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Virulence and antimicrobial resistance genes detected in *Staphylococcus* spp. isolated from clinical and non-clinical mastitis using whole-genome sequencing

Genes de virulência e resistência antimicrobiana detectados em *Staphylococcus* spp. isolados de mastite clínica e não clínica usando sequenciamento do genoma completo

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Highlights

Ninety-four virulence genes were observed in this study.
Important resistance genes were also detected.
Strains carrying virulence and resistance genes pose a risk to public health.
Controlling its transmission from farm to fork consumer table is very important.

Abstract

Staphylococcus spp. are among the most isolated bacteria in clinical and subclinical mastitis cases in dairy cattle. The genus comprises biofilm-forming bacteria capable of producing toxins and acquiring resistance to multiple drugs. This work aimed to evaluate the genetic profile related to virulence and antimicrobial resistance characteristics of *Staphylococcus* spp., isolated from clinical mastitis and non-clinical fresh cows using whole genome sequencing (WGS). The bacterial collection consisted of 29 *Staphylococcus* strains isolated from clinical cases of mastitis (n = 7), as well as milk samples collected from fresh cows (n = 22). Strains were identified as *Staphylococcus aureus* (n = 2), *Staphylococcus chromogenes* (n = 19), and *Staphylococcus haemolyticus* (n = 8). A total of 94 virulence genes were

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observed, including *pvl, icaA, icaD* genes, and microbial surface components that recognize adhesive matrix molecules (MSCRAMMs). We also detected important resistance genes such as *blaZ, ant*(4), *erm*(B), *fexA, lnu*(D), *tet*(L), and *tet*(M). The phylogenetic tree listed the species as expected and presented four clades. A variety of virulence and resistance genes were detected. In addition, the expression of important genes such as those responsible for the formation of biofilms and enterotoxins may represent a risk to the health of consumers. being a concern for public health.
Key words: Genetic analysis. Staphylococci. Dairy cattle. Food safety.

Resumo

Staphylococcus spp. estão entre as bactérias mais isoladas em casos de mastite clínica e subclínica em bovinos leiteiros. O gênero compreende bactérias formadoras de biofilme capazes de produzir toxinas e adquirir resistência a múltiplos medicamentos. Este trabalho teve como objetivo avaliar o perfil genético relacionado às características de virulência e resistência antimicrobiana de *Staphylococcus* spp., isolados de mastites clínicas e vacas recém paridas não clínicas, utilizando sequenciamento do genoma completo (WGS). A coleta bacteriana foi composta por 29 *Staphylococcus* isolados de casos clínicos de mastite (n = 7), além de amostras de leite coletadas de vacas recém paridas (n = 22). Os isolados foram identificadas como *Staphylococcus aureus* (n = 2), *Staphylococcus chromogenes* (n = 19) e *Staphylococcus haemolyticus* (n = 8). Foram observados um total de 94 genes de virulência, incluindo genes *pvl, icaA, icaD* e componentes de superfície microbiana que reconhecem moléculas de matriz adesiva (MSCRAMMs). Também foram detectados importantes genes de resistência como *blaZ, ant*(4), *erm*(B), *fexA, lnu*(D), *tet*(L) e *tet*(M). A árvore filogenética listou as espécies conforme o esperado e apresentou quatro clados. Uma variedade de genes de virulência e resistência foram detectados. Além disso, a expressão de genes importantes como os responsáveis pela formação de biofilmes e enterotoxinas pode representar um risco à saúde dos consumidores. sendo uma preocupação para a saúde pública.
Palavras-chave: Análise genética. Estafilococos. Gado leiteiro. Segurança de alimentos.

Introduction

Bovine mastitis is the most significant disease in the dairy chain and is associated with pain and reduced well-being of affected animals. Mastitis causes economic losses due to reduced milk production, milk discard, premature slaughter, impairment of reproductive performance, veterinary costs, and antibiotic usage (McDougall et al., 2009; Haran et al., 2012).

Staphylococci are responsible for numerous infections in humans and animals (Capurro et al., 2010), including cellulitis, bacteremia, endocarditis, pneumonia, and mastitis. *Staphylococcus aureus* is the most pathogenic species in the genus (Weese & van Duijkeren, 2010) and is considered one of the major pathogens of bovine mastitis (Silva et al., 2013). *S. aureus* is known to invade, survive, and even multiply within a large variety of eukaryotic cells, such as the epithelial cells of the mammary gland or immune cells (Almeida et al., 1996; Kerro Dego et al., 2002). The intracellular survival protects the bacteria from the effects of

antibiotics commonly used in mastitis treatment. Furthermore, another noteworthy pathogenicity mechanism is that *S. aureus* is capable of forming biofilms on different surfaces (Fitzpatrick et al., 2005; Garzoni & Kelley, 2009).

