

Molecular characterization and virulence factor profiles of *Staphylococcus aureus* isolated from bovine mastitis in Brazilian herds

Caracterização molecular e perfil de fatores de virulência de *Staphylococcus aureus* isolados de mastite bovina em rebanhos brasileiros

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Highlights

The severity of *S. aureus* infection depends on several virulence genes.
PFGE is considered the gold standard for genotyping *S. aureus* isolates.
Proteins encoded by virulence genes show potential in vaccine development.

Abstract

Staphylococcus aureus is the main etiological agent of bovine mastitis worldwide and knowledge about its diversity and virulence factors is vital in controlling infections caused by this pathogen. The present study aimed to perform molecular characterization of a population of *S. aureus* ($n=153$ strains isolated from 1994 to 2014 in seven Brazilian states) by pulsed-field gel electrophoresis (PFGE) and evaluate their virulence profiles via polymerase chain reaction (PCR). PFGE identified 93 pulsotypes, with the

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isolates organized into 26 clusters and 20 unique pulsotypes. Predominant pulsotypes were observed, with variations according to the years of isolation and geographic origin of the isolates. Based on the PCR results for the genes encoding agglutination factors (*ClfA* and *ClfB*), binding proteins (fibronectin binding protein - *FnBPA*, elastin binding protein - *Ebps*, collagen binding protein - *Cna*), and toxins (*Hla*, *Hlb* and *Luk-ED*), 40 virulence profiles were detected. The frequency of virulence genes ranged from 58 to 98% (*clfA*:84.3%; *clfB* and *hly* both 81%; *hla*:71.2%; *fnBA*:82.3%; *Can*:94.7%; *ebps*:58%; and *lukED*:98%). The existence of prevalent genotypes in some of the Brazilian states and the time period studied suggests that these genotypes are better adapted, with favorable characteristics in host/pathogen relationships. Genes of proven importance for *S. aureus* pathogenesis in bovine mastitis were widely distributed in genetically divergent populations, suggesting that most of these genes may be interesting candidates in the development of vaccines to control bovine mastitis in Brazil.

Key words: Genotyping. Intramammary infection. PFGE. Population diversity. Virulence genes.

Resumo

Staphylococcus aureus destaca-se como o principal agente etiológico da mastite bovina em todo o mundo, e o conhecimento sobre sua diversidade e fatores de virulência é de grande importância para o controle das infecções causadas por este patógeno. Este estudo teve como objetivo realizar a caracterização molecular de uma população de *S. aureus* (n=153 linhagens isoladas entre 1994 e 2014 de sete estados brasileiros), por análise de Pulsed-field Gel Electrophoresis (PFGE); e avaliar os perfis de virulência por PCR. PFGE apontou 93 pulsotipos com os isolados organizados em 26 agrupamentos e 20 pulsotipos únicos. Foram observados pulsotipos predominantes, com variações conforme os anos de isolamento e origem geográfica dos isolados. De acordo com os resultados da PCR para os genes que codificam fatores de aglutinação (*ClfA* e *ClfB*), proteínas de ligação (proteína de ligação à fibronectina - *FnBPA*, proteína de ligação à elastina - *Ebps*, proteína de ligação ao colágeno - *Cna*) e toxinas (*Hla*, *Hlb* e *Luk-ED*), foram observados 40 perfis de virulência. As frequências dos genes de virulência variaram de 58 a 98% (*clfA*:84,3%; *clfB* e *hly* ambos 81%; *hla*:71,2%; *fnBA*:82,3%; *Cna*: 94,7%; *ebps*:58%; e *lukED*:98%). A existência de genótipos prevalentes em alguns Estados estudados e ao longo do período sugere que esses genótipos são mais bem adaptados, possuindo características que os favorecem nas relações hospedeiro/patógeno. Ampla distribuição de genes de comprovada importância para a patogênese de *S. aureus* na mastite bovina foi observada em populações geneticamente divergentes, sugerindo que a maioria deles podem ser candidatos interessantes para o desenvolvimento de vacinas para controle da mastite bovina no Brasil.

Palavras-chave: Diversidade populacional. Genes de virulência. Genotipagem. Infecção intramamária. PFGE.

Introduction

Brazil is the leading producer of milk and dairy products in Latin America, second on the American continent and sixth in the world. The country produced around 36 billion liters of milk in 2022 on more than 1 million dairy farms, with family farms responsible for the largest share of milk production (Food and Agriculture Organization [FAO], 2023). A major obstacle in national dairy farming is mastitis, a disease responsible for the greatest losses to dairy farmers and the dairy industry. The inherent impacts of this disease for dairy producers are the costs of medicines and veterinary services, early slaughter or animal death, milk discard, and lower production in affected animals. Losses to the dairy industry are due to changes in milk composition, thus decreasing the yield and quality of dairy products (Acosta et al., 2016).

Bovine mastitis is a multifactorial disease caused by different viruses, algae, fungi and primarily bacteria. Among bacterial agents, *Staphylococcus aureus* is considered the main bovine mastitis-inducing pathogen in several countries (Hoque et al., 2018). In Brazil its occurrence at herd level has been reported in several states, with isolation rates ranging from 12.6 to 70.9% (Costa, 2008; Langoni et al., 2009; Bandeira et al., 2013; Mesquita et al., 2019).

S. aureus is involved in clinical and subclinical mastitis, causing chronic infections and higher milk somatic cell counts (Botaro et al., 2015). In addition, *S. aureus*-infected cows can act as reservoirs, promoting dissemination of the pathogen

to other animals in the herd. The zoonotic potential of the pathogen means it can cause several diseases in humans (Howden et al., 2023). Moreover, previous studies reported high levels of antimicrobial resistance in *S. aureus* populations (Mesquita et al., 2019; Zhang et al., 2022), which act as reservoirs of resistance genes that can be transferred horizontally within the species, or to other important pathogens in collective health (Rossi et al., 2020).

Several virulence factors are involved at different times in the *S. aureus*-induced infectious process. At the onset of infection, for adherence to host cells, the pathogen expresses surface antigens such as teichoic acid, protein A (*SpA*), agglutination factors (*CifA* and *CifB*), fibronectin-binding proteins (*FnBPA* and *FnBPB*), collagen (*Cna*), fibrinogen (*Efb*), vitronectin and elastin (*Ebps*) (Foster et al., 2014). After colonization, different virulence genes can be expressed, with functions related to invasion, obtaining nutrients, invasion, immune system evasion and dissemination in host tissues. These genes include enzymes and exotoxins such as α , β , γ and δ hemolysins, leukotoxins and enterotoxins, capable of damaging host cells through cytolytic action (Pérez et al., 2020). Although many of these virulence factors are known to contribute to mammary gland colonization and infection, the mechanisms involved in disease evolution to the various clinical manifestations have yet to be fully understood (Peton & Le Loir, 2014). Studies have shown that the pathogenesis of staphylococcal infections depends on the simultaneous occurrence of different virulence factors, making it important to better understand this synergistic action.

Genotyping studies demonstrate that *S. aureus* strains that cause bovine mastitis can be genetically heterogeneous, albeit with the existence of predominant genotypes (Costa et al., 2012; Klein et al., 2012). Better characterization of the strains circulating in dairy herds can provide essential epidemiologic information for the implementation of more effective measures to control the intramammary infections caused by this pathogen. It is noteworthy that diversity and virulence profile data of *S. aureus* associated with intramammary infections in several Brazilian States are scarce.

