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Association between *coa* gene and enterotoxin gene in *S. aureus* from dairy cattle in Brazil

Mylena Karoline VALMORBIDA[1*], Marita Vedovelli CARDOZO[2], Camila Chioda de ALMEIDA[1], Natália PEREIRA[1], Diogenes DEZEN[3], Marcella Zampoli de ASSIS[3], Newton Valério VERBISCK[4], Eliete GRIEBELER[3], Lucas José Luduverio PIZAURO[5], Fernando Antônio de ÁVILA[6]

Abstract

Staphylococcus aureus is an important agent in bovine mastitis, and some specific virulence factors may be implicated in this disease. Therefore, this study aimed to investigate the importance of the presence of coagulase, superantigens, genotypic and phenotypic resistance, and pulsotypes in 65 *S. aureus* isolates from bovine clinical and subclinical mastitis in the Southeast of Brazil. A high correlation was observed between the genes *coa* and *see,* as well as between the *sei* and the *see* and *seh*. High resistance rates were observed for penicillin (95.4%), tetracycline (89.2%), cefoxitin (86.1%), oxacillin (84.6%), erythromycin (84.6%), clindamycin (84.6%), chloramphenicol (81.5%), ceftriaxone (80.0%), and ampicillin (80.0%). Analysis of antimicrobial resistance profiles showed that 89.2% of isolates were multi-drug-resistant. No *mecA*-positive *S. aureus* isolates were detected. It was observed that seven isolates were resistant to all the β-lactam tested while being susceptible to cefoxitin, which could be indicative of borderline methicillin resistance in *S. aureus*. High genetic diversity with no specific virulence profile being predominant was observed. Thus, this study observed a high correlation between the *coa* and enterotoxins genes, and demonstrates that there is no predominant pulsotype causing intramammary infection and that there is a high rate of antibiotic resistance in *S. aureus* isolates from dairy farms in the southeast regions of Brazil.

Keywords: mastitis; toxins; resistance; pulsed-field gel electrophoresis; dairy.

Practical application: Data analyses have shown a correlation between coagulase (*coa*) and enterotoxin (*sec*, *see*, *sei*, and *seh*) genes in *S. aureus* from intramammary infection in dairy cattle and that borderline oxacillin resistance in *S. aureus* may be present among beta-lactam multi-resistant isolates from dairy farms in Brazil.

1 INTRODUCTION

Milk production has been growing worldwide with mastitis as one of the costliest diseases in dairy farms due to decreased production, discard of milk, treatment cost, reduced product quality, anticipated culling, and increased labor (Heikkilä et al., 2012). *Staphylococcus aureus* is reported to be responsible for about 40% of mastitis cases worldwide (Ren et al., 2020). This predominance is attributed to this agent possessing a varied array of virulence factors such as hemolysin, exfoliative toxins, enterotoxins, toxic shock syndrome toxins, and coagulase that hinder treatment and disease control in the affected herds (Luo et al., 2018). Also, according to Vitale et al. (2018), more than 20 enterotoxins produced by *S. aureus* strains are known to date to be the main cause of food poisoning. Furthermore, the toxic shock syndrome toxin produces clinical signs such as hypotension, fever, and rash, and after a week or two of infection, causes skin peeling (Tam & Torres, 2019). Also, coagulase is one of the main virulence factors produced by *S. aureus*, causing coagulation of the host's blood plasma, and it is believed that coagulase producer isolates are more pathogenic and mostly the ones that cause bovine mastitis worldwide (Javid et al., 2018). Due to the indiscriminate use of antibiotics, it is now possible to observe that strains of *S. aureus* have been developing mechanisms to survive even in the presence of antimicrobial agents. The most notable examples of this acquired resistance are methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) (Dan et al., 2019). There are several other types of resistance mechanisms to various other classes of antimicrobials, and the resistance can be transferred from strains that affect animals to those that affect humans, causing a major public health problem (Dan et al., 2019).

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[1]*Universidade Estadual Paulista "Júlio de Mesquita Filho", Postgraduate Program in Agricultural Microbiology, Faculty of Agricultural and Veterinary Sciences, Department of Veterinary Pathology, Jaboticabal, SP, Brazil.*
[2]*Universidade do Estado de Minas Gerais, Department of Biomedical Sciences and Health, Passos, MG, Brazil.*
[3]*Instituto Federal Catarinense, Veterinary Microbiology Laboratory, Concórdia, SC, Brazil.*
[4]*Empresa Brasileira de Pesquisa Agropecuária, Embrapa beef cattle, Campo Grande, MS, Brazil.*
[5]*Universidade Estadual de Santa Cruz, Department of Agricultural and Environmental Sciences, Ilhéus, BA, Brazil.*
[6]*Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculty of Agricultural and Veterinary Sciences, Department of Preventive Veterinary Medicine and Animal Reproduction, Jaboticabal, SP, Brazil.*
*Corresponding author: mylikarolyne15@gmail.com

In this regard, it has been demonstrated that the staphylocoagulase (*coa*) gene is located outside of genomic islands and known to be polymorphic, which, in combination with virulence genes that are also present outside of genomic island such as enterotoxins (*se*) genes that may only be present in certain types of *S. aureus* strains, could demonstrate a specific virulence profile that could be implicated in causing intramammary infections (IMI) (Baba et al., 2008). Also, it has been shown that borderline oxacillin-resistant *S. aureus* (BORSA), whose first documented underlying mechanism of borderline resistance is the hyper-production of beta-lactamase, may be a more pronounced problem than it may have been anticipated (Hryniewicz & Garbacz, 2017).