On the other hand, other staphylococci species known as coagulase-negative staphylococci (CoNS) have emerged as important bacteria associated with bovine mastitis (Freitas Guimarães et al., 2013; Frey et al., 2013; Tomazi et al., 2015; Levison et al., 2016). *S. chromogenes, S. haemolyticus, S. epidermidis, S. simulans, S. sciuri,* and *S. xylosus* are among the CoNS species commonly associated with mastitis (Frey et al., 2013; Tomazi et al., 2014; Vanderhaeghen et al., 2014; Mahmmod et al., 2018). Some species of CoNS can persist in the udder for months, or even throughout the lactation (Aarestrup et al., 1995; Thorberg et al., 2009). The ability to form biofilm was also reported in CoNS isolated from bovine milk (Tremblay et al., 2013). Moreover, CoNS present a high number of virulence factors, and the control of mastitis is complicated because CoNS is composed of many different species (Thorberg et al., 2009).

Although CoNS and *S. aureus* share the same genus, they have different forms of pathogenicity in the course of mastitis (Taponen & Pyörälä, 2009), and further studies are still needed to elucidate the genetic mechanisms of infection associated with these pathogens. Studies evaluating the expression of virulence and resistance genes of staphylococcal species, as well as the evaluation of the animal's clinical results, are necessary to understand the potential that certain species within this group have to cause diseases.

Next-generation sequencing (NGS) has provided relevant information on virulence and resistance genes among mastitis-causing bacteria (Vélez et al., 2017; Naushad et al., 2019). Although there are studies in which whole genome sequencing (WGS) was used to genetically characterize major pathogens of mastitis, there are few reports on CoNS isolated from milk (Naushad et al., 2016, 2019). Thus, this study aimed to evaluate the genetic profile related to virulence and antimicrobial resistance characteristics of *Staphylococcus* spp. isolated from bovine milk using WGS and build a phylogenetic tree with gene sequences of *Staphylococcus* spp. isolated from clinical mastitis and non-clinical fresh cows.

Material and Methods

Origin and isolation of strains

The *Staphylococcus* spp. strains evaluated in this study belonged to a bacteria collection from the Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca. The strains were isolated from 22 cows postpartum with no clinical signs of mastitis, in addition to 7 cows with clinical mastitis. Clinical mastitis isolates were defined as those from cows with clinical signs of intramammary infection (alteration of milk and or udder normal appearance). Non-clinical isolates were those identified from cows postpartum without visual clinical signs of mastitis.

The strains were isolated from milk samples collected in a large commercial dairy farm located in Scipio, New York, and sequenced, as described below. The farm

had an average milk production per cow of 40.4 kg (42.2 kg of energy-corrected milk) and bulk tank SCC of 135.330 cells/mL during the milk samples collection.

Strains from mastitic cows were isolated during a contemporary clinical trial evaluating the efficacy of 4 protocols for the treatment of clinical mastitis caused by Gram-positive pathogens (Tomazi et al., 2021). In the study, total Gram-positive bacterial counts were performed using AccuTreat® quadplates (FERA Animal Health LCC, Ithaca, NY), which contain selective and differential culture medium for Gram-positive pathogens. *Staphylococcus* isolates were selected based on the colony color and morphology observed in the plates. Pink and orange colonies were selected for species confirmation and microbiological procedures.

For non-clinical fresh cows, milk samples were collected from all functional quarters with no clinical symptoms of mastitis (e.g., alteration of normal milk and udder appearance) or other diseases at 10 ± 3 days postpartum. Milk samples were aseptically collected and kept on ice until further laboratory procedures were performed in the Department of Population Medicine and Diagnostic Sciences at Cornell University. At the laboratory, each quarter milk sample was streaked onto one partition of the AccuTreat® quadplate using a sterile cotton swab (Puritan Medical Products, Guilford, ME), followed by incubation at 37 °C overnight. Then, the colonies were selected based on bacterial morphological features.

Upon selection, a single colony was streaked onto CHROMagar™ Mastitis GP base (Springfield, NJ) and incubated at 37 °C for 24 hours. This step was repeated at least two more times to confirm that a pure colony was obtained.

