pore-forming exotoxins that can induce inflammatory changes in mammalian cells, inactivating the immune system, their direct cytotoxic effect degrades tissues, providing nutrients for bacteria and facilitating their dispersion to new sites (Haveri et al., 2007).

A high number of *S. aureus* genotypes from cattle herds worldwide have been studied to develop better strategies for the treatment of mastitis (Kot et al., 2016). Regulatory circuitry that controls virulence and adaptation of *S. aureus* to the environment is complex. This regulatory system can receive signals from the external environment to modulate biofilm formation and produce various exoproducts in a manner appropriate at the infection site (Ster et al., 2005). One of the regulatory systems, *agr*, encodes a constituent component of quorum sensing that is activated by bacterial density through auto-induced peptide secretion. Activation of *agr* follows the production of the molecule RNA III, which is an effector molecule of the agr system. RNA III follows the reduction of surface proteins, such as adhesins required for colonization. At the same time, RNA III allows the production of secreted proteins, such as nucleases and proteases, both of which participate in the release of biofilm bacteria (Novick & Geisinger, 2008; Otto et al., 2013).

Understanding the clonal relationship among strains is essential to determine the source and routes of infection, identify an outbreak, trace-route of transmission, recognize particularly virulent strains and evaluate the effectiveness of control measures (Pérez-Losada et al., 2013).

Methods commonly used to type and subtype *S. aureus* strain include Pulsed Field Gel Electrophoresis (PFGE) (Middleton et al., 2002), *spa* gene typing (Shopsin et al., 1999) and Multilocus Sequence Typing (MLST) (Smith et al., 2005). Although PFGE is considered a gold standard as a strain typing method for this genus and species, this method has greater discriminatory power in relation to other techniques (Adkins et al., 2016). The present study aimed to evaluate the
genetic diversity of a collection of *S. aureus* isolated from bovine mastitis through the screening for VFs, agr and *spa* typing, PFGE and MLST.

**Material and methods**

**Sampling**

A total of 120 milk samples were collected from cows presenting subclinical mastitis identified by California Mastitis Tests (CMT) from October to November 2012, from seven dairy farms located in the cities of Barra do Piraí, Vassouras, Passa Três, Paraíba do Sul, Rio das Flores and Carmo in the state of Rio de Janeiro, Brazil. Fifty-three strains of *S. aureus* were recovered and identified by phenotypic tests (Koneman et al., 2012) and confirmed by amplification of the coa gene (Hookey et al., 1998) and *nuc* (Ciftci et al., 2009) as showed in Table 1. A total of 17 isolates of *S. aureus* were randomly selected for the present study, which were from four dairy farms initially selected, considering the virulence profile observed.

**DNA extraction**

Bacterial total DNA extraction was performed according to the protocol established by Tito et al. (2015).

**Virulence genes**

The analysis of VFs comprised the detection of the genes *icaA* and *icaD* (Vasudevan et al., 2003), implicated in biofilm production, *fnbA* and *fnbB*, that encodes fibronectin-binding proteins (El-Sayed et al., 2006) and the hemolysin genes *hlA* and *hlB* (Nilsson et al., 1999), that encodes α- and β-hemolysins were performed by PCR (Polimerase Reaction Chain) as shown at Table 1. ATCC 29213 *S. aureus* was used as quality control.