Different techniques have been used for genotypic characterization of *S. aureus* (Adkins et al., 2016), with pulsed-field gel electrophoresis (PFGE) considered the gold standard technique. It is used to characterize *S. aureus* isolates from bovine mastitis (Bonsaglia et al., 2018), other strains of animal and human origin (Liu et al., 2018), antimicrobial-resistant isolates (Gökmen et al., 2018), and foodborne-associated strains (Vitale et al., 2018). The widespread use of PFGE is due to its high discriminatory power and epidemiological agreement, and good intra-laboratory reproducibility (Sabat et al., 2013).

Although studies investigating population diversity and virulence factors in *S. aureus* isolated from different milk-producing regions are scarce, they are important in gaining a better understanding of epidemiological knowledge about the pathogen and establishing broader prevention and control strategies. As such, this study aimed to genotypically characterize a population of 153 *S. aureus* strains isolated from 1994 to 2014 in seven Brazilian states, according to their diversity and virulence profiles.

Material and Methods

Bacterial isolates and cultivation conditions

One hundred and fifty-three *S. aureus* strains isolated from milk samples from cows with subclinical and clinical mastitis, collected between 1994 and 2014 in 76 municipalities across seven Brazilian states (Minas Gerais, Paraná, Rio de Janeiro, Rio Grande do Sul, Rondônia, São Paulo and Santa Catarina), were used in this study (Figure 1 and Table 1). The isolates were selected based on the importance of each state in terms of national milk production, in order to best represent the main dairy basins in Brazil. The *S. aureus* strains belong to the Collection of Microorganisms of Interest for Agroindustry and Livestock, based at Embrapa Dairy Cattle in Juiz de Fora, Minas Gerais, Brazil.

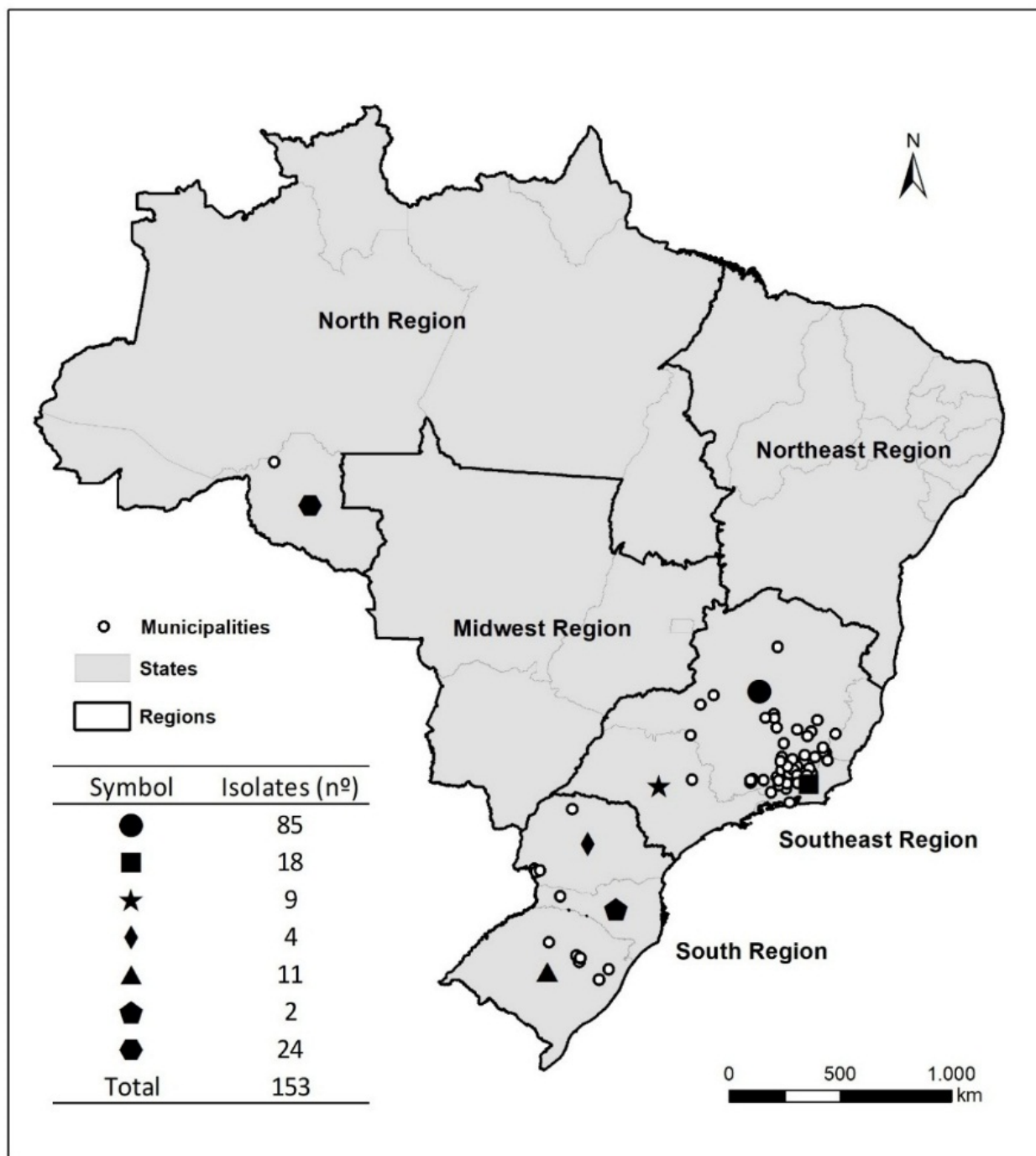


Figure 1. Map indicating the origin of the 153 *Staphylococcus aureus* strains analyzed in the present study. The solid black geometric shapes represent the seven different states of origin of the isolates, and small circles the dispersion of the 76 municipalities with representative strains.

Table 1

Distribution of 153 *Staphylococcus aureus* strains isolated from subclinical mastitis cases in 76 Brazilian municipalities, according to the year of isolation and state of origin

Years of isolation	States of origin (number of isolates)
1994	MG (1)
1995	MG (3)
1996	MG (13); RJ (1)
1997	MG (6); RJ (2)
1998	MG (1); RJ (1)
1999	MG (4); RJ (1)
2000	MG (10); RJ (1)
2001	MG (7); RJ (3); SP (2)
2002	MG (3); RJ (1)
2011	RJ (1)
2013	MG (29); RJ (7); SP (7); PR (10); SC (2)
2014	MG (8); RS (5); RO (24)

MG – Minas Gerais, SP – São Paulo, SC – Santa Catarina, RS – Rio Grande do Sul, PR – Paraná, RO – Rondônia.

Genomic DNA macrorestriction and pulsed-field gel electrophoresis (PFGE) conditions

For this experiment, the isolates were thawed and cultivated in BHI (brain heart infusion) agar (Sigma-Aldrich®, India) for 18h at 37°C. After incubation, a single colony from each strain was transferred to tubes containing 3 mL of BHI broth (Sigma-Aldrich®, India) and incubated at 37°C for 20 to 24 hours. Pellets were obtained by centrifugation of 650 µl of the cultures at 12,000g for three minutes and re-suspended in 350 µl of TE (10mM Tris HCl, 5 mM EDTA, pH 8.0).