Identification of strains by 16S rDNA gene sequencing

DNA was extracted from each bacterial isolate using the DNAasy PowerFood Microbial Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The 16S ribosomal DNA gene was then amplified through PCR and the PCR products were purified using Gel/PCR Fragments Extraction Kit (IBI Scientific, Peosta, IA), following the manufacturer's instructions. The purified DNA samples were submitted to the Cornell University Institute of Biotechnology for Sanger sequencing using 8 pmol of primer fD1 and 300 ng of PCR products (Weisburg et al., 1991). For species identification, we compared our FASTA sequences with the sequences stored in GenBank, using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This step was performed with bacterial species before whole genome sequencing.

Whole genome sequencing (WGS)

The concentration of total gDNA of the samples was determined using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA), and then DNA samples were standardized. Subsequently, library preparation was carried out according to the manufacturer's protocol of Nextera® DNA Library Prep Reference Guide. Pair-end sequencing was then performed using a MiSeq Reagent Kit v3 (600 cycles) through the MiSeq Platform (Illumina Inc., San Diego, CA).

Genome sequence and phylogenetic analysis of Staphylococcus spp.

The quality of the raw reads was evaluated using FASTQC. Potential contamination of the sequences was checked using Kraken2. Sequencing reads were submitted to the comprehensive genome analysis service using the Pathosystems Resource Integration Center (PATRIC) (Wattam et al., 2017). Reads were assembled using SPAdes (Bankevich et al., 2012) and the genomes were annotated using the Rast tool kit found in PATRIC (PATRIC 3.2.96), which are part of the all-bacteria Bioinformatics Resource Center available online (Brettin et al., 2015).

In addition, all sequences were submitted to the online software available at the Center of Genomic Epidemiology website (https://cge.cbs.dtu.dk/services/KmerFinder/), Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/analyze/rgi), and ResFinder for the Center of Genomic Epidemiology (https://cge.cbs.dtu.dk/services/ResFinder).

The combined *tuf, rpo*B, and *16s* datasets were selected to infer the species phylogeny (Lamers et al., 2012). Unweighted Parsimony analysis was conducted by using a heuristic search option with 1000 random addition sequences and tree bisection reconnection branch swapping in PAUP 4.0b10 (Sinauer Associates, Sunderland, MA, USA). Gaps were treated as missing data. The Consistency Index (CI) and the Retention Index (RI) were calculated to indicate the amount of homoplasy present. Neighbor-joining and maximum likelihood analyses were performed in PAUP 4.0b10 using an appropriate nucleotide substitution model determined by JModelTest (University College Dublin, Dublin, Ireland). Clade stability was assessed via Maximum Parsimony Bootstrap Proportions (MPBS) in PAUP 4.0b10, using 1,000 heuristic search replications with random sequence addition. The datasets were rooted with *S. hyicus*, as it is considered a suitable outgroup. The phylogenetic trees were visualized using FigTree v.1.4 (University of Edinburgh, Edinburgh, United Kingdom).

Results and Discussion

Seven strains were isolated from mastitic milk and identified as *S. aureus* (n=1), *S. chromogenes* (n=4), and *S. haemolyticus* (n=2). Isolates from non-clinical fresh cows (n=22) included *S. chromogenes* (n=15), *S. haemolyticus* (n=6), and *S. aureus* (n=1). Sequences from four strains identified as S. chromogenes and two as *S. haemolyticus* failed during the quality control after WGS and were not included in the dataset.

CoNS has become a concern among milk producers, especially in farms where major pathogens of mastitis were controlled. Studies have reported that some species of CoNS are commensal to mammary gland microbiota and could also be more resistant to antibiotics than *S. aureus* (Pyörälä, 2009; Valckenier et al., 2019). Moreover, some reports have suggested that these microorganisms have specific antibacterial activities that benefit them while competing with other bacteria. Therefore, quarters infected with CoNS would be more resistant to subsequent infections by major pathogens, such as *S. aureus* (Matthews et al., 1990).

In this study, we also evaluated the prevalence of virulence and resistance

genes of *Staphylococcus* spp. (Tables 1 and 2). In total, 94 virulence genes (Table 1) were identified using Patric, ResFinder v2.1 (Zankari et al., 2012) and Virulence Finder v1.5 (Joensen et al., 2014). The most frequent genes identified in strains isolated from mastitic cows (n=7) were *rec*A (100%) and *mgr*A (100%), whereas *trp*B (75%) and *rec*A (70%) were the most prevalent genes among isolates from non-clinical fresh cows. The gene *rec*A is known as a reference in *Staphylococcus* spp. and is related to the contribution of homologous recombination and DNA repair (Mei et al., 1997), as well as part of SOS response against stress (Goerke et al., 2006). The *mgr*A gene is a significant global virulence gene regulator in *S. aureus* and mediates host-pathogen interactions and virulence (Li et al., 2019).

