

Whole genome sequence analysis of *Staphylococcus* spp. isolated from clinical mastitis and non-clinical fresh cows

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Abstract

Background

Staphylococcus spp. are among the most isolated bacteria from cases of clinical and subclinical mastitis in dairy cattle. Also, *Staphylococcus* is commonly isolated from bovine mammary secretions, not exclusively from intramammary infections. The genus comprises bacteria capable to form biofilm, produce toxins and acquire multi-drug resistance. The aims of this work were to evaluate the genetic virulence and antimicrobial resistance features of *Staphylococcus* spp. isolated from bovine milk using whole genome sequencing, and build a phylogenetic tree with gene sequences of *Staphylococcus* spp. isolated from clinical mastitis and non-clinical fresh cows.

Results

The bacterial collection comprised 29 *Staphylococcus* strains isolated from clinical mastitis cases (n = 7) and 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). After sequences quality control, twenty-three strains had good quality of contigs to be included in the further analysis.

Conclusions

Ninety-four virulence genes were observed, including *pvl*, *icaA*, *icaD* and MMSCRAMS genes. We also detected important resistance genes as *blaZ*, *ant(4)*, *erm(B)*, *fexA*, *Inu(D)*, *tet(L)* and *tet(M)*. The phylogenetic tree related the species as expected and presented four clades.

Background

Bovine mastitis is the most important disease in the dairy industry worldwide, and it is associated with pain and reduced welfare of affected animals (1, 2). Mastitis causes economic losses due to reduced milk production, milk discard, premature slaughter, impairment of reproductive performance, veterinary costs, and antibiotic usage (3, 4).

Staphylococci are responsible for numerous infections in humans and animals (5), including cellulitis, bacteremia, endocarditis, pneumonia, and mastitis. *Staphylococcus aureus* is the most pathogenic species in the genus (6) and it is considered one of the major pathogens of bovine mastitis (7). This bacterium 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 the immune cells (8, 9). The intracellular survival protects the bacteria from the effect of antibiotics commonly used in mastitis treatment, but also enables them to persist in the host for a long time without causing apparent inflammation (10).

Other important mechanism is that *Staphylococcus aureus* is capable of forming biofilms on both body and surfaces (11). The adherence and biofilm formation occur because the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and genes that encode for biofilm formation (*icaA*, *icaD* and *bap*) (12–14).

Focused management practices to prevent infections by contagious pathogens have reduced the incidence of *S. aureus* (15). On the other hand, other staphylococci species comprising a group known as coagulase-negative staphylococci (CoNS) emerged as important bacteria associated with bovine mastitis (16–19). Among the CoNS species commonly associated with mastitis are *S. chromogenes*, *S. haemolyticus*, *S. epidermidis*, *S. simulans*, *S. sciuri* and *S. xylosus* (18, 20–22). Some species of CoNS have the capacity to persist in the udder for months or even throughout the lactation (23, 24). The ability to form biofilm was also reported in CoNS isolated from bovine milk (25). Moreover, CoNS present a high number of virulence factors and the control of mastitis is complicated by the fact that CoNS is a group composed by a large number of different species (24).

The antimicrobial resistance in bacteria has increased during the last decades, which can reduce the efficacy of antimicrobial treatments against infectious diseases. Studies have described numerous antimicrobial resistance genes in CoNS isolated from mastitic milk (18, 26, 27), which can facilitate the persistence of intramammary infections in cows. Moreover, multiresistant CoNS strains have been described, which could impair the cure rates of mastitis caused by those pathogens (26).

Although CoNS and *S. aureus* share the same genus, they have different pathogenicity in the course of mastitis (28), and further studies are needed to elucidate the genetic mechanisms of infection associated with these pathogens. Studies assessing the genomic, resistance and virulence features of staphylococci species are necessary to understand the species relatedness within this group as well as their capacity to cause disease.

Next generation sequencing (NGS) technologies have provided relevant information about virulence and resistance genes among mastitis-causing bacteria (29, 30). Although there are studies where WGS was used to genetically characterize major pathogens of mastitis, there are few reports on CoNS isolated from milk (29, 31). Thus, the objectives of this study were to evaluate the genetic virulence and antimicrobial resistance features of *Staphylococcus spp.* isolated from bovine milk using whole genome sequencing, and build a phylogenetic tree with gene sequences of *Staphylococcus spp.* isolated from clinical mastitis and non-clinical fresh cows.

Results And Discussion

Twenty-nine *Staphylococcus spp.* were isolated from the farm's bank of strains and identified by Sanger sequencing. Seven strains were isolated from mastitic milk and were identified as *S. aureus* (n = 1), *S. chromogenes* (n = 4) e *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). The bacterial genomes were analyzed using the PATRIC software (<https://www.patricbrc.org>), and the quality and consistency of genomes are

showed in Table 1. Sequences from five strains identified as *S. chromogenes* and one *S. haemolyticus* failed during the quality control after WGS and were not included in the present dataset.