**Table 1.** List of primers used to identify *Staphylococcus aureus* specie and virulence factors (VFs).

| Gene/ PCR Product | Sequence (5’ -3’) | Program/ Reference |
|-------------------|------------------|--------------------|
| **coa** | ATA GAG ATG CTG GTA CAG G | 94°C 4 min (94ºC 1 min, 60°C 1 min, 72°C 1 min) x 30 and 72°C 5 min (Hookey et al., 1998). |
| *(Variável)* | GCT TCC GAT TGT TCG ATG C | |
| **nuc** | GCG ATT GAT GGT GAT ACG GTT | 94°C 5 min (94°C 45 s, 68°C 45 s, 72°C 90 s) x 30 and 72°C 10 min (Ciftci et al., 2009). |
| *(279 bp)* | AGC CAA GCC TTG ACG AAC TAA AGC | |
| **pan-agr** | ATG CAC ATG GTG CAC ATG C | (94°C 1 min, 55°C 1 min, 72°C 1 min) x 25 (Shopsin et al., 2003). |
| **icaA** | CCT AAC TAA CGA AAG GTA G | (92°C 45s, 49°C 45s, 72°C 1 min) x 30 and 72°C 7min (Vasudevan et al., 2003). |
| *(1315bp)* | AAG ATA TAG CGA TAA GTG C | |
| **icaD** | AAA CGT AAG AGA GGT GG | (92°C 45s, 49°C 45s, 72°C 1 min) x 30 and 72°C 7min (Vasudevan et al., 2003). |
| *(381bp)* | GGC AAT ATG AAG ATA C | |
| **fnbA** | GCG GAG ATC AAA GAC AA | (94°C 30s, 60°C 30s, 72°C 1 min) x 30 (El-Sayed et al., 2006). |
| *(1279 bp)* | CCA TCT ATA GCT GTG TGG | |
| **fnbB** | GGA GAA GGA ATT AAG GCG | (94°C 30s, 50°C 30s, 72°C 1 min) x 30 (El-Sayed et al., 2006). |
| *(812 bp)* | GCC GTC GCC TCG AGC GT | |
| **hlA** | CTG ATT ACT ATC CAA GAA ATT CGA T | 94°C 5min, (94°C 1 min, 50°C 1 min, 72°C 1 min) x 30 and 72°C 7min (Nilsson et al., 1999). |
| *(210 bp)* | CTT TCC AGC CTA CTT TTT TAT CAG T | |
| **hlB** | GTG CAC TTA CTG ACA ATA GTG C | 94°C 5min, (94°C 1 min, 50°C 1 min, 72°C 1 min) x 30 and 72°C 7min (Nilsson et al., 1999). |
| *(300 bp)* | GTT GAT GAG TAG CTA CCT TCA GT | |
Molecular typing

agr system

Classification of agr system groups was based on the hyper variable domain of agr locus according to Shopsin et al. (2003), as showed at Table 2. A sensu primer, pan-agr, corresponding to conserved sequences of the agrB gene, was used in all reactions, combined with four anti-sensu primers, each one specific for the amplification of a single agr group based on the agr locus polymorphism.

spa typing

spa typing was performed according to Shopsin et al. (1999), as showed at table 2, and ATCC 29213 S. aureus was used as quality control. PCR products were purified using Exo-SAP-IT® (USB Corporation, Cleveland, Ohio) as recommended by the manufacturer and then sequenced. The sequences generated were edited using the Bioedit program (Hall, 1999) and Mega version 7.0 (Kumar et al., 2016), and later were analyzed using the DNAGear program (Faroq et al., 2012) for spa type designation.

Pulsed Field Gel Electrophoresis (PFGE)

PFGE typing was performed according to protocol established by the CDC (Centers for Disease Control and Prevention, 2013). Salmonella Braenderup H9812 was used as standard control. Gel images were imported into BioNumerics (Applied Maths®) for analysis. Macrorestriction patterns were compared using the BioNumerics Fingerprinting software (Version 6.5, Applied Math, Austin, TX). The similarity index of the isolates was calculated using the Dice correlation coefficient option of the software with a position tolerance of 1% and an optimization of 0.5%. The unweighted-pair group method using average linkages (UPGMA) was used to construct a dendrogram.