Therefore, the ingestion of food contaminated with *S. aureus* enterotoxins can cause intoxication with symptoms such as vomiting, diarrhea, and inappetence (Käppeli et al., 2019). Thus, due to these and other factors, this study aimed to investigate the importance of the presence of the coagulase, superantigens (enterotoxins), and genotypic and phenotypic resistance of *S. aureus* in clinical and subclinical bovine cases from the Southeast of Brazil.

2 MATERIALS AND METHODS

2.1 Isolates origin

In this study, 65 *S. aureus* isolates were obtained from 2016 to early 2019 from 345 milk samples that were collected from cows with clinical or subclinical mastitis. The animals were from 70 dairy herds located in 16 municipalities (Alto Bela Vista, Arabutã, Capinzal, Concórdia, Descanso, Ipira, Irani, Jaborá, Lindóia do Sul, Modelo, Peritiba, Pinhalzinho, Ponte Serrada, Presidente Castelo Branco, Seara, and Xavantina) in the southern region of Brazil (Figure 1). These isolates were received at the Veterinary Microbiology Laboratory of the Instituto Federal Catarinense and were previously isolated and identified according to their morphological, microscopic, and biochemical patterns (Markey et al., 2013). A brief interview was conducted regarding the mastitis state of the animal when the samples were received. After the isolates were identified as *Staphylococcus* spp., a rabbit plasma coagulation assay was performed with a commercial kit (Coagu-Plasma, Laborclin®, Pinhais, Brazil) according to manufacturer instructions, with *S. aureus* ATCC 25293 and *S. epidermidis* ATCC 12228 used as positive and negative controls, respectively. Coagulase-positive isolates were considered suspects of *S. aureus*. All isolates were stored at -20°C and brought to the Sao Paulo State University for further analysis.

2.2 DNA extraction

DNA extraction was performed after well-isolated colonies were obtained in 5% sheep blood agar and were used to inoculate tubes containing 5 mL of BHI broth. After incubation at 37°C for 18 h, genomic DNA extraction was done