Table 1

Quality and parameters of genome sequences of 23 strains of *Staphylococcus* spp. isolated from healthy fresh cows (n = 17) and cows with CM (n = 6).

Lab ID	Specie	Origin of milk samples	Coarse Consistency*	Fine Consistency**	Contigs	Genome Length (bp)
282M	<i>S. aureus</i>	Mastistic cows	87.5	87.1	442	1,119,826
6M	<i>S. chromogenes</i>	Mastistic cows	97.9	97.4	79	2,192,580
294M	<i>S. chromogenes</i>	Mastistic cows	99.2	98.8	122	2,262,071
132M	<i>S. chromogenes</i>	Mastistic cows	99	98.7	230	2,325,883
155M	<i>S. haemolyticus</i>	Mastistic cows	88.7	87.4	346	1,288,193
44M	<i>S. chromogenes</i>	Mastistic cows	97.7	97.2	189	2,165,475
29A	<i>S. chromogenes</i>	Fresh cows	99.2	98.7	158	2,229,609
16A	<i>S. chromogenes</i>	Fresh cows	88	87.7	181	1,140,948
6A	<i>S. chromogenes</i>	Fresh cows	98.8	98.2	178	2,236,198
56A	<i>S. chromogenes</i>	Fresh cows	97.9	96.3	281	2,061,418
32A	<i>S. chromogenes</i>	Fresh cows	97.5	96.8	179	2,135,793
33A	<i>S. chromogenes</i>	Fresh cows	95.3	94.1	192	1,843,540
9A	<i>S. chromogenes</i>	Fresh cows	98.3	97.6	213	2,138,703
17A	<i>S. chromogenes</i>	Fresh cows	94.7	94.5	154	1,886,569
2A	<i>S. chromogenes</i>	Fresh cows	89	89	100	932,130
11A	<i>S. chromogenes</i>	Fresh cows	98.8	98.2	162	2,197,988
28A	<i>S. chromogenes</i>	Fresh cows	91.8	91.5	125	1,868,433

Lab ID	Specie	Origin of milk samples	Coarse Consistency*	Fine Consistency**	Contigs	Genome Length (bp)
57A	<i>S. aureus</i>	Fresh cows	89.1	88.8	236	1,741,203
31A	<i>S. haemolyticus</i>	Fresh cows	91	89.7	306	1,645,480
4A	<i>S. haemolyticus</i>	Fresh cows	94.7	94.2	263	1,853,994
24A	<i>S. haemolyticus</i>	Fresh cows	96.4	95.3	233	2,124,565
13A	<i>S. haemolyticus</i>	Fresh cows	89.7	88.2	458	1,401,570
14A	<i>S. haemolyticus</i>	Fresh cows	89.2	89.1	198	998,556
*Fine Consistency: This is the percentage of roles whose exact number of occurrences was correctly predicted by EvalCon. A higher number indicates the genome is more self-consistent. A lower number means the genome is less self-consistent.						
**Coarse Consistency: This is the percentage of roles whose presence or absence was correctly predicted by EvalCon. A higher number indicates the genome annotation is more self-consistent. A lower number means the genome annotation is less self-consistent.						

A higher frequency of CoNS strains was observed when compared to *S. aureus*, which denotes the high prevalence of these species in the mammary gland of dairy cows, especially during the first stages of lactation. 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 of mammary gland microbiota, and could also be more resistant to antibiotics than *S. aureus* (32, 33). 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* (34, 35).

Only three of the 22 fresh cows were further diagnosed with clinical mastitis, one of them had isolation of *S. aureus* and two were isolated with *S. chromogenes* at 10 ± 3 days in milk. Due the low frequency of clinical mastitis cases, it was not possible to perform an association analysis between the isolation of *Staphylococcus* spp. during the fresh period and occurrence of clinical mastitis within the next 3 months.

Among CoNS, five species are commonly found in milk samples: *S. chromogenes*, *S. simulans*, *S. xylosus*, *S. haemolyticus*, and *S. epidermidis* (21, 22). In our study, only two of those species were identified (*S. chromogenes* and *S. haemolyticus*); however only fresh cows with isolation of *S. chromogenes* developed clinical mastitis. Among the most prevalent CoNS causing mastitis, *S. chromogenes* is considered the most host-adapted species, especially because of its high prevalence and uncommon isolation from the environment (36–38).

In this present study we also evaluated the prevalence of virulence and resistance genes of *Staphylococcus* spp. (Tables 2 and 3). In total, 94 virulence genes (Table 2) were identified using Patric, ResFinder v2.1 (39) and Virulence Finder v1.5 (40). The most frequent genes identified in strains isolated from mastitic cows (n = 7) were *recA* (100%) and *mgrA* (100%), whereas *trpB* (75%) and *recA* (70%) were the most prevalent virulence genes among isolates from non-clinical fresh cows. The gene *recA* is known as a reference in *Staphylococcus* spp. and is related with the contribution of homologous recombination and DNA repair (41) and it is part of SOS response against stress (42). The *mgrA* gene is an important global virulence gene regulator in *Staphylococcus aureus* and mediates host-pathogen interactions and virulence (43).