Multilocus sequence Typing-MLST

MLST typing was performed according to protocol described by Enright et al. (2000), considering the amplification of housekeeping genes (arcC, aroE, glpF, gmk, pta, tpi e yqiL), as showed at Table 2. ATCC 29213 S. aureus was used as quality control. PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, Ohio) as recommended by the manufacturer and then sequenced. Sequences were edited using the program BioEdit (Hall, 1999) and Mega version 7.0 (Aanensen & Spratt, 2005) and the allele and sequence types (STs) were determined using the MLST website (Trust Pharmacy, 2020).

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Table 2. List of primers used to typing Staphylococcus aureus.

| Gene    | Sequence (5’-3’) | Program                  | Reference            |
|---------|------------------|--------------------------|----------------------|
| pan-agr | ATG CAC ATG GTG CAC ATG C | (94°C 1 min, 55°C lmin, 72°C 1min)x 25 | Shopsin et al. (2003) |
| agrI    | GTC ACA AGT ACT ATA AGC TGC GAT | (94°C 1 min, 55°C lmin, 72°C 1min)x 25 | Shopsin et al. (2003) |
| agrII   | GTA TTA CTA ATT GAA AAG TGC CAT AGC | (94°C 1 min, 55°C lmin, 72°C 1min)x 25 | Shopsin et al. (2003) |
| agrIII  | CTG TTG AAA AAG TCA ACT AAA AGC TC | (94°C 1 min, 55°C lmin, 72°C 1min)x 25 | Shopsin et al. (2003) |
Results

Detection of VF’s

The characteristics of *S. aureus* isolates in this study are shown in Table 3. The analyses of VFs demonstrated the presence of majority of the genes evaluated. The distribution of VFs generated 11 virulence profiles, and the most prevalent were profile 6 (*icaA*+/*icaD*+/*fnbA*-/*fnbB*-/*phtA*+ and *hB*+) presenting 17.6% (3/17) of the isolates followed by profile 1 (positive for all virulence genes), profile 3 (*icaA*+/*icaD*+/*fnbA*-/*fnbB*+/*hA*+ and *hB*+), profile 4 (*icaA*-/*icaD*+/*fnbA*-/*fnbB*+/*hA*+ and *hB*+) and profile 11 (*icaA*-//*icaD*-/*fnbA*-/*fnbB*-/*hA*+ and *hB*+) which were observed in 11.7% (2/17) of the isolates (Table 3).

Considering the presence of VFs was also possible to observe that most of the strains tested presented the genes *icaA* and/or *icaD* (profiles 1, 2, 3, 4, 5, 6, 7, 8 and 9) (Table 3), responsible to biofilm production, presenting 82.3% (14/17) of the isolates the *icaD* gene and 47% (8/17) of the isolates the *icaA* gene.