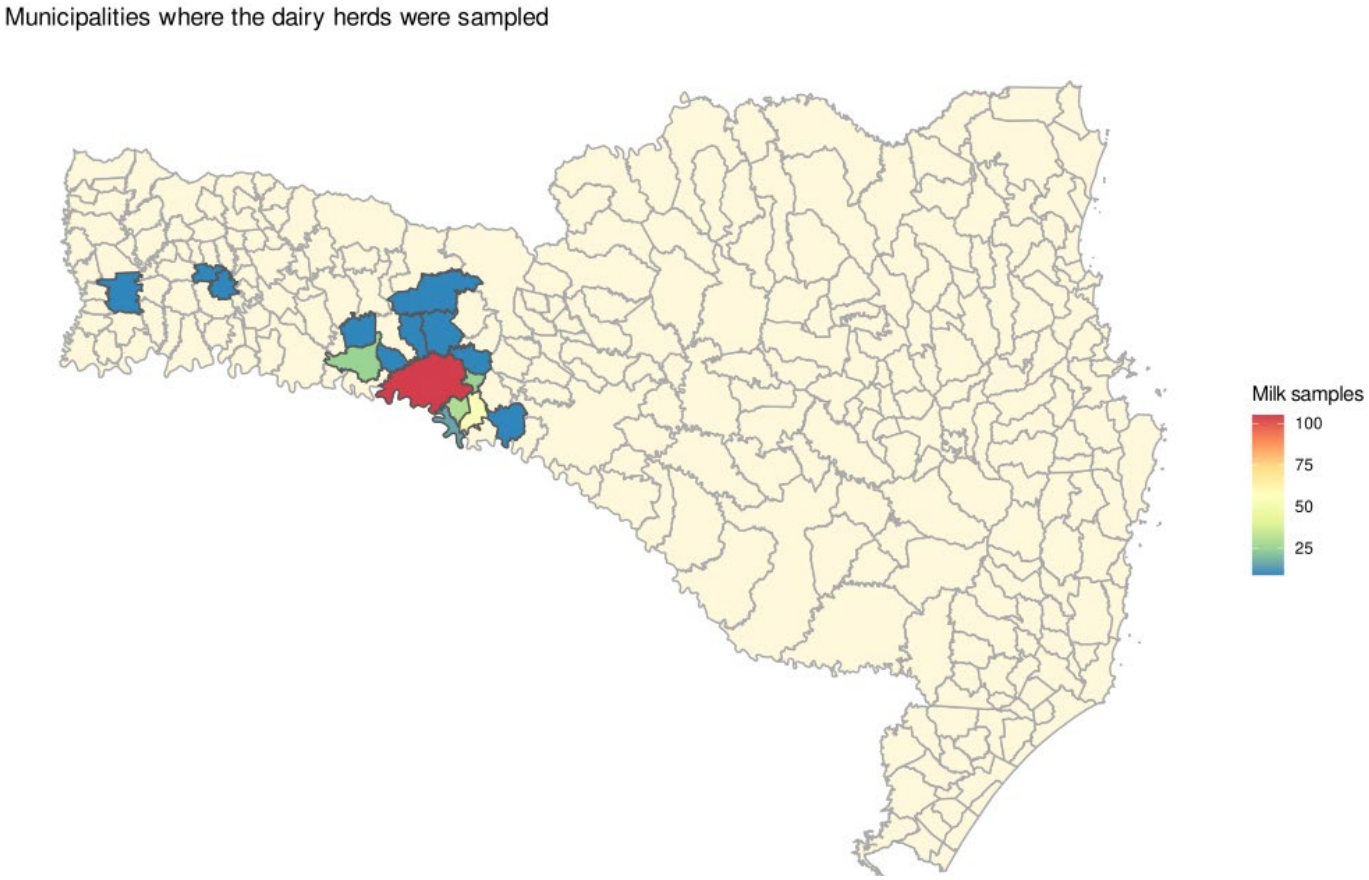

Figure 1. Spatial distribution of the municipalities where the dairy herds were sampled.

using a modified (Kuramae-Izioka, 1997) method. A 1 mL of bacterial culture was transferred to 2.0 mL tubes, centrifuged at 13,400 × g for 5 min, and the cell pellet re-suspended in 700 µL of extraction buffer [160 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 20 mM NaCl, and SDS 0.5% (w/v)]. After that, the cell suspensions were vortexed and maintained in a water bath at 65°C for 40 min. Subsequently, 300 µL of 5 M potassium acetate was added and then incubated in ice for 30 min. The samples were removed from ice, and 650 µL of chloroform and isoamyl alcohol [24:1 (v/v)] were added. The samples were then mixed by inversion for 2 min and centrifuged at 13,400 × g for 10 min at 4°C. Following centrifugation, the supernatants were transferred to new tubes, and the DNA was precipitated by the addition of 1,000 µL of ice-cold absolute ethanol. The DNA precipitate mixtures were mixed by inversion and placed at -20°C overnight prior to recovery of the DNA by centrifugation at 13,400 × g for 10 min at 10°C. The resultant DNA pellets were air dried for 30 min and suspended in 30 µL 10 µM Tris-HCl, pH 7.3, 0.1 µM EDTA (TE).

2.3 Species identification by molecular biology and MALDI-TOF mass spectrometry

Polymerase chain reaction (PCR) identification for the staphylococci genus was performed by the TStag PCR (Martineau et al., 2001), and the specie level of *S. aureus* was performed by the *cydB* gene (De Almeida et al., 2018) as follows: a DNA aliquot (2 µL) was added to the mixture containing 0.4 µL of dNTP (10 mM), 2 µL of 10´ buffer solution (100 mm Tris-HCl, 500 Mm KCl, 0.8% Nonidet P40), 0.8 µL of 50 mM $MgCl_2$, 1.0 µL of each 10 pM primer, and a Taq DNA polymerase unit (Fermentas, Europe). The cycles consisted of an initial stage at 95°C for 3 min, followed by 30 cycles, each containing denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final extension cycle was at 72°C for 7 min. The PCR products were visualized in an agarose gel containing 1.5% SYBR-safe (Thermo Fisher Scientific) by exposing the gel to UV light and were photographed. *S. aureus* ATCC 25923 was used as a positive control, and *S. epidermidi*s ATCC 12228 was used as a negative control.

2.4 MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry was performed in a MALDI Biotyper system (Bruker Daltonics, Billerica, MA, USA), and the results were analyzed using the Biotyper 3.1 software (Bruker Daltonics, Bremen, Germany). Bacterial isolates were prepared by an initial fixation step with 100% ethanol (PA), followed by 70% formic acid and acetonitrile extraction. For spectra acquisition, 1 µL of bacterial proteins was transferred to a spot of a 96 polished steel target plate, then 1 µL of α-cyano-4-hydroxycinnamic acid was added, and spotted samples in triplicates were air-dried 1–2 min at room temperature. The acquired mass spectra were analyzed and compared with the reference spectra at MBT Compass Library DB-7311 (Bruker Daltonics, Bremen, Germany). Bacterial identification to the species level was achieved if the score value was between 2.30 and 3.00. A score of 2.00–2.29 was accepted as an accurate genus identification, values of 1.70–1.99 had probable genus identification, and for values below 1.70, the identification was not reliable.