The PFGE technique was used to evaluate the genetic diversity of the strains, using a homogeneous electric field device (CHEF DRII, Bio-Rad®, United States). Each microtube containing the isolate cells was

added with 350 µL of low melting agarose (Bio-Rad®, USA), melted at 2% and maintained at 55°C, and the mixture was then used to fill the molds. After solidification, the plugs were incubated for at least 4 hours at 37°C in EC Buffer (6mM Tris HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij-L23, 0.2% sodium deoxycholate, 0.5% laurosylsarcosine) containing lysostaphine (1 mg/mL). Next, the plugs were washed five times in TE (10 mM Tris HCl, 5 mM EDTA, pH 8.0) at room temperature (28 °C), and stored in 3 mL of TE at 4°C until use. A 3-mm slice of each plug was incubated in 100 µl of *Sma*I endonuclease buffer (Sigma-Aldrich®, India) at 37°C for 30 minutes. The buffer was then discarded and the plug digested in 100µl of restriction endonuclease solution containing 20U of the *Sma*I enzyme (Sigma-Aldrich®, India) for 24 hours, in line with the manufacturer's recommendations.

After restriction, the plugs were placed in 1.5% agarose gel wells with 0.5X TBE buffer (0.9 M Tris Base, 0.9 M Boric Acid, 1 mM EDTA pH 8.0) at 14 °C. The Lambda PFG Marker (New England Biolabs®, USA) was used as a ladder. The gel ran at 6 V/cm for 23 hours, with initial and final pulse times of 5 and 40 seconds, respectively. The gels were stained with ethidium bromide (50 µg/mL) and photographed under ultraviolet light in an L-Pix Chemi Photo Digitizer (Loccus Biotecnologia®, Brazil).

DNA fingerprint analysis

The band pattern was visually inspected and analyzed using Bionumerics software, version 7.5 (Applied Maths®, Belgium). The bands were automatically assigned by the program and manually corrected after the original images were visually evaluated. Cluster analysis was performed based on the Dice coefficient and unweighted pair-group method with arithmetic mean (UPGMA). The dendrogram was constructed with 1.5% optimization and 2% tolerance. Phylogenetic trees [Minimum Spanning Tree (MST)] were generated to assess the association between the grouping patterns of the isolates by PFGE, genetic virulence profiles, and the state of origin of the isolates. The MST used has the highest overall reliability score and was constructed using UPGMA to calculate the distance matrix in Prim's Algorithm, associated with the priority rule and permutation resampling (Feil et al., 2004). Strains with 100% similarity were considered indistinguishable and those with similarity > 90% were arranged in the same cluster.

Extraction and purification of genomic DNA for PCR

The isolates were previously cultivated in plates containing BHI Agar (Sigma-Aldrich®, India) for 18h at 37°C. After incubation, a single colony from each strain was transferred to tubes containing 1-2 mL of BHI broth (Sigma-Aldrich®, India) and incubated at 37°C for 20 to 24 hours. The cultures were centrifuged at 12,000g for three minutes and used for DNA extraction according to the phenol/chloroform method (Chen et al., 2001).

PCR conditions and analysis of virulence gene frequencies and profiles

The presence of virulence genes encoding agglutination factors (*CifA* and *CifB*), binding proteins (*FnBPA*, *Ebps*, and *Cna*) and toxins (*Hla*, *Hlb* and *Luk-ED*) was evaluated by PCR (Table 2). Individual reactions of 20 µL were performed for each target DNA region, containing 1X buffer, 0.25mM of each dNTP, 2.5mM of MgCl₂, 0.25mM of each primer, 1.5U Taq polymerase and 50 ng of DNA. Amplifications were performed in a GeneAmp®PCR System 9700 thermal cycler (Applied Biosystems®, United States) programmed for initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30seconds, 53°C for 30seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. The amplified DNA fragments were visualized in agarose gel (1.5% w/v) stained with ethidium bromide solution (50 µg/mL) after horizontal electrophoresis (80 volts). Five µL of 100 bp Ladder solution (Ludwig®, Brazil) was applied to the first well

of each gel as a molecular size standard. Images were recorded in an L-Pix Chemi Photo Digitizer (Loccus Biotecnologia®,

Brazil). The frequencies of each gene in the total population were determined and the results grouped in virulence profiles.

Table 2

Primers and PCR conditions used to detect virulence genes in *Staphylococcus aureus* strains isolated from subclinical mastitis cases in Brazilian herds

Target gene	Primer	Primer sequence	Amplicon size (bp)/ Position	Reference
ClfA	Forward	TGCAACTACGGAAGAAACGCCG	315–336	Ster et al. (2005)
	Reverse	CCTCCGCATTTGTATTGCTTGATTG	394–418	
ClfB	Forward	TGCAAGTGCAGATTCCGAAAAAAC	344–367	Ster et al. (2005)
	Reverse	CCGTCCGTTGAGGTGTTTCATTTG	142–165	
FnBPA	Forward	CGACACAACCTCAAGACAATAGCGG	371–395	Ster et al. (2005)
	Reverse	CGTGGCTTACTTTCTGATGCCGTTTC	479–503	
Ebps	EBP-1	CATCCAGAACCAATCGAAGAC	186	Tristan et al. (2003)
	EBP-2	CTTAACAGTTACATCATCATGTTTATCTTTG		
Cna	Forward	AAAGCGTTGCCTAGTGGAGA	192	Arciola et al. (2005)
	Reverse	AGTGCCTCCCAAACCTTTT		
Hla	Forward	GCGAAGAAGGTGCTAACAAAAGTGG	284–308	Ster et al. (2005)
	Reverse	CGCCAATTTTTCCTGTATCATCACC	454–478	
Hlb	Forward	CGACCGTTTTGTATCCAAACTGGG	161–184	Ster et al. (2005)
	Reverse	TTTGTCCACCCCTGATTGAGAACG	337–360	
Luk-ED	LUKDE-1	TGAAAAAGGTTCAAAGTTGATACGAG	269	Jarraud et al. (2002)
	LUKDE-2	TGTATTCGATAGCAAAGCAGTGCA		

Results and Discussion

Analyses of the DNA fingerprints generated by PFGE identified 93 different genotypes among the 153 strains studied here, representing almost 61% non-redundant genes. Only isolates with an identical electrophoretic profile (100% band similarity) were considered to belong to the

same genotype. There were 71 different genotypes with just one isolate each, 10 with two, three with three, four with four, one genotype with five and six isolates each, two with seven and one with 12. For eight different genotypes observed in PFGE, strains with a difference of up to 18 years of isolation were grouped in the same genotype (Figure 2).