| Gene | Oligonucleotide Sequence | Temperature/Time | Ref. |
|------|--------------------------|------------------|------|
| agrIV | CGA TAA TGC CGTA ATA CCC G | (94ºC 1 min, 55ºC 1min, 72ºC 1min)x 25 | Shopsin et al. (2003) |
| arc | TTG ATT CAC CAG CGC GTA TTG TC | 95ºC 5min (95ºC 1min, 58ºC 1min, 72ºC 1min) 30X | Enright et al. (2000) |
| aro | AGG TAT CTG CTT CAA TCA GCG | 72ºC 5min | Enright et al. (2000) |
| glp | ATC GGA AAT CCT ATT TCA CAT TC | 95ºC 5min (95ºC 1min, 58ºC 1min, 72ºC 1min) 30X | Enright et al. (2000) |
| gmk | CTA GGA ACT GCA ATC TTA ATC C | 95ºC 5min (95ºC 1min, 58ºC 1min, 72ºC 1min) 30X | Enright et al. (2000) |
| pta | TGG TAA AAT CGC ATG TCC AAT TC | 72ºC 5min | Enright et al. (2000) |
| tip | CTA TTA ACT ACA ACG TAA TCG TA | 72ºC 5min | Enright et al. (2000) |
| yqi | GAC CCT TGT GAA AAG CTT AA | 72ºC 5min | Enright et al. (2000) |
| spa | TGG TTA TCG CCA TGG CCA CT | 95ºC 5min (95ºC 1min, 58ºC 1min, 72ºC 1min) 30X | Enright et al. (2000) |
| yqi | GGT GTT GTA TTA ATA ACG ATA TC | 95ºC 5min (95ºC 1min, 58ºC 1min, 72ºC 1min) 30X | Enright et al. (2000) |
| spa | GAC CCT TTT GTT GAA AAG CTT AA | 72ºC 5min | Enright et al. (2000) |
| tip | GAC CCT TTT GTT GAA AAG CTT AA | 72ºC 5min | Enright et al. (2000) |
| yqi | CGT TGA GGA ATC GAT ACT GGA AC | 72ºC 5min | Enright et al. (2000) |
| spa | AGA CGA TCC TTC GGT GAG C | 95ºC 10 min (95ºC 30s, 60ºC 30 s, 72ºC 45s) 30X | Shopsin et al. (1999) |
| GCT TTT GCA ATG TAA TCT ACT G | 72º 10 min. | Shopsin et al. (1999) |
Considering the presence of fibronectin-binding proteins genes (*fbn*A and *fbn*B) the majority of the isolates presenting one or both of them (profiles 1, 2, 3, 4, 5, 6 and 7) as showed at Table 3. In this study, 41% (7/17) of the strains were positive for *fbn*A gene and 47% (8/17) were positive for *fbn*B gene.

The detection of the genes responsible for the production of hemolysins α and β, *hl*A and *hl*B respectively, demonstrated that only one profile (profile 5) did not present this capacity, as showed at Table 3. 94% (16/17) of the strains were positive for the *hl*A gene and 70.5% (12/17) were positive for the *hl*B gene.

### Molecular typing of *S. aureus*

The *agr* typing classified the *S. aureus* isolates predominantly as *agr* type-II, 58.8% (10/17). It should be noted that 41.2% (7/17) of the strains were classified as non-typeable.

The *spa* typing detected seven different *spa*-types (t605, t359, t23, t693, t432, t17, t10) among the 17 strains tested. *spa*-type 605 was the most prevalent, 59.2% (9/17), being found in isolates recovered from three different farms (Table 3).

Through PFGE technique it was possible to observe high genetic heterogeneity among the strains examined (Figure 1). All patterns were relatively unique to the strains examined. Strains 208 and 225 appear to be closely related as they differ in only one fragment. In addition, both strains share similar virulence profiles (Table 3) and were recovered from the same farm (farm C).