Bacterial pathogens have developed pathogenic strategies to survive in well-protected host microenvironments. Mechanisms of adherence and internalization into host cells are strategies that permit bacterial pathogens to defeat defense mechanisms functional at mucosal surfaces. Besides, after internalization, pathogens need to overcome intracellular bacteriostatic/bactericidal mechanisms such as endosome acidification and endosome-lysosome fusion (Almeida et al., 1996). Thus, virulence factors (as well as the genes responsible for mediating these factors) play important roles, as their presence can make these microorganisms more or less pathogenic.

Herein, some isolates had a high frequency of virulence genes. One *S. aureus* isolated from a non-clinical fresh cow presented 44 virulence genes. Other strains with high frequency of virulence genes were one *S. chromogenes* isolated from a cow with CM that had 43 virulence genes, one *S. aureus* isolated from another mastitic cow with 38 virulence genes, and three *S. haemolyticus* isolated from non-clinical fresh cows that had 16 virulence genes in total. The fresh cow infected with *S. aureus* with 44 virulence genes progressed to subclinical mastitis according to the monthly somatic cell count test performed at the cow level. This strain was the only one positive for lukF-PV, which is a gene associated with a cytolytic toxin Panton-Valentine leukocidin (PVL). PVL is associated with tissue necrosis and leukocyte destruction (Nawrotek, 2018).

The *msr*A and *sdr*D genes were among those observed in strains from both clinical and non-clinical strains. The *msr*A gene encodes the mechanism which involves a macrolide efflux pump. The protein produced by this gene can export 14 macrolides and streptogramin B antibiotics from bacterial cells (Leclercq, 2002). In addition, this gene is responsible for producing methionine sulfoxide reductases in oxidative stress tolerance and was reported as an important virulence factor in *S. aureus* (Singh et al., 2015). The *sdr*D gene encodes the cell surface-associated calcium-binding protein, which plays an important role in adhesion ability and bacteria pathogenesis. This gene contributes to the resistance against the innate immune components (such as neutrophils present in the blood) and attenuates bacterial clearance (https://www.uniprot.org/uniprot/O86488). Both *msr*(A) and *sdr*D could hinder the antibiotic treatment as well as the immune response, facilitating the infection onset and its persistence.

Table 1
Prevalence of virulence genes in Staphylococcus spp. isolated from non-clinical fresh cows and mastitic cows

Virulence Genes	*S. aureus* (n=2)		*S. chromogenes* (n=15)		*S. haemolyticus* (n=6)	
	non-clinical fresh cows n (%)	mastitic cow n (%)	non-clinical fresh cows n (%)	mastitic cow n (%)	non-clinical fresh cows n (%)	mastitic cow n (%)
*trp*B	0	0	11 (73.3%)	4 (26.7%)	4 (66.7%)	0
*rec*A	1 (50%)	1 (50%)	11 (73,3%)	4 (26.7%)	4 (66.7%)	2(33,3%)
*mgr*A	0	1 (50%)	9 (60%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
*opp*D	0	0	8 (53.3%)	4 (26.7%)	2 (33.3%)	0
SA1453	0	0	8 (53.3%)	4 (26.7%)	3 (50%)	0
*clp*X	1 (50%)	0	5 (33.3%)	4 (26.7%)	0	0
Asd	0	0	4 (26.7%)	3 (20%)	5 (83.3%)	1 (16.7%)
Lip	1 (50%)	1 (50%)	3 (20%)	1 (6.7%)	0	0
*fem*B	0	0	3 (20%)	3 (20%)	3 (50%)	0
*msr*A	0	0	3 (20%)	3 (20%)	0	0
*esx*A	1 (50%)	0	1 (6.7%)	0	0	0
*car*B	0	0	1 (6.7%)	0	0	0
*pyr*AA, *purl*	1 (50%)	1 (50%)	0	0	5 (83.3%)	1 (16.7%)
Fbp	1 (50%)	1 (50%)	0	0	4 (66.7%)	1 (16.7%)
*clp*P	0	1 (50%)	0	1 (6.7%)	2 (33.3%)	0
*lys*A	0	0	0	0	5 (83.3%)	1 (16.7%)
*cit*B	1 (50%)	0	0	0	4 (66.7%)	0
SA1061	0	0	0	0	5 (83.3%)	0
SAHV_0914	1 (50%)	1 (50%)	0	0	1 (16.7%)	1 (16.7%)
*ess*C, *sdr*D, *ads*A, *aur*, cap8D, cap8E, cap8F, cap8G, cap8L, cap8M, cap8N, cap8O, cap8P, *clf*B, *esa*A, *esa*B, *ess*A, *ess*B, *fnb*A, *geh*, *hlg*B, *opp*F, *sdr*E, *til*S	1 (50%)	1 (50%)	0	0	0	0
*ica*D, cap8A, cap8B, *clf*A, *esa*C, *esx*B, *ica*A, *ica*B, *ica*C, lukF-PV, *sbi*	1 (50%)	0	0	0	0	0
*atm*B, *ccp*A, *cia*R, *cps*Y, *cyd*A, *fba*, *gid*A, *gln*A, *gua*A, *has*C, *lep*A, *leu*S, *lux*S, *per*R, *pur*B, *pur*H, *pur*N, *rpo*E, *sod*A, SP_0095, SP_0111, SP_0310, SP_0494, SP_0819, SP_0856, SP_0916, SP_1396, SP_1398, SP_1970, SP_1086, SPy_1633, *vic*K	0	0	0	1 (6.7%)	0	0
*hlg*C, *hys*A, *map*, *odh*B, *sdr*C, *ssp*B	0	1 (50%)	0	0	0	0
*trp*A	0	0	0	0	1 (16.7%)	0