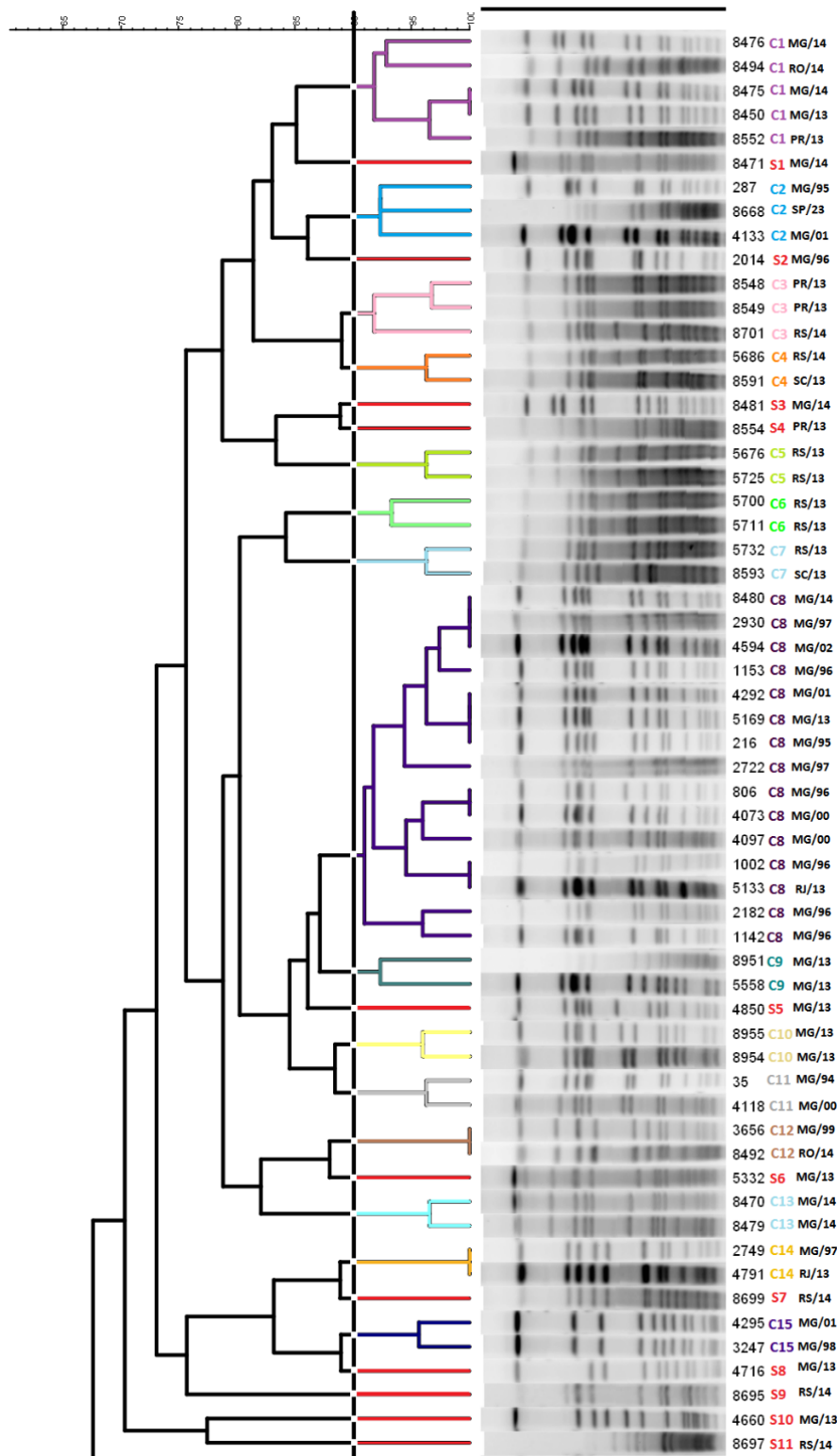


Figure 2. (part 1): Pulsed field gel electrophoresis (PFGE) dendrogram of 153 *Staphylococcus aureus* isolates obtained from cows with subclinical mastitis in Brazilian herds. Vertical lines indicate the cutoff points of 100 and 90% similarity (different colors).

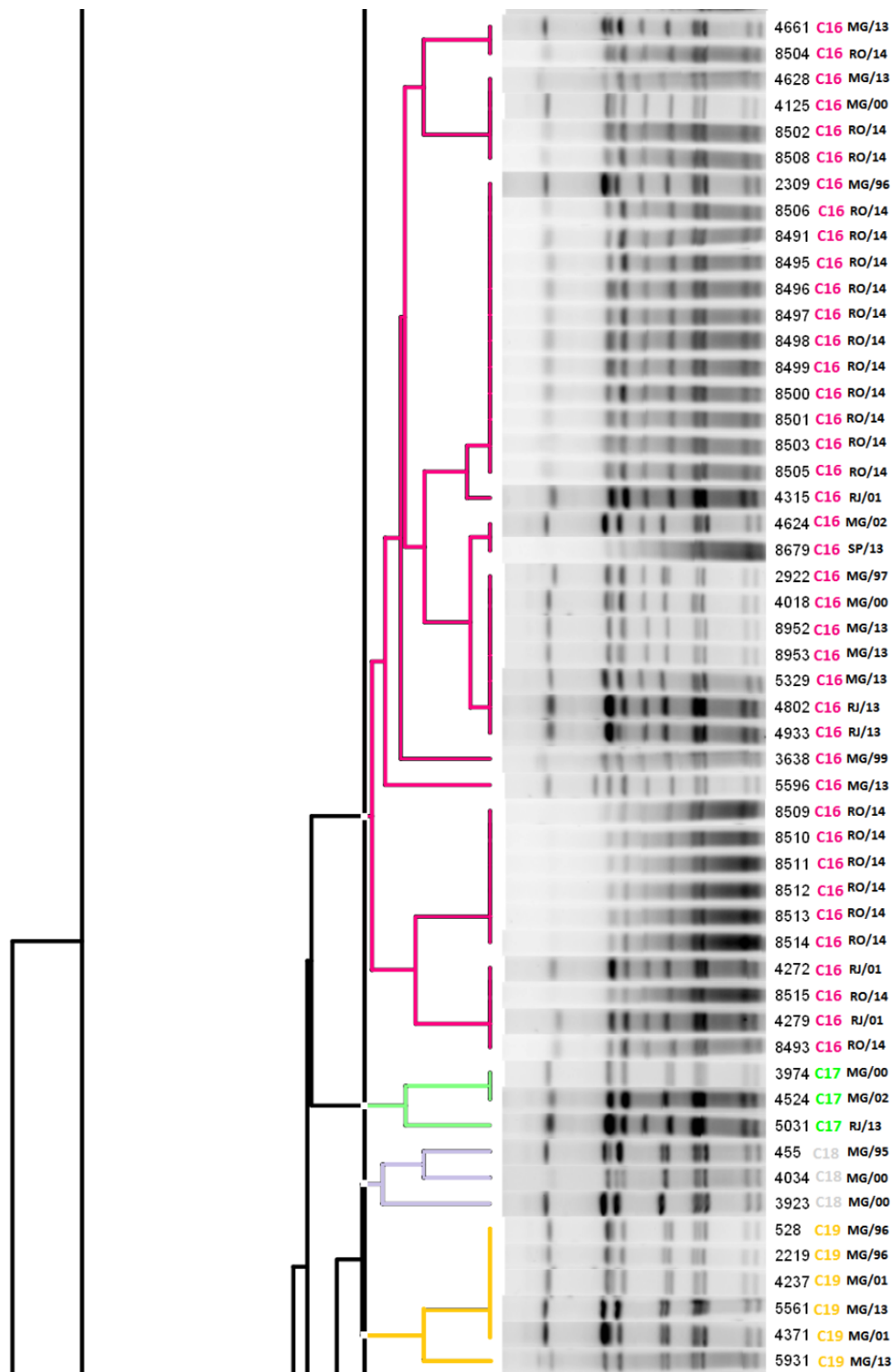


Figure 2. (part 2): Pulsed field gel electrophoresis (PFGE) dendrogram of 153 *Staphylococcus aureus* isolates obtained from cows with subclinical mastitis in Brazilian herds. The vertical lines indicate the cutoff points of 100 and 90% similarity (different colors).