2.5 Coagulase, enterotoxin, and antibiotic resistance genes detection

The detection of the coagulase gene (*coa*) of *S. aureus* was performed by PCR of its conserved region, according to De Almeida et al. (2018). Also, the detection of staphylococcal superantigens was performed by PCR as follows. The toxic shock syndrome toxin (TSST-1) gene (*tst*) was detected as described by Paniagua-Contreras et al. (2012), and the enterotoxin-encoding (SEA, SEB, SEC, SED, SEE, SEG, SEJ, and SEI) genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *sej*, and *sei*) were detected as described by Kim et al. (2011). In addition, PCR for the detection of antimicrobial resistance genes was performed to detect the *mecA* (methicillin resistance) (Mehrotra et al., 2000), *aacA-D* (aminoglycoside resistance) (Kumar et al., 2010), *blaZ* (penicillinase resistance), *tet(M)* (tetracycline resistance), and *erm(A)* (erythromycin resistance) genes (Duran et al., 2012).

2.6 Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed using the disk diffusion method established by Bauer et al. (1966), following the standards established by the Clinical & Laboratory Standards Institute (CLSI, 2018). The inoculates were prepared in tubes containing 3 mL of 0.85% saline and with solution turbidity at OD_{625nm} ranging from 0.1 to 0.2 nm. Subsequently, this inoculum was seeded with the aid of a sterile swab in Mueller-Hinton plates (MH, Himedia, India), and after dying, the following antibiotics were distributed on the plate: amoxicillin + clavulanic acid 30 µg (AMC), ampicillin 10 µg (AMP), cefepime 30 µg (COM), cefazolin 30 µg (CFZ), ceftriaxone 30 µg (CRO), cefoxitin 30 µg (CFO), ciprofloxacin 5 µg (CIP), chloramphenicol 30 µg (CLO), clindamycin 2 mg (CLI), erythromycin 15 µg (ERI), gentamicin 10 mg (GEN), oxacillin 1 µg (OXA), penicillin G 10UI (PEN), rifampicin 30 µg (RIF), sulfazotrin 25 µg (SUT), tetracycline 30 µg (TET), and teicoplamine 30 µg (TEC). The plates were incubated for 18 h in an oven at 37°C, the inhibition halos formed were measured, and the strains were classified as sensitive, resistant, or of intermediate sensitivity for the respective active principle using the parameters adopted by the Clinical & Laboratory Standards Institute (CLSI, 2018).

2.7 Pulsed-field gel electrophoresis

The isolates pulsotypes were characterized by the standard PFGE protocol from PulseNet, as described by Ribot et al. (2006), with modifications. The system used was the CHEF DR-III (Bio-Rad, USA), the chromosomal DNA was digested with the enzyme SmaI (Invitrogen, USA), and the electrophoresis was performed on a Pulsifield Certified 1% agarose gel (Bio-Rad, USA) with a voltage of 6 V cm^{-1}, at an angle of 120°, with an initial pulse time of 2.2 s and a final pulse time of 54.2 s. The gels were subjected to electrophoresis for 21 h at a temperature of 14°C. The Salmonella Braenderup H9812 strain was used as a standard for the experiment, and their DNA fragments were used as molecular size markers. DNA

fragment similarities were compared using the Dice coefficient at 1% tolerance and 0.5% optimization. The dendrogram was calculated using the UPGMA clustering method using the BioNumerics Software, version 7.1 (Applied Mathematics, Sint-Mastens-Latem, Belgium).

2.8 Data analysis

All data based on the presence and absence of the genes were used to perform Hierarchical clustering analysis by PAST software v4.03 (Hammer et al., 2001). Also, the correlation between the presence/absence of superantigen-associated genes, T-distributed stochastic neighbor embedding analysis, and gene profiling heatmap were obtained using NumPy (Van Der Walt et al., 2011), Matplotlib (Hunter, 2007), and R (R Core, 2016).

3. RESULTS AND DISCUSSION

The production of coagulase is important to distinguish staphylococcal strains into coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS) (Rossi et al., 2020). In this regard, reports of typically coagulase negative staphylococci producing coagulase have been made before (De Almeida et al., 2018; Santos et al., 2016), thus highlighting the importance of other means for the specific identification of *S. aureus*, such as PCR. The use of the (*cydB*) gene has been reported as a new approach to complement MALDI-TOF MS (Szafraniec et al., 2020). In this study, the use of this approach for *S. aureus* identification demonstrated an 88% (57/65) agreement with the results obtained by MALDI-TOF MS and the *cydB* gene PCR. Similar results have been observed by Pérez-Sancho et al. (2018) with an 81% success rate for *S. aureus* identification by MALDI-TOF MS; other studies observed a much higher identification rate (>90%) (Manukumar & Umesha, 2017; Nix et al., 2020) that could be attributed to differences in the software spectra (Biotyper), especially considering that the database could have a number of different standards for each specific species, as well as the isolates proteomic characteristics.