Table 2
Prevalence of resistance genes in *Staphylococcus* spp. isolated from milk of non-clinical fresh cows and mastitic cows

Resistance Genes	*S. aureus* (n=2)		*S. chromogenes* (n=15)		*S. haemolyticus* (n=6)	
	non-clinical fresh cows n (%)	mastitic cow n (%)	non-clinical fresh cows n (%)	mastitic cow n (%)	non-clinical fresh cows n (%)	mastitic cow n (%)
EF-G, EF-Tu, *gid*B, *gyr*A, *gyr*B, *mur*A, *rpo*B, *rpo*C, S12p	1 (50%)	1 (50%)	11 (73.3%)	4 (26.7%)	5 (83.3%)	1 (16.7%)
*fol*P	1 (50%)	1 (50%)	11 (73.3%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
*kas*A	0	0	11 (73.3%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
*nor*A	1 (50%)	0	11 (73.3%)	4 (26.7%)	5 (83.3%)	1 (16.7%)
Rho	1 (50%)	0	11 (73.3%)	4 (26.7%)	1 (16.7%)	0
*tca*B	0	0	11 (73.3%)	4 (26.7%)	2 (33.3%)	0
tet(38)	1 (50%)	1 (50%)	11 (73.3%)	4 (26.7%)	0	0
*inh*A, *fab*I	0	1 (50%)	10 (66.6%)	4 (26.7%)	4 (66.7%)	0
S10p	1 (50%)	1 (50%)	11(73.3%)	4 (26.7%)	5 (83.3%)	1 (16.7%)
ddl, *pgs*A	0	0	9 (60%)	4 (26.7%)	1 (16.7%)	0
*gdp*D	0	0	9 (60%)	4 (26.7%)	3 (50%)	1 (16.7%)
iso-tRNA	0	0	9 (60%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
*vrar*R, *vra*S	0	0	9 (60%)	4 (26.7%)	0	0
*mgr*A	0	1 (50%)	9 (60%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
pare	0	0	9 (60%)	4 (26.7%)	3	0
*tca*R	0	0	9 (60%)	3 (20%)	2 (33.3%)	1 (16.7%)
Alr	1 (50%)	0	8 (53.3%)	4 (26.7%)	4 (66.7%)	0
Dxr	0	0	8 (53.3%)	3 (20%)	0	0
*vra*F	0	0	8 (53.3%)	4 (26.7%)	0	0
*mpr*F	0	0	7 (46.7%)	4 (26.7%)	3 (50%)	0
sav1866	1 (50%)	0	7 (46.7%)	4 (26.7%)	1 (16.7%)	0
folA, *dfr*	0	0	6 (40%)	4 (26.7%)	1 (16.7%)	0
*bla*Z	0	0	1 (6.7%)	0	0	0
ant(4')-Ib, *erm*(B), *mep*A, *tet*(L)	1 (50%)	0	0	0	0	0
*arl*R, *bce*A, *bce*B	0	0	0	0	2 (33.3%)	0
*bce*R, *bce*S	0	0	0	0	2 (33.3%)	1 (16.7%)
*dfr*C	0	0	0	0	1 (16.7%)	0
*fab*K, *fexA family*	0	0	0	1 (6.7%)	0	0
lnu(A)	0	0	0	0	0	1 (16.7%)
lnu(D)	0	0	0	1 (6.7%)	0	0
*mep*R	1 (50%)	1 (50%)	0	0	0	0
*rlm*A(II)	0	0	0	1 (6.7%)	0	0