### Table 3. Characterization of *Staphylococcus aureus* isolates and virulence and typing analyses.

| Strain | Cow   | Farm | City         | *icaA* | *icaD* | *fbn*A | *fbn*B | *hl*A | *hl*B | Virulence profile | Typing analyses |
|--------|-------|------|--------------|--------|--------|--------|--------|-------|-------|------------------|-----------------|
| 47     | 363   | A    | Barra do Pirai | -      | -      | -      | +      | +     |       | 11               | NT t693 XVII CC97 |
| 54     | 369   | A    | Barra do Pirai | -      | -      | -      | +      | +     |       | 11               | NT t605 VIII ST3087 |
| 76     | 614   | A    | Barra do Pirai | +      | -      | +      | +      | -     | +     | 10               | NT t359 XV ST126 |
| 360    | Serena| B    | Passa Três    | +      | +      | -      | -      | +     | +     | 6                | 2 t432 X ST97    |
| 208    | 1110  | (39933) | C   | Carmo   | +      | +      | +      | +      | +     | +    | 1                | 2 t605 VI ST126  |
| 225    | 57    | C    | Carmo         | +      | +      | +      | -      | +     | +     | 6                | 2 t605 VII CC126 |
| 241    | 551   | C    | Carmo         | +      | +      | +      | -      | +     | +     | 6                | 2 t605 IX ST126  |
| 262    | 518   | C    | Carmo         | +      | +      | +      | +      | +     | +     | 4                | 2 t605 I CC126   |
| 281    | 219   | C    | Carmo         | -      | +      | -      | +      | +     | +     | 4                | NT t23 IV ST747  |
| 295    | 858   | (30976) | C  | Carmo   | -      | +      | -      | -      | +     | -     | 8                | NT t17 XIII ST5  |
| 300    | 391   | C    | Carmo         | +      | -      | +      | -      | -     | +     | 5                | NT t10 III ST5   |
| 310    | 872   | C    | Carmo         | +      | +      | +      | +      | +     | +     | 1                | 2 t359 XVI ST126 |
| 311    | 683   | C    | Carmo         | +      | -      | +      | +      | +     | +     | 3                | NT t23 II ST126  |
| 325    | Abeia | C    | Carmo         | +      | -      | -      | +      | +     | +     | 7                | 2 t605 XI ST126  |
| 336    | Pintura | D     | Vassouras     | +      | -      | +      | +      | +     | -     | 2                | 2 t605 XIV ST126 |
| 338    | Germana | D    | Vassouras     | +      | -      | +      | +      | +     | +     | 3                | 2 t605 XII ST126 |
| 339    | Germania | D     | Vassouras     | +      | +      | +      | +      | +     | +     | 9                | 2 t605 V ST126   |

NT: nontypeable; (+): number of isolates that amplified; (-): number of isolates that not amplified.
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They also share ST 126 and spa type - spa 605 and were both classified as agr type-II but were isolated from different animals. Analyses of MLST typing demonstrated five different ST/CC types (ST/CC126, ST5, ST/CC97, ST3087 and ST747) (Table 3). Most of the strains, 64.7% (11/17), were identified as belonging to ST/CC 126 and this was distributed on three different farm properties.

**Discussion**

**Analyses of VFs detection of *S. aureus***

Most *S. aureus* strains have the genetic capacity to produce biofilm. Our results corroborate with Marques et al. (2017) that showed a high abundance of genes icaA and icaD in *S. aureus* isolates, producing biofilm in samples from bovine intramammary infections in Brazil.

Acosta et al. (2018) reported a high frequency of fnbA (94%), fnbB (81.3%), blaA (88%) and hIB (88.3%) genes in *S. aureus* from milk samples, and these genes were more abundant in samples from bovine milk than goat milk in a recent study developed in cow and goat herds raised in three different geographical regions of the state of Pernambuco, Brazil, and these results corroborate with our data.

The importance of monitoring biofilm production (icaA and icaD), fibronectin-binding proteins (FBN) A and B (fnbA and fnbB) and α- and β-hemolysins (blaA and hIB) genes in *S. aureus* isolated from dairy environment is reinforced due to its high frequency amongst the isolates investigated (Table 3). It supports the idea that these VFs are strictly related to severe mastitis cases.

**Analyses of molecular typing of *S. aureus***

Agr typing of *S. aureus* isolates showed a predominance of agr type-II and non-typeable. Similar results were reported by Fabres-Klein et al. (2015) who also detected the prevalence of agr type-II suggesting that this type is better adapted to the dairy environment, and by Marques et al. (2017) that also noted that not all *S. aureus* strains examined could be classified, when analyzing *S. aureus* from bovine mastitis in Brazil.

spa type 605 has been reported in several studies of bovine mastitis (Guinane et al., 2010; Sakwinska et al. 2011). This spa type has also been reported as an ordinary strain associated