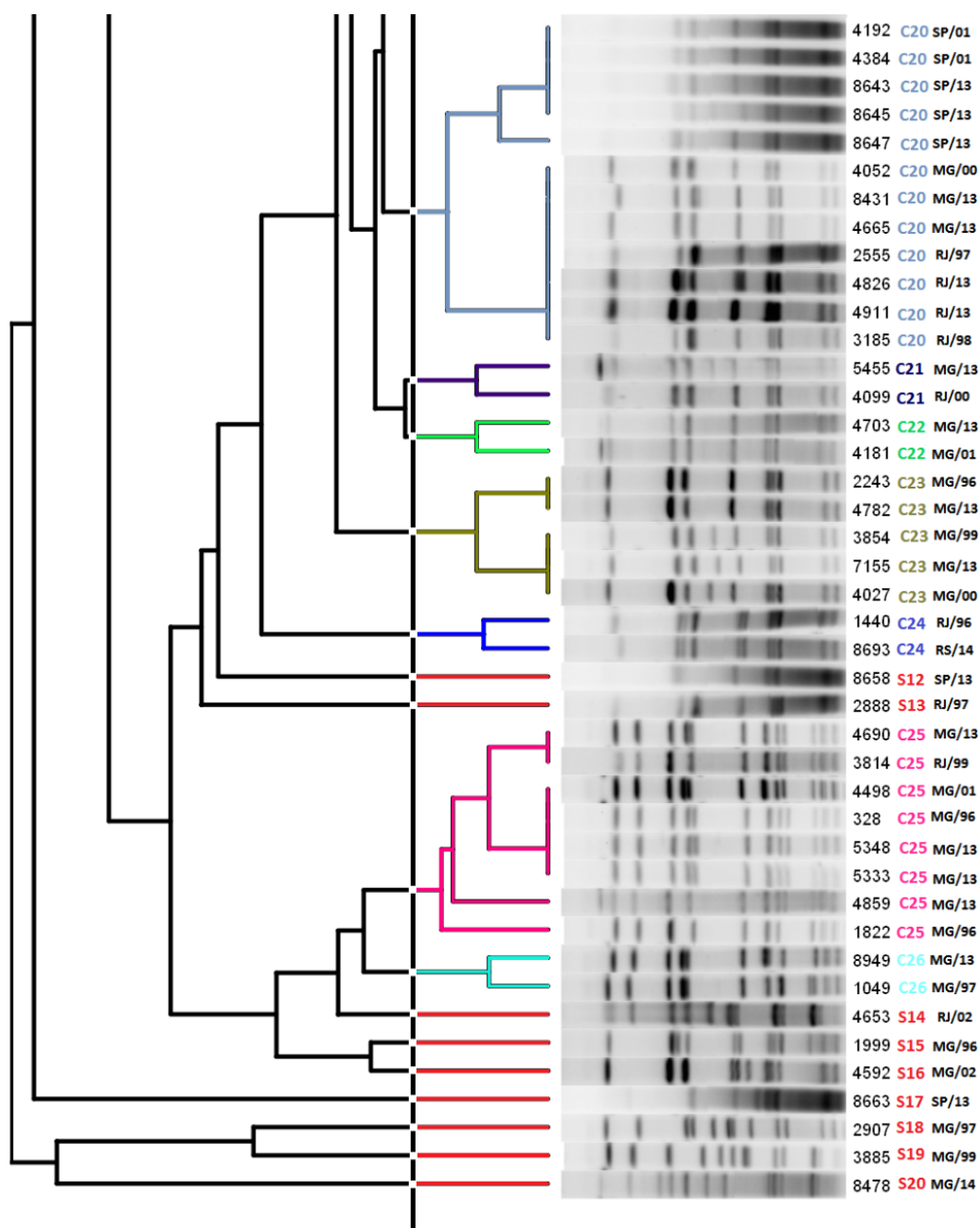


Figure 2. (part 3): Pulsed field gel electrophoresis (PFGE) dendrogram of 153 *Staphylococcus aureus* isolates obtained from cows with subclinical mastitis in Brazilian herds. The vertical lines indicate the cutoff points of 100 and 90% similarity (different colors).

Based on a cutoff point of 90% similarity, the isolates were organized into 26 groups with a varying number of isolates and 20 genotypes with only one. A predominant

cluster was observed, comprising 40 strains (C16), most (22 strains) isolated from milk samples collected in 2014 from herds in Rondônia state. The remaining isolates in

this group (C16) were from the states of Minas Gerais, São Paulo and Rio de Janeiro and the years of isolation varied from 1996 to 2013. The strains in group C8, containing 15 isolates, were predominantly from Minas Gerais (14), with the year of isolation between 1996 and 2014. C20 (12 isolates) contained isolates from Minas Gerais (3), Rio de Janeiro (4) and São Paulo (5), with a large variation in years of isolation (1997 to 2013) (Figure 2).

Genotype distribution in relation to the state of origin presented in the MST (Figure 3) showed greater diversity among isolates from Minas Gerais and Rio de Janeiro. The strains from these states were positioned at distant points of the MST. On the other hand, isolates from the states of São Paulo, Rondônia, Rio Grande do Sul, Santa Catarina and Paraná generally showed less genetic diversity, although some were more genetically distant.