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*tca*A	0	0	0	0	2 (33.3%)	0
*tca*B2	1 (50%)	0	0	0	3 (50%)	1 (16.7%)
tet(M)	0	0	0	1 (6.7%)	0	0
*Ykk*CD	0	0	0	0	5 (83.3%)	1 (16.7%)

The Venn diagram was constructed using the 15 most prevalent genes identified in strains isolated from non-clinical fresh cows and animals with clinical mastitis (http://bioinformatics.psb.ugent.be/webtools/Venn/). Twelve genes were concomitant in both categories of animals (Figure 1). On the other hand, the genes msrA, sdrD, and clpP were identified only in clinical cows, while the genes lysA, SA1062, and citB were observed only in non-clinical fresh cows.

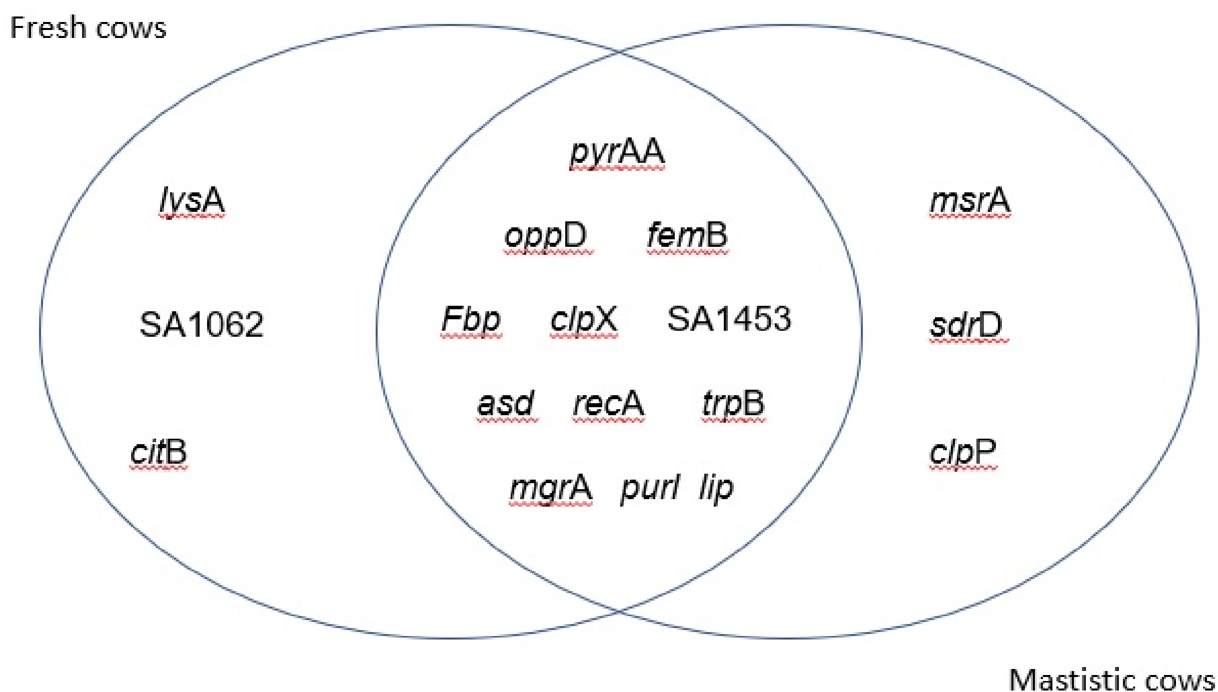

Figure 1. Correlation of the most prevalent virulence genes in mastitic and non-clinical fresh cows. Venn diagram illustrating the most prevalent virulence genes across all *Staphylococcus* spp. isolates from milk of non-clinical fresh cows and cows affected with mastitis.

Genes involved with biofilm formation and MSCRAMM (i.e., icaD, sdrD, clfB, sdrE, and clfA) were also identified in our strains. CoNS is a heterogeneous group and its epidemiology on mastitis is still not clear, but the importance of biofilm formation during infection was considered in this group of bacteria (Osman et al., 2015). The biofilm-producing CoNS were reported to be less susceptible to antibiotics than planktonic cells (Tremblay et al., 2014), which could be a factor in increasing the persistence of certain species in this group. Another group of proteins involved in the adhesion of bacteria to the host cells is the staphylococcal MSCRAMM (microbial surface components recognizing adhesive matrix molecules), as bacteria with this virulence factor are more likely to adhere to specific components of the extracellular matrix of a wide variety of human or animal tissues (Cucarella et al., 2002).