The tube coagulase test is used to detect the free coagulase, which is encoded by the *coa* gene. This enzyme promotes the conversion of fibrinogen to fibrin that forms a coat on the surface of the bacteria, protecting it from phagocytosis and other host defenses (Rossi et al., 2020). In this study, it was observed that 75.4% (49) of the isolates cloth rabbit plasma and 94.0% of isolates were *coa* positive, with only one tube test-positive isolate being *coa* negative. Also, it was observed that 11 *coa*-positive strains did not cloth rabbit plasma. This result may indicate that although some isolates may have the coagulase gene, they did not express it. This behavior was also found in the study by De Almeida et al. (2018), in which strains of *S. aureus* with the *coa* gene also did not cloth rabbit plasma and came from buffalo IMI. One highlight to be made is that the *coa* gene is highly polymorphic due to the presence of tandem repeats at its 3' end, thus generating different coagulase genotypes that were proven to be more resistant to neutrophil activities (Javid et al., 2018). In the study by Silva and Silva (2005), the authors demonstrate that although mastitis was caused by *S. aureus* strains that have many variants of the *coa* gene, only a few *coa* gene variants predominate. This is important, especially when associated with the fact that the staphylocoagulase gene is located outside of genomic islands and known to be polymorphic, which, in combination with virulence genes that are present only in certain types of *S. aureus* strain, could demonstrate a specific virulence profile (Baba et al., 2008).

In this study, five out of nine surveyed superantigenic toxins such as the enterotoxins genes were identified, namely: *sec* (3.07%), *seg* (6.15%), *see* (7.69%), *seh* (23.1%), and *sei* (53.8%) (Table 1). It was possible to observe a high correlation between the detection of the *coa* gene and the presence of the *see* and *sei* genes (Figures 2 and 3) and between the presence of the *sec* gene and the *see* and *seh* genes. In this regard, the use of data analyses has demonstrated the importance and association of virulence genes in staphylococci related to mastitis, but they were focused on adherence and biofilm genes (Pizauro et al., 2021). The high

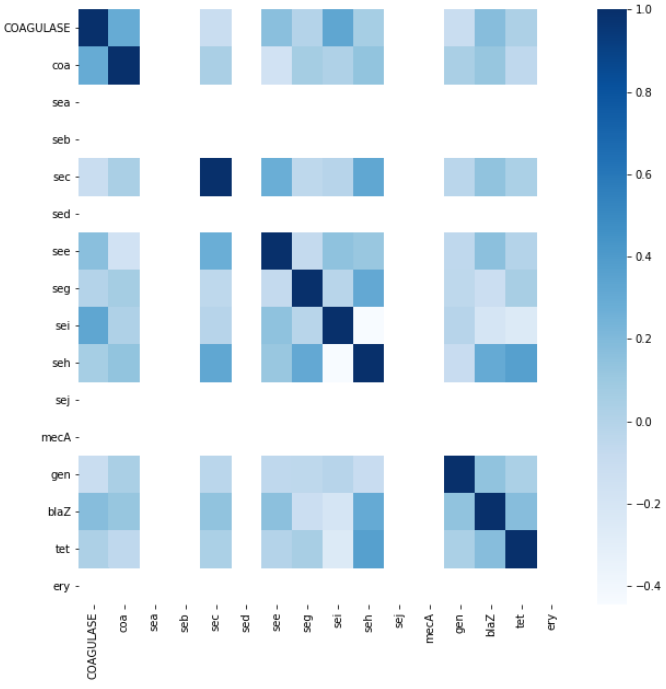

Figure 2. Correlation between the presence of virulence genes in *S. aureus* from IMI from dairy farms in Southeast of Brazil. Strong correlation is observed when their coefficient is closest to 1.0.

Table 1. Coagulase, virulence, and antibiotic gene profile of the 65 *Staphylococcus aureus* isolates from IMI.

Values	Coagulase	*seb*	*sec*	*sed*	*see*	*seg*	*sei*	*seh*	*sei*	*mecA*	*AacA-D*	*blaZ*	*tet(M)*	*erm(A)*
Absolute	49.0	0.00	2.00	0.00	5.00	4.00	35.0	15.0	0.00	0.00	2.00	12.0	26.0	0.00
Relative	75.4	0.00	3.08	0.07	7.69	6.15	53.8	23.1	0.00	0.00	3.08	18.4	40.0	0.00