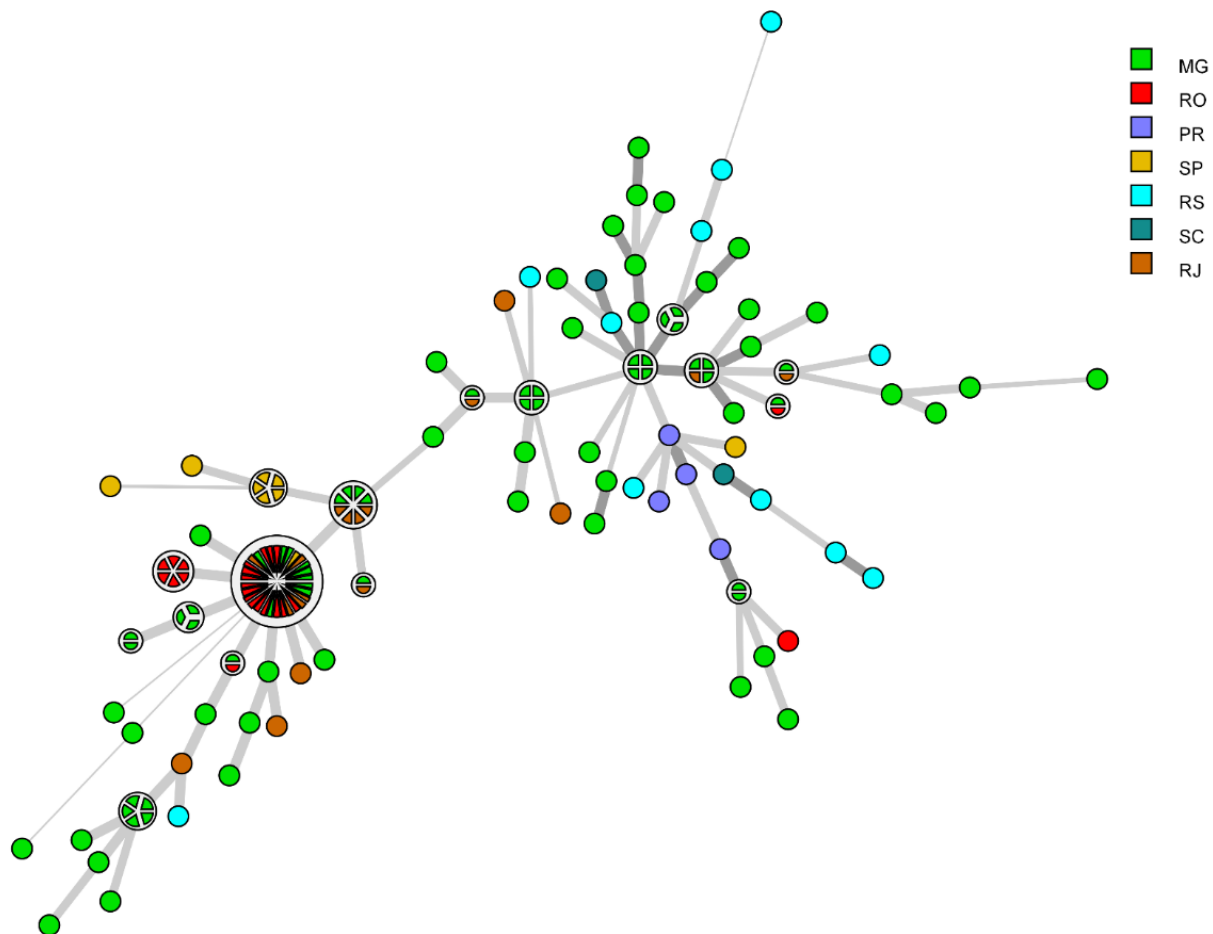


Figure 3. Minimum spanning tree (MST) grouping 153 *Staphylococcus aureus* isolates obtained from cows with subclinical mastitis in Brazilian herds. This MST has the highest overall reliability score and was calculated using the UPGMA (unweighted pair-group with arithmetic mean) associated with the priority rule and permutation resampling. Colors represent the different states of origin of the isolates.

The wide diversity observed in the *S. aureus* population studied here can be attributed to the extensive temporal variation of the strains studied (1994-2014), and the heterogeneity in the geographical origin of the isolates, which came from seven Brazilian states in the South, Southeast and North of the country. Different studies have demonstrated considerable genotypic variation in bovine mastitis-inducing *S. aureus* isolates in Brazil and worldwide (Klein et al., 2012; Bonsaglia et al., 2018; Monistero et al., 2018).

The isolates from the southern states (Paraná, Santa Catarina and Rio Grande do Sul) showed greater genetic similarity by the PFGE (Figure 3), both when the states were analyzed together and individually. This may be because herds in these southern states are predominantly highly technified and produce their own replacement animals, thus avoiding the need to introduce external animals as generally occurs in herds from other regions (Fernandes et al., 2004; Marques et al., 2016). Greater genetic variation is expected in farms that purchase animals when compared to closed herds (Fessler et al., 2010). However, it should be noted that the isolates from these three southern states were from a shorter time interval (2013 to 2014), which may also justify the lower genetic variation observed. Additionally, the small number of isolates from these states (SC=2; PR=4; RS=11) and their fewer herds of origin may also have contributed to the lower diversity observed in these cases.

Clusters with 90% similarity indicated that group C8 (Figure 2) contained isolates obtained across a longer time period (1995 to 2014) and the three largest groups (C16, C8 and C20) contained isolates

from different geographical regions. This suggests that these genotypes may be better adapted to the bovine mammary gland, with characteristics that may favor greater survival and dissemination among herds.

Widely disseminated genotypes have often been reported among herds and regions of different countries (Fessler et al., 2010; Castelani et al., 2013; Srednik et al., 2018). Some studies have identified *S. aureus* strains with a combination of virulence genes that enable wider dissemination and possibly greater contagiousness (Magro et al., 2017).

To colonize the host, *S. aureus* depends on the expression of different virulence factors that are secreted or present on the cell surface, escaping the immune system and causing cell damage (Pérez et al., 2020). These variable gene frequencies and combinations in mastitis-inducing isolates in different geographic regions (Fournier et al., 2008; Adkins et al., 2016; Monistero et al., 2018) enable the pathogen to cause different types of infection. The increase or loss of virulence genes through mobile genetic elements can occur rapidly or gradually slow and is related to clonal stability (Feil et al., 2003).

The virulence genes analyzed were widely disseminated in the bacterial population, with different frequency rates: *clfA* (84.3%); *clfB* and *hly* (both 81%); *hly* (71.2%); *fnBA* (82.3%); *cna* (94.7%); *ebps* (58%) and *lukED* (98%). The frequency of virulence genes also varied according to the geographic origin of strains (Table 3). The strains were grouped into 40 virulence profiles based on the virulence genes in their genomes (Figure 4).

Table 3

Relative frequency of the virulence genes encoding agglutination factors (*CifA* and *CifB*), the fibronectin binding protein (*FnBPA*), elastin binding protein (*Ebps*), binding protein collagen (*Cna*) and toxins (*Hla*, *Hlb* and *Luk-ED*) in the 153 *Staphylococcus aureus* isolates evaluated

State	CLFA	CLFB	HLA	HLB	FNBPA	CNA	EBPS	LUKED	Total no. of strains
MG	85.8%	84.7%	70.6%	80.0%	82.3%	91.7%	64.7%	98.8%	85
RJ	77.7%	77.7%	66.6%	77.7%	94.4%	94.4%	61.1%	100%	18
SP	88.8%	44.4%	88,8%	100%	88.8%	100%	22.2%	88.8%	9
SC	50.0%	50.0%	100%	100%	50.0%	100%	50.0%	100%	2
RS	90.9%	81.8%	90.9%	90,9%	72,7%	100%	72.7%	100%	11
PR	25.0%	50.0%	25.0%	25.0%	50.0%	100%	50.0%	75.0%	4
RO	91.6%	91.6%	66.6%	83.3%	83.3%	100%	41.6%	100%	24
Average	72.8%	68.6%	69.9%	77.6%	74.8%	98.0%	51.7%	94.6%	-

MG – Minas Gerais, SP – São Paulo, SC – Santa Catarina, RS – Rio Grande do Sul, PR – Paraná, RO – Rondônia.

The MST (Figure 5) was generated to assess virulence profile distribution in the *S. aureus* fingerprints determined by PFGE. Isolates belonging to the same profile were located at distant points, not grouped in accordance with the genotype clusters determined by PFGE. All the isolates tested exhibited at least two of the virulence genes studied. Profile 5 (P5), which encompassed isolates carrying all the virulence genes investigated in the present study, and P4, with isolates lacking only the *ebps* gene, were

the most frequent, comprising 33 and 30 isolates, respectively. The states of origin and the year of isolation for isolates belonging to these profiles were quite variable within the population. P5 contained strains from Minas Gerais, Rio de Janeiro, Rondônia, Paraná and Rio Grande do Sul, isolated between 1996 and 2013, while strains in P4 were from Minas Gerais, Rio de Janeiro, São Paulo, Rio Grande do Sul, Rondônia and Santa Catarina, isolated from 1996 to 2014.