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**Figure 1.** Similarity dendogram generated from data obtained from *Staphylococcus aureus* typing using PFGE technique (Pulsed Field Gel Electrophoresis) using the Dice grouping method, BioNumerics.
Considering PFGE analyses, the similarity between the strains 208 and 225 could be occurred by a small-scale mutation or by the presence of mobile genetic elements. The fact that they shared several characteristics and only differed at the animal origin suggests that there may be similar strains circulating and causing mastitis in this particular herd. Previous studies conducted by our research group on distinct farms of the present study showed similar results, high genetic heterogeneity, and the absence of clonally related strains (Marques et al., 2013). Rabello et al. (2007) also reported similar results when using the same technique to evaluate *S. aureus* strains from Rio de Janeiro state. A possible explanation for the high genetic heterogeneity of *S. aureus* strains in properties in the state of Rio de Janeiro can be explained by the fact that the production is carried out on small properties with distinct characteristics for milking and breed. This high heterogeneity would also imply the difficulty of obtaining effective prophylactic measures to control mastitis because of the diversity of strains implicated.

ST 126 has been reported in several studies associated with bovine mastitis outbreaks (Guinane et al., 2010; Sakwinska et al., 2011) and has also been reported as implicated in bovine mastitis in the state of Rio de Janeiro (Aires-de-Sousa et al., 2007; Rabello et al., 2007). A study by Smyth et al. (2009) showed that ST126 is usually associated with *agr* type-II and *spa* type t605. A similar result was observed in our study that presenting a prevalence of *spa* type t605. However, some strains were also identified as t359 and t23, suggesting that *spa* type and ST are not exclusively linked. This can be explained by using a single locus method such as *spa* typing for macroepidemiologic investigations, which can distort the underlying clonal relationships (Nübel et al., 2008).

ST 97 has also been detected in previous studies of bovine mastitis in the state of Rio de Janeiro, this ST appears to be widely distributed in several countries around the world, in addition to being related to LA-MRSA strains and has also been implicated as a causative agent of bovine mastitis (Meemken et al., 2010; Smith et al., 2005).

### Comparative analysis of the genetic profiles generated by different molecular typing techniques

The clustering of strains, according to the virulence genes distribution, yielded 11 distinct profiles. Most strains shared the same virulence profile but not the same type through other typing and subtyping techniques, as already reported by Melles et al. (2004) in a study evaluating the population dynamics and expansion of pathogenic clones of *S. aureus* in healthy humans. Their study suggested that not all clones of *S. aureus* share the same VFs because the strains may be under different environmental pressure that results in strains from some clonal lineages being more virulent than others.

Regarding *spa*-type analysis of the strains examined in this study, seven different *spa* types were detected among the 17 strains examined. Genotypic characterization based on the investigation of a locus of the highly variable tandem repeat region of the *spa* gene is another successful tool for the genotypic characterization of *S. aureus*. *spa* gene typing also considers the recombination of *X* region events. Since recombination occurs more frequently within this region, this typing method typically provides more types (*spa*) than MLST. Although *spa* typing and MLST are usually concordant and allow a similar classification for distinct genotypes, there are also several cases of sorting errors by the *spa* typing technique, which are most likely caused by recombination events. Thus, *spa* typing is a useful method to obtain information about the genotype of the strains but should not replace the MLST technique (Strommenger et al., 2008).

Regarding the detection of STs by MLST technique, five different sequence types (ST) or clonal complexes (CC) were detected. According to MLST technique, a clone is defined based on the sequence type, so an isolate is defined as a clone if it shares the same ST. This definition of clones using the MLST technique is very useful for investigating the evolutionary history of bacteria. However, it may lead to misconception since the term clone is defined as bacterial isolates with indistinguishable genotypes. However, in the case of bacteria, it is not possible to define clones based only on this definition, since high rates of mutation and recombination, as well as the acquisition or loss of mobile genetic elements, can lead (depending on the species) to a bacterial genome that is highly variable. Permanent diversification of the ancestral genome results in an increasingly diverse set of genotypes. The MLST method, however,
provides an efficient method to identify genetic lineages that share the same ancestor, due to MLST method being based on the amplification of multiple internal fragments in the DNA sequence of relatively conserved housekeeping genes (with approximately 450-500 bp for each gene) that are present in all strains of the same species. Genetic relationships between the strains is determined by the analysis of these housekeeping genes sequences, which are compared by analysis of nucleotide substitution in the sequence. Based on this definition, clones (MLST) include isolates of the same sequence type but are not necessarily genetically identical (Ranjbar et al., 2014; Spratt, 2004).