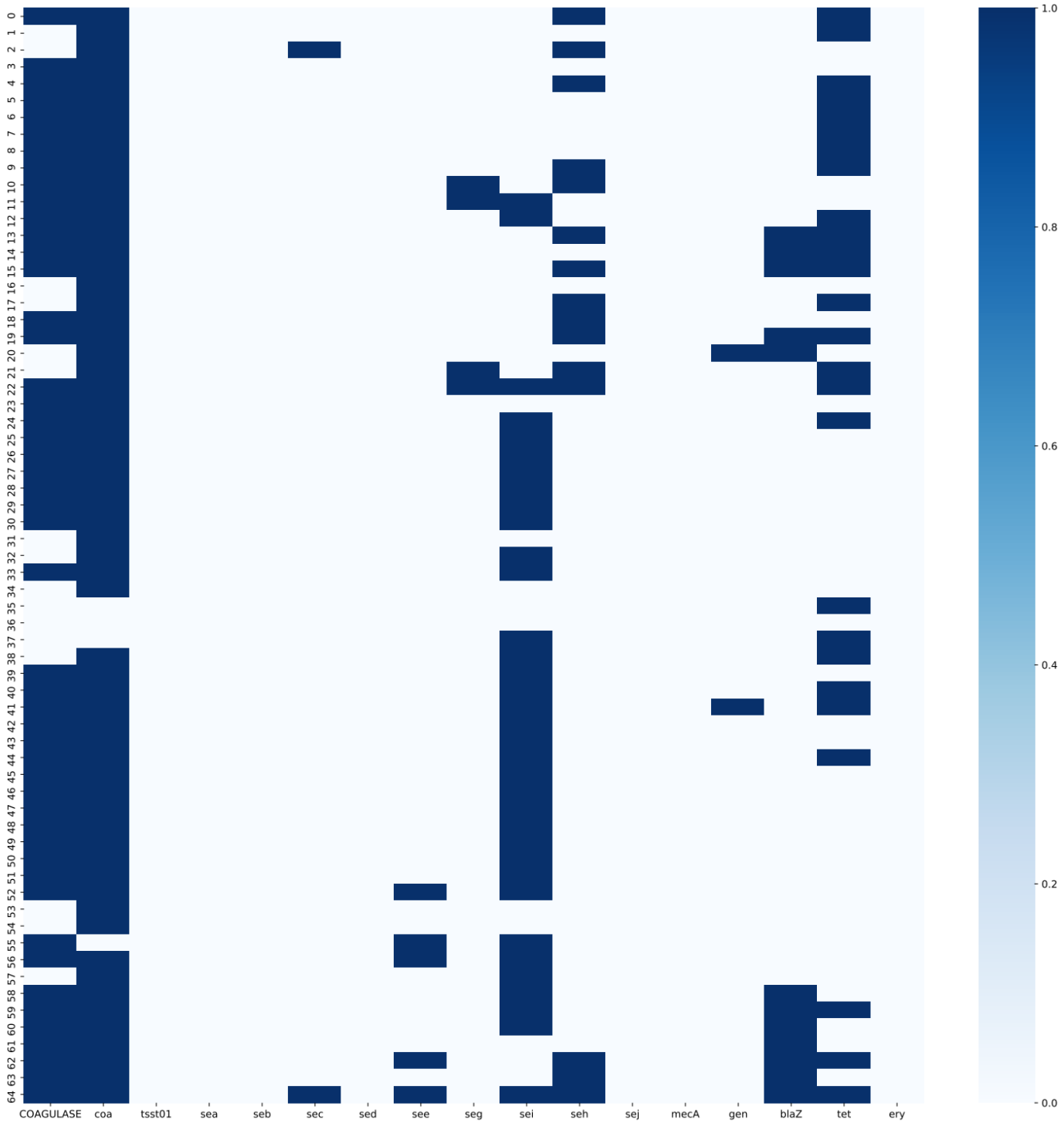

Figure 3. Heatmap based on the presence/absence of coagulase and virulence genes in *S. aureus* from IMI from dairy farms in Southeast of Brazil.

correlation observed between these genes could be attributed to the fact that the *sec* gene, which encodes enterotoxin C, is thought to have an important role in mastitis infection due to its ability to induce significant inflammation with inflammatory cell infiltration and tissue damage in a dose-dependent manner (Fang et al., 2019).

Fursova et al. (2018) observed that in 60 strains of *S. aureus* obtained from subclinical mastitis cases in farms in Central Russia, the occurrence of the enterotoxin genes *sec* (50%), *see* (46.6%), and *sei* (10%) was also associated with the presence of cytotoxin genes, mainly *hlA*, *psmA*, *psmB*, and *hld*. In addition, Ren et al. (2020) observed that the 65 *S. aureus* isolates from subclinical bovine mastitis from dairy farms in southern Xinjiang, China, had the *sec* (33.8%), *seh* (41.5%), and *sei* (41.5%) genes that were distributed among 44 distinct sequence types (Sts). These findings also contribute to the fact that their presence may

be correlated with and attributed to certain types of *S. aureus* strains carrying specific virulence traits. It is important to notice that the presence of classic enterotoxins (SE) determinants in *S. aureus* is known to cause sporadic food poisoning episodes and food-borne outbreaks, especially when considering that these enterotoxins are thermoresistant and can resist pasteurization temperature (Wang et al., 2018).