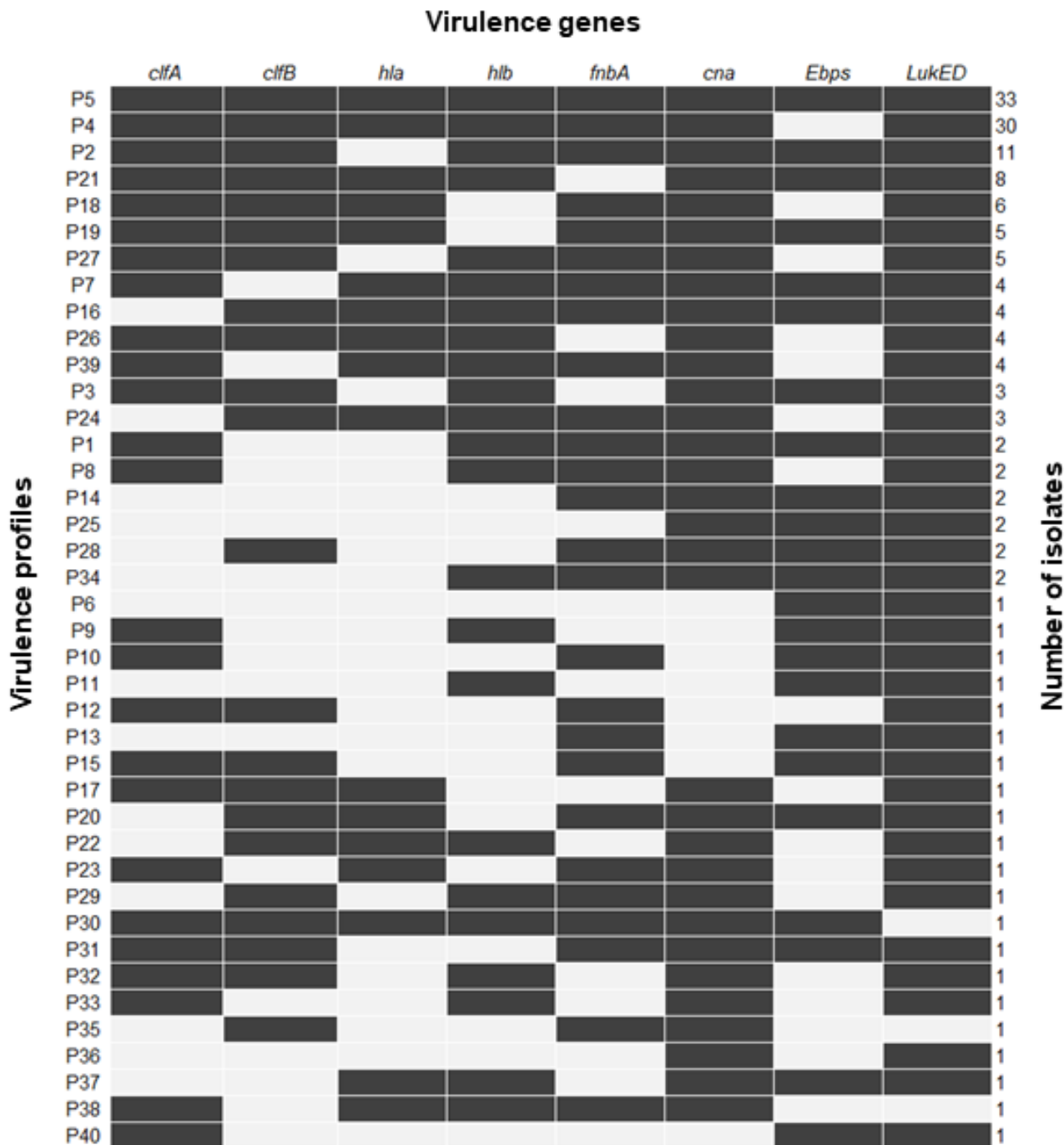


Figure 4. Forty virulence profiles and their frequencies in 153 *Staphylococcus aureus* strains obtained from subclinical mastitis cases in cows from Brazilian herds. The black rectangles indicate the presence of the respective gene.

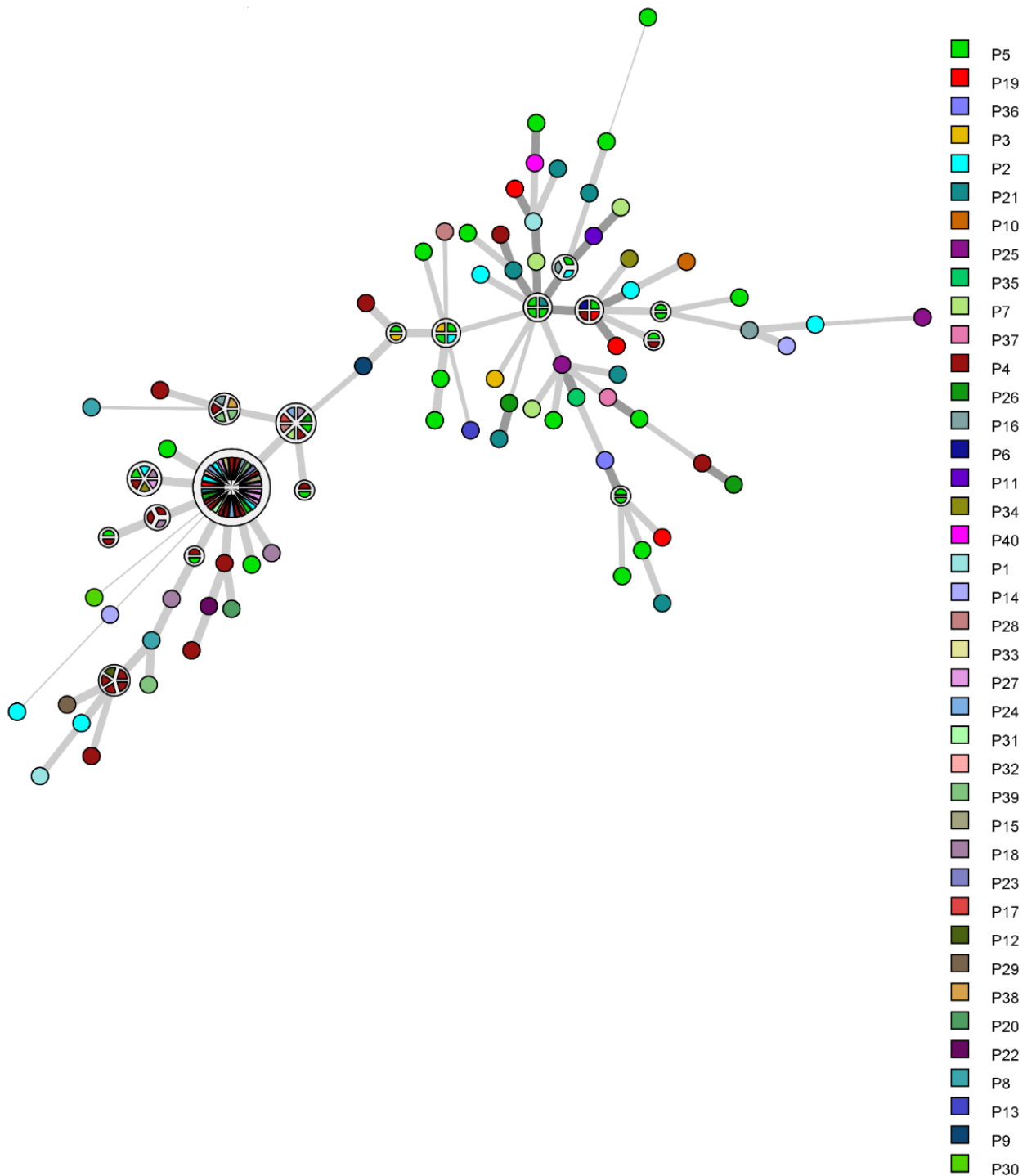


Figure 5. Minimum spanning tree (MST) grouping 153 *Staphylococcus aureus* strains obtained from cows with subclinical mastitis in Brazilian herds. This MST has the highest overall reliability score and was calculated using the UPGMA (unweighted pair-group method with arithmetic mean) associated with the priority rule and permutation resampling. Colors represent the 40 virulence profiles obtained through PCR.