The bacterial resistance compromises mastitis treatment and is a problem for public health. Organisms can acquire resistance to antibiotics by different mechanisms. The antimicrobial resistance determinants can be classified into acquisition of foreign DNA (when bacteria acquire the DNA by transduction), transformation, and conjugation; mutations of preexisting genetic determinants that affect structural or regulatory genes; and mutations in acquired genes (Maja Babic, 2009).

Beta-lactams, macrolides, and lincosamides are antimicrobials used for the prevention and treatment of mastitis (Pyörälä, 2009). The resistance to lincosamides, streptogramins, macrolides, tetracycline, beta-lactams, and ciprofloxacin in bacterial strains isolated from bovine with mastitis have been previously reported (Silva et al., 2013, 2014; Souza et al., 2019). Resistance to beta-lactams is a known public health problem worldwide (Harkins et al., 2017; Souza et al., 2019). Resistance in the Staphylococcus genus is explained by the production of the beta-lactamase enzyme encoded by the gene blaZ and synthesis of the penicillin-binding protein 2A (PBP2A) with a low affinity for binding to penicillin coded by the gene mecA (Fuda et al., 2005; Olsen et al., 2006). Although the blaZ gene is common (Aslantaş & Demir, 2016), only one strain presented this gene in our study and none had a resistance gene against other beta-lactam.

The resistance to lincosamides and streptogramins could be a result of the acquisition of endogenous mutations or horizontally transmitted resistance genes (Schwarz et al., 2016). The mechanisms in which the bacteria resist these antibiotics are enzymatic inactivation of active efflux and/or structural changes at the ribosomal target site (Schwarz & Kehrenberg, 2006). The strains genetically evaluated in our present study had the lnu(A) and lnu(D) genes, which are associated with bacterial

resistance to lincomycin and pirlimycin. In addition, clindamycin resistance has also been suggested (Morar et al., 2009; Lozano et al., 2012) (Table 2).

At least 35 different tetracycline resistance (tet) genes and 3 oxytetracycline resistant (otr) genes have been characterized. Generally, tetracycline resistance occurs by active efflux resulting from the acquisition of these genes or a protein that protects bacterial ribosomes from the action of tetracyclines (Roberts & Schwarz, 2017). In our sequencing results, 5 genes that confer resistance to tetracycline were detected, mepA, tet(L), tet(M), tet(38), and S10p. SP10 is a ribosomal protein that requires mutations to confer resistance to tet.

In our study, we also performed a phylogenetic analysis of the isolates (Figure 2), and four clades were detected: two clades of S. chromogenes, one of S. haemolyticus, and one of S. aureus. No relationship was observed between clinical isolates and non-clinical isolates even when the strains belong to the same species. The tuf, rpoB, and 16s dataset consisted of 55 taxa and 2626 nucleotides with 624 parsimony informative characters (PICs). The analysis resulted in a most parsimonious tree (CI = 0.60, RI = 0.70) (Figure 2). No topological differences were detected between trees derived from neighbor-joining, maximum likelihood, and parsimony phylogenetic inferences. The isolates found in this study clustered within S. aureus, S. haemolyticus, and S. chromogenes lineages. The main clades were resolved and supported by MPBS.

Naushad et al. (2016) observed five clades in their study, in which they built a tree with non-aureus staphylococci species isolated from bovine intramammary infection, with consistent interspecies relationships within clades in WGS phylogenetic reconstructions (Naushad et al., 2016).

CoNS or non-aureus staphylococci are a heterogeneous group, and it is common for the phylogeny between species to be conflicting since generally only one gene is used for their determination (Ghebremedhin et al., 2008; Naushad et al., 2016). It is important to consider that the use of WGS sequences in phylogenetic trees offers great accuracy in reconstructing evolutionary relationships for identification and elucidation of evolutionary histories of bacterial organisms (Naushad et al., 2016). S. aureus strains in our study were more related to S. haemolyticus in the phylogenetic tree, but the S. chromogenes strains presented a higher number of virulence and resistance genes, being more virulent than S. haemolyticus.