PFGE analysis generated quite distinct profiles demonstrating the heterogeneity of the strains studied. Compared with MLST results, this technique allows detection of more frequent genomic alterations. Thus, using PFGE it is possible to demonstrate the diversity of S. aureus strains sharing the same sequence type and thus show that clones based on MLST definition are not necessarily genetically identical (Murchan et al., 2003).

PFGE and MLST techniques are considered as some of the best methods for molecular typing of S. aureus but did not have a reasonable correlation when applied individually. However, when combined, it is possible to obtain an accurate overview of the population of S. aureus present in dairy herds. In view of the results generated from this study, it can be concluded that the choice of the typing method and its application depends on the type of epidemiological study that is to be carried out. However, it should be emphasized that in order to carry out the study, the application of multiple typing techniques that can be combined provides higher power to the analysis and the research question.

Although there is much still that remains to be investigated and understand regarding recombination events in S. aureus it seems clear that it occurs frequently enough to be detectable but not often enough to destroy vertical or clonal signals. Understanding how the mix of horizontal and vertical processes of clonal evolution contributes to the structuring and evolution of this bacterial species is a goal to be achieved, and techniques such as Whole Genome Sequencing (WGS) can clarify these aspects.

However, for these studies to be reliably validated, epidemiological and clinical biases need to be eliminated. Currently, most of the phylogenomic studies of S. aureus consist of sample collection and analysis related to clonal complexes of medical interest, which hinders an evolutionary view of these transformations (Planet et al., 2016). Regarding isolates from animal production, especially those of bovine origin, these studies are still emerging and point to a highly diversified phylogenetic structure. Possibly, due to the intense selection pressure exerted by the use of antimicrobials and sanitizing agents in the production environment. Also, the great diversity of bacterial species in a constant competition that induces elaboration of strategies for survival and adaptation to the host, fomites, and the environment as a whole.

Conclusions

A high genetic variety of S. aureus strains associated of the presence of different virulence factors justified the absence of clonal strains at the properties evaluated. Besides that, the definition of S. aureus clonal strains is not fully understood since there are a limited number of studies in S. aureus associated with bovine mastitis. Therefore, for a better understanding of phylogenetic diversity of this agent more studies are necessary to clarify the role of population dynamics and clones of S. aureus at the pathogenicity of mastitis in bovines, improving the monitoring and the control of this disease.

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Ethics statement

The study followed all criteria established by Ethical Research Committee at UFRRJ for research involved non-human vertebrate (CEUA Nº 3664040915).
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Conflict of interests

BSS, CCM, NLB, DAM, MAG, TAA, SMOC, ISC, CML and MMSS- No conflict of interests.

Authors’ contributions

BSS, CCM, NLB, DAM, MAG, TAA, SMOC, ISC, CML and MMSS - All authors made substantial contributions to the conception, design, acquisition, analysis, or interpretation of data for this work. BSS, CCM, DAM, MAG, TAA- visited the milk farms, collected, and processed all samples. BSS, NLB, CML, worked at the typification of S. aureus isolates using PFGE technique.

Availability of complementary results

All information obtained as a result of the study is included in the manuscript.

The study was carried out at Laboratório de Bacteriologia Veterinária, Departamento de Microbiologia e Imunologia Veterinária, Universidade Federal Rural do Rio de Janeiro - UFRJR, Seropedica, RJ, Brasil, and Bacterial Pathogenesis Laboratory, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Iowa, USA.

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