Recent studies (Liu et al., 2017; Zhao et al., 2016) have reported a discrepancy between the genotypic and phenotypic antibiotic resistance profiles. This behavior was attributed to the fact that there are different types of genes related to resistance, in addition to variations of the same type of gene (Liu et al., 2017; Zhao et al., 2016). In this study, there was a very low association between the presence of resistance genes assessed (*mecA*, *aacA-D*, *blaZ*, *tet(M)*, and *erm(A)*) and their respective expected antibiotic resistance phenotypes (Figure 4), with the exception of the *tet(M)* gene and tetracycline resistance. This could be attributed to the variations in antibiotic resistance genes and the types of genes involved in antibiotic resistance. Also, the genotypic evaluation by PCR of the strains showed a low presence of *aacA-D* (3.1%), *blaZ* (18.5%), and *tet(M)* (40.0%). No strain was positive for the *erm(A)* gene. A high phenotypic resistance was observed for penicillin (95.4%), tetracycline (89.2%), cefoxitin (86.1%), oxacillin (84.6%), erythromycin (84.6%), clindamycin (84.6%), chloramphenicol (81.5%), ceftriaxone (80.0%), and ampicillin (80%) (Table 2). Only seven isolates (10.8%) did not present resistance to more than four antibiotic classes simultaneously and thus were not considered multi-drug resistant, with 89.2% being considered multi-drug resistant. High resistance to penicillin is not uncommon and is attributed to the fact that β-lactams are widely prescribed to treat bovine mastitis cases worldwide such as in China, as well as in Turkey (Ren et al., 2020), Brazil (Freitas et al., 2018), and Egypt (Ameen et al., 2019).

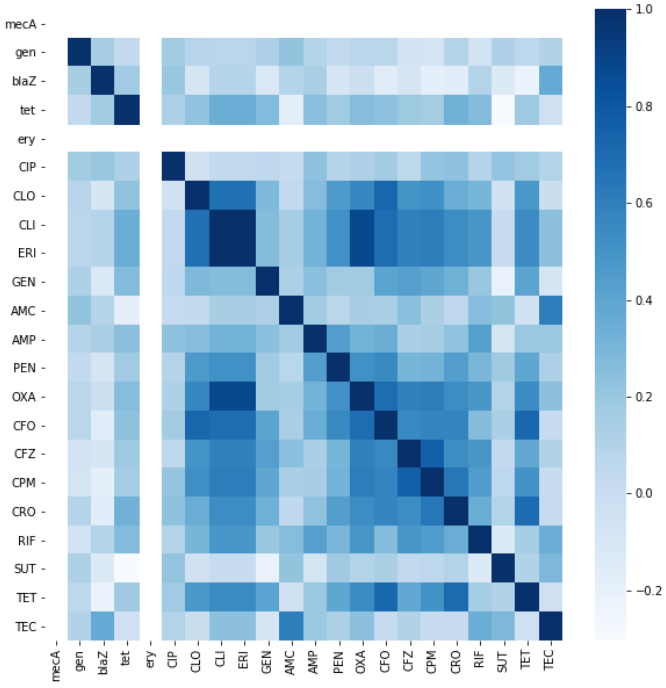

Figure 4. Correlation between the presence of antibiotic resistance genes and phenotypic resistance in *S. aureus* from IMI from dairy farms in Southeast of Brazil. Strong correlation is observed when their coefficient is closest to 1.0.

The high resistance rates observed in this study to tetracycline, cefoxitin, erythromycin, clindamycin, chloramphenicol, ceftriaxone, and ampicillin were found to be different from those observed elsewhere (Ameen et al., 2019; Freitas et al., 2018; Ren et al., 2020) and are supported by the fact that resistance rates of *S. aureus* are influenced by regional antimicrobial agent usage and could even vary from farm to farm (Ren et al., 2020). Although in this study no *S. aureus* isolates were positive for the *mecA* gene PCR and only 18.4% were positive for the *blaZ* gene, almost 70% of the isolates were resistant to the seven β-lactams tested (ampicillin, penicillin, oxacillin, cefoxitin, cefazolin, cefepime, and ceftriaxone) with a most accentuated resistance to penicillin (95.4%). This could indicate that these strains could be composed of borderline oxacillin-resistant *S. aureus* (BORSA), which is characterized by the hyperproduction of beta-lactamase (Hryniewicz & Garbacz, 2017). Although susceptibility to cefoxitin cannot be considered a marker for borderline methicillin resistance, it is a good indicator of staphylococcal sensitivity to penicillinase-resistant penicillins and thus contributes to their possible classification as BORSA (Hryniewicz and Garbacz, 2017). Using this criteria, seven *S. aureus* strains would fit the BORSA description and could indicate that these strains may also be present in intramammary infections, thus increasing the difficulty of mastitis treatment alongside the multi-drug resistance observed.

PFGE analysis (Figure 5) indicated that the isolates presented a high diversity of pulsotypes, which in turn reflect their high genetic diversity, which also highlights that no specific virulence profile is predominant and that only a certain type of *S. aureus* strain may demonstrate a specific virulence profile and that antibiotic resistance and multidrug resistance are more persistent traits in *S. aureus* from IMI than initially thought in the southeast regions of Brazil, especially when considered that they did not belong to a specific pulsotype. In this regard, the occurrence of herd-specific strains has also been reported, and different strains are associated with different clinical outcomes in the host, which include the severity and persistence of infection and response to antibiotic treatment (Schmidt et al., 2017).

Table 2. Phenotypic antibiotic resistance of the 65 *Staphylococcus aureus* from IMI.