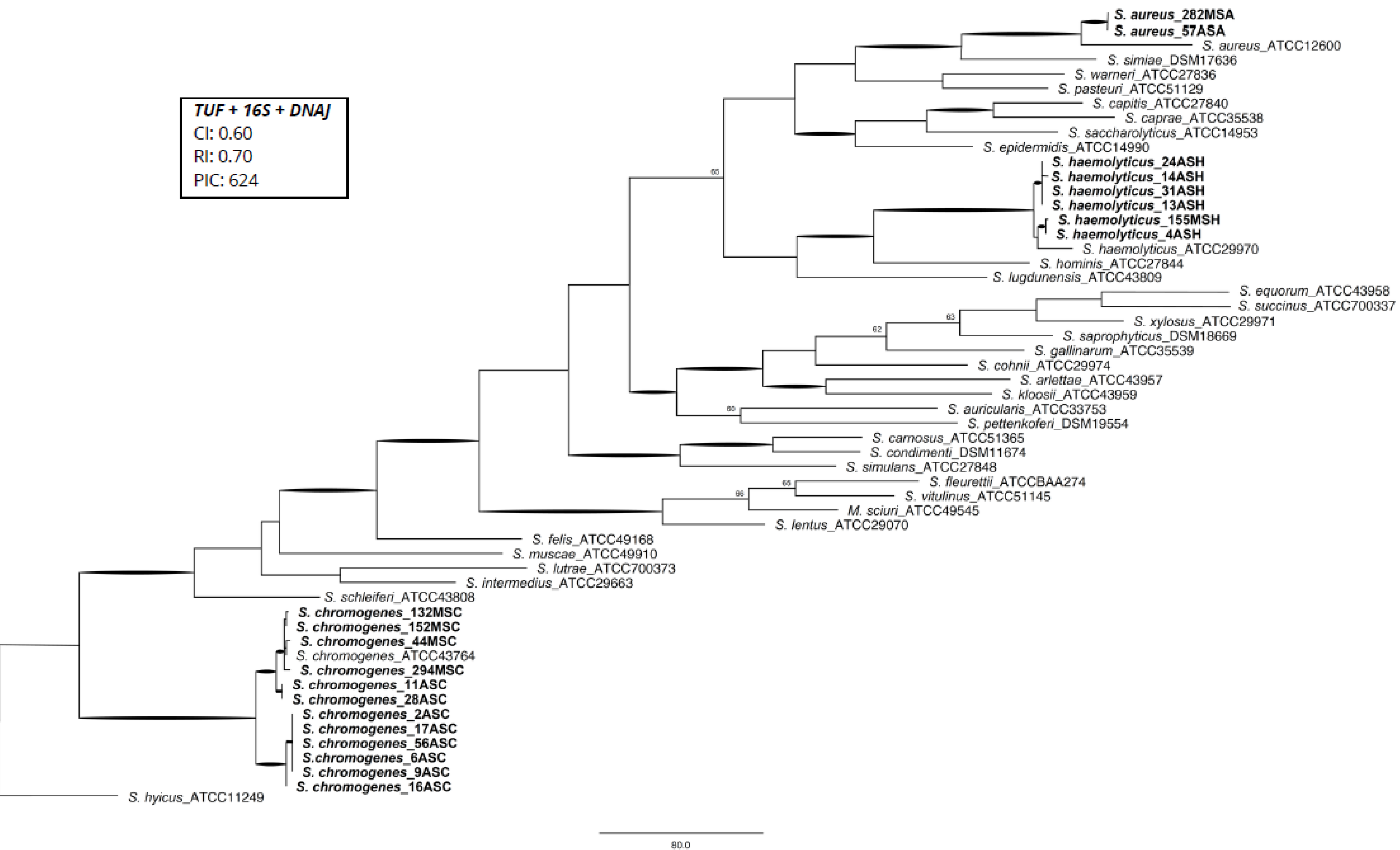

Figure 2. Phylogeny inferred in this study. Most-parsimonious tree for the combined *tuf*, *rpo*B, and *16s* data sets, including 54 ingroup taxa with *S. hyicus* ATCC11249 as the outgroup. Bootstrap intervals (1,000 replications) greater than 70% are indicated as branches in bold in the phylogenetic tree.

Conclusion

The phylogenetic tree showed the relation among the species, with four clades being observed. Relation between clinical isolates and non-clinical isolates was not observed. The presence of biofilm formation genes and toxin genes is a concern for public health and the food industry. *Staphylococcus* sp. must be controlled to prevent mastitis and, consequently, avoid the transmission farm to fork.

Disclosure Statement

The authors declare no competing interests.

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