In general, the population studied here showed a high frequency of the virulence genes investigated, except for *ebps* (58%). However, there was no association between the genotypes identified by PFGE and the genetic virulence profiles (Figure 5). Bonsaglia et al. (2018) also found no relationship between molecular and virulence profiles in *S. aureus* strains of bovine origin. Nevertheless, some studies have demonstrated an association between molecular genotype and genetic virulence profile (Piccinini et al., 2012).

The wide distribution of virulence genes in the population (58 to 97.7%), and the fact that the most frequent virulence profiles (P4 and P5) contained strains from the seven states evaluated obtained between 1996 and 2014, suggest that these genes play an important role in the pathogenesis of mastitis in Brazilian dairy herds. This hypothesis is supported by the fact that all the genes analyzed here have been linked to mammary gland pathogenesis (Klein et al., 2012; Foster et al., 2014) or tissue damage in humans (Howden et al., 2023), and several studies have reported their presence in isolates from Brazil and worldwide (Srednik et al., 2018; Pérez et al., 2020).

The collagen binding protein (*Cna*) encoded by the *cna* gene interacts with the collagen present in host tissues and has been reported as an important factor in the pathogenesis of staphylococcal infections in heart valves (Hienz et al., 1996) and bones (Elasri et al., 2002). In the present study, among the adhesion genes, *cna* was the most frequent (94.7%), suggesting that this protein may be important in the pathogenesis of bovine staphylococcal mastitis in Brazil, and its interaction with bovine mammary gland

tissue should be further investigated. The high presence of *cna* in our study contrasts with a previous investigation that attributed little importance to this gene in *S. aureus* strains causing intramammary infections in Brazil (Klein et al., 2012). It is noteworthy that *S. aureus* virulence profiles are temporally and geographically variable and may be influenced by the number of herds and strains analyzed. In our study, a higher number of genetically different isolates from a larger area of the country was analyzed, making the results more reliable. *FnBPs* are important in *S. aureus* adhesion and internalization in the mammary epithelial cells of cattle, preventing the bacteria from being eliminated by milk flow during milking (Campos et al., 2022). The present study showed the high presence of the *fnBPA* gene (82.3%) among the isolates, corroborating previous research (Pereyra et al., 2016).

ClfA and *ClfB* agglutination factors also play a key role in *S. aureus* adhesion to host cells, allowing the bacteria to bind to fibrinogen at the beginning of the infectious process (Rodríguez et al., 2023). Detected in 91% of isolates from southeastern Brazil (Klein et al., 2012), the average frequency of the *clfB* gene in this study was high (81%). In addition to being an adhesion factor, *ClfA* is important in preventing phagocytosis, contributing to the pathogen's escape from the immune system (Pidwill et al., 2021) and has been deemed a good vaccine antigen for mastitis prevention in mice (Gong et al., 2010). The *clfA* gene is widely distributed in *S. aureus* strains of mastitis origin and high frequencies (96.6 to 100%) have been related in isolates from Belgium (Ote et al., 2011) and Argentina (Pereyra et al., 2016). In the present study it was found in 84.3% of isolates.

The elastin-binding protein (Ebps) enables *S. aureus* to bind to elastin in the extracellular matrix of host cells (Pizauro et al., 2021). In this study, Ebps exhibited the lowest frequency (58%) among the genes assessed, varying considerably in *S. aureus* strains according to strain origin. In a study by Kot et al. (2016) with *S. aureus* isolated from bovine mastitis in Poland, the gene was detected in 25% of isolates. Another study reported high Ebps occurrence in subclinical mastitis staphylococcal isolates from China (Yang et al., 2020), and recent genomic research using data from the National Center for Biotechnology Information database (NCBI) confirmed high occurrence in clinical and subclinical mastitis isolates from several countries, including Brazil (Pizauro et al., 2021; Rodrigues et al., 2022).

Genes *hla* and *hlb* encode *Hla* and *Hlb* toxins, respectively, which favor the spread of bacteria throughout the host body, causing pore formation and pro-inflammatory changes in mammalian cells, in addition to immune system inactivation and degradation of infected tissues (Bownik & Siwicki, 2008). Both genes have been related to adherence and cytotoxicity in mammary epithelial cells of bovines. Prince et al. (2012) also demonstrated that *Hla* can rapidly kill defense cells. In our study, *hla* was detected in 71.2% of isolates and *hlb* in 81%, frequencies very similar to those found in previous studies with isolates from Rio de Janeiro state (94.5 and 89.1%, respectively) (Soares et al., 2017).

Leukocidins are pore-forming exotoxins and several types have been associated with the degradation of polymorphonuclear leukocytes such as LukS, LukF, LukD, LukE, LukM and LukF-PV

in cattle (Vrieling et al., 2016). In the present study, only the LukDE leukocidin encoding gene was investigated, and identified in 98% of isolates. This gene has also been reported as widely disseminated among herds in Finland (Haveri et al., 2008) and isolates from Swedish herds (Artursson et al., 2016).

Several vaccines against *S. aureus* have been developed and evaluated, both experimentally and commercially, albeit with no satisfactory results (Pereira et al., 2011). Monitoring diversity and virulence genes in *S. aureus* populations is essential in identifying factors that may be associated with the greater prevalence of certain pathogenic strains and their related proteins, allowing the development of more effective strategies to control bovine mastitis caused by these strains, such as vaccines. Vaccine antigens should be genetically conserved and expressed by most strains, adhesins being the most interesting targets. According to Klein et al. (2012), developing an effective *S. aureus* vaccine should be based on a combination of proteins, which may vary according to the genetic profiles of a given geographical region. Detailed genetic studies may facilitate the development of more effective vaccines for use in herds from different regions.

The virulence factor genes assessed were widely disseminated among the isolates studied, all of which contained two or more of the prospected genes. Among the isolates, 125 (81.7%) contained at least six of the eight genes analyzed (Figure 4) and none of the genes were absent in any of the states included in the study. The lowest relative frequency (22.2%) was recorded for ebps in the São Paulo isolates (Table 3).

The high frequency of virulence genes (clfa, clfb, fnbpa, ebps, can, hla, hlb and luk-ed) in the *S. aureus* population studied here, whose strains have temporally and geographically distinct origins, suggests that the set of proteins encoded by these genes may be interesting candidates for the development of a vaccine to prevent and control *S. aureus*-induced mastitis in Brazilian herds, since this study involved isolates from socially and economically important milk-producing regions of the country.

Conclusions

The occurrence of predominant genotypes in different states and over time suggests that these genotypes are better adapted, with characteristics that favour their persistence in the bovine mammary gland.

Seven of the eight virulence genes analyzed were widely distributed in the population. Since these genes encode important proteins for the pathogenesis of *S. aureus* in bovine mastitis, they may be interesting candidates for the development of vaccines to control bovine mastitis in Brazilian dairy herds.

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Conflicts of interest/Competing interests

The authors declare that they have no conflicts of interest.

Consent for publication

All the authors consented to publication.

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