Values	CIP	CLO	CLI	ERI	AMC	AMP	PEN	OXA	CFO	CFZ	CPM	CRO	RIF	SUT	TET	TEC
Absolute	35.0	53.0	55.0	55.0	7.00	52.0	62.0	55.0	56.0	43.0	44.0	52.0	42.0	46.0	58.0	16.0
Relative	53.8	81.5	84.6	84.6	10.8	80.0	95.4	84.6	86.1	66.1	67.7	80.0	64.6	70.8	89.2	24.6

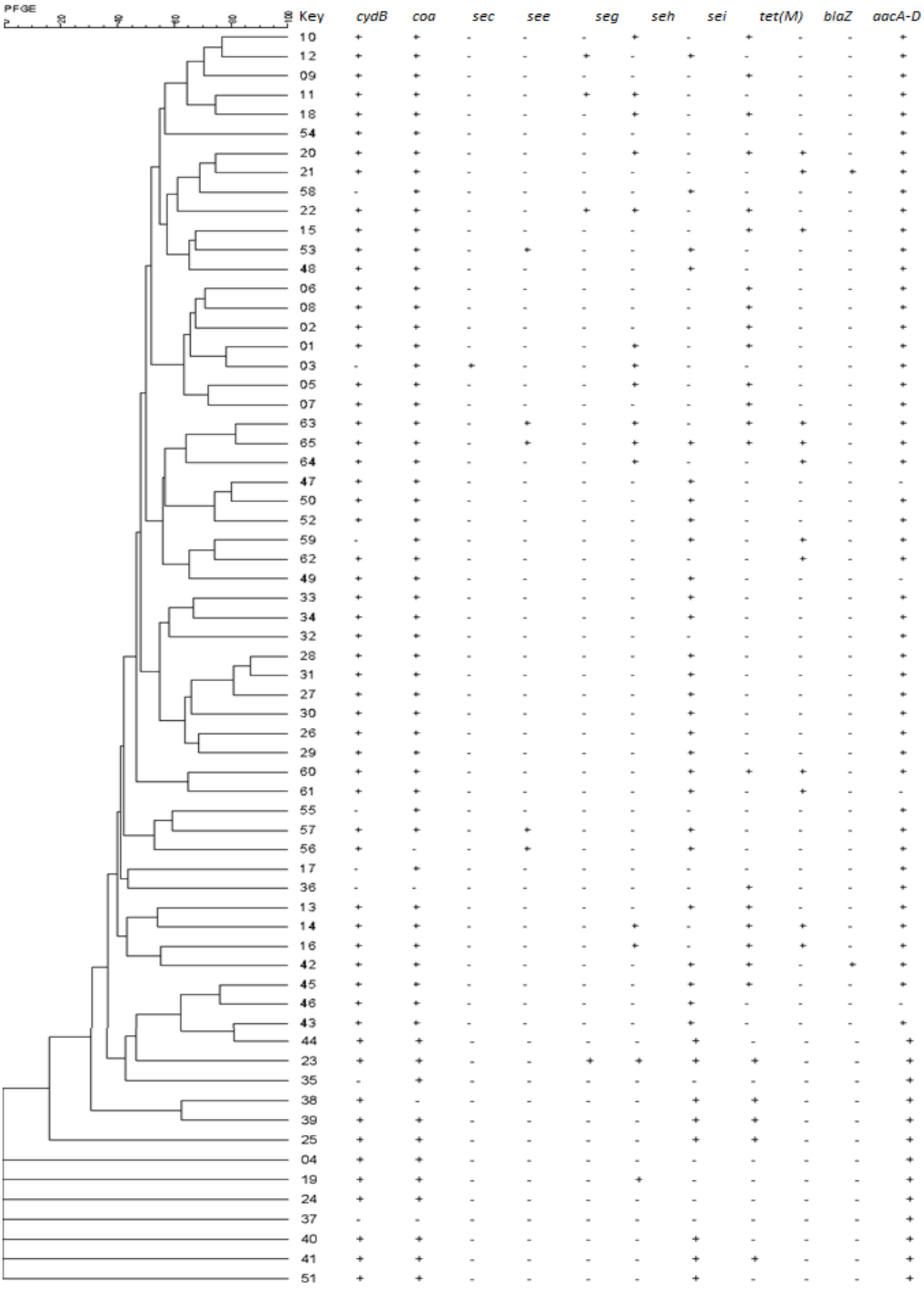

Figure 5. Dendrogram of the 65 isolates of *Staphylococcus aureus* from IMI, their genetic diversity, and frequency of virulence and resistance genes.

4 CONCLUSION

In this study, data analysis of the *S. aureus* isolates from IMI in the southern region of Brazil suggested an association between the presence of the *coa* gene and the presence of the *see* and *sei* and the presence of the *sec* gene and *see* and *seh* genes was observed, which are worthy future genomic studies. Also, phenotypic evaluation suggests that borderline oxacillin-resistant *S. aureus* may be present in mastitis isolates with multi-drug resistance from dairy farms in Brazil and that no specific pulsotype is implicated in causing mastitis in the southeast region of Brazil, but they might have a specific virulence gene content.

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