Table 2

Prevalence of virulence genes in *Staphylococcus* spp. isolated from fresh cows and mastitis cows.

Virulence Genes	<i>S. aureus</i> (n = 2)		<i>S. chromogenes</i> (n = 15)		<i>S. haemolyticus</i> (n = 6)	
	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)
<i>trpB</i>	0	0	11 (73.3%)	4 (26.7%)	4 (66.7%)	0
<i>recA</i>	1 (50%)	1 (50%)	9 (60%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
<i>mgrA</i>	0	1 (50%)	9 (60%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
<i>oppD</i>	0	0	8 (53.3%)	4 (26.7%)	2 (33.3%)	0
SA1453	0	0	8 (53.3%)	4 (26.7%)	3 (50%)	0
<i>clpX</i>	1 (50%)	0	5 (33.3%)	4 (26.7%)	0	0
<i>Asd</i>	0	0	4 (26.7%)	3 (20%)	5 (83.3%)	1 (16.7%)
<i>Lip</i>	1 (50%)	1 (50%)	3 (20%)	1 (6.7%)	0	0
<i>femB</i>	0	0	3 (20%)	3 (20%)	3 (50%)	0
<i>msrA</i>	0	0	3 (20%)	3 (20%)	0	0
<i>esxA</i>	1 (50%)	0	1 (6.7%)	0	0	0
<i>carB</i>	0	0	1 (6.7%)	0	0	0
<i>pyrAA, purL</i>	1 (50%)	1 (50%)	0	0	5 (83.3%)	1 (16.7%)
<i>fbp</i>	1 (50%)	1 (50%)	0	0	4 (66.7%)	1 (16.7%)
<i>clpP</i>	0	1 (50%)	0	1 (6.7%)	2 (33.3%)	0
<i>lysA</i>	0	0	0	0	5 (83.3%)	1 (16.7%)
<i>citB</i>	1 (50%)	0	0	0	4 (66.7%)	0

Virulence Genes	<i>S. aureus</i> (n = 2)		<i>S. chromogenes</i> (n = 15)		<i>S. haemolyticus</i> (n = 6)	
	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)
SA1061	0	0	0	0	5 (83.3%)	0
SAHV_0914	1 (50%)	1 (50%)	0	0	1 (16.7%)	1 (16.7%)
<i>essC, sdrD, adsA, aur, cap8D, cap8E, cap8F, cap8G, cap8L, cap8M, cap8N, cap8O, cap8P, clfB, esaA, esaB, essA, essB, fnbA, geh, hlgB, oppF, sdrE, tilS</i>	1 (50%)	1 (50%)	0	0	0	0
<i>icaD, cap8A, cap8B, clfA, esaC, esxB, icaA, icaB, icaC, lukF-PV, sbi</i>	1 (50%)	0	0	0	0	0
<i>atmB, ccpA, ciaR, cpsY, cydA, fba, gidA, glnA, guaA, hasC, lepA, leuS, luxS, perR, purB, purH, purN, rpoE, sodA, SP_0095, SP_0111, SP_0310, SP_0494, SP_0819, SP_0856, SP_0916, SP_1396, SP_1398, SP_1970, SP_1086, SPy_1633, vickK</i>	0	0	0	1 (6.7%)	0	0
<i>hlgC, hysA, map, odhB, sdrC, sspB</i>	0	1 (50%)	0	0	0	0
<i>trpA</i>	0	0	0	0	1 (16.7%)	0

Table 3

Prevalence of resistance genes in *Staphylococcus* spp. isolated from milk of fresh cows and mastitic COWS.

Resistance Genes	<i>S. aureus</i> (n = 2)		<i>S. chromogenes</i> (n = 15)		<i>S. haemolyticus</i> (n = 6)	
	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)
<i>EF-G, EF-Tu, gidB, gyrA, gyrB, MurA, rpoB, rpoC, S12p</i>	1 (50%)	1 (50%)	11 (73.3%)	4 (26.7%)	5 (83.3%)	1 (16.7%)
<i>folP</i>	1 (50%)	1 (50%)	11 (73.3%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
<i>kasA</i>	0	0	11 (73.3%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
<i>NorA</i>	1 (50%)	0	11 (73.3%)	4 (26.7%)	5 (83.3%)	1 (16.7%)
<i>rho</i>	1 (50%)	0	11 (73.3%)	4 (26.7%)	1 (16.7%)	0
<i>TcaB</i>	0	0	11 (73.3%)	4 (26.7%)	2 (33.3%)	0
<i>Tet(38)</i>	1 (50%)	1 (50%)	11 (73.3%)	4 (26.7%)	0	0
<i>inhA, fabI</i>	0	1 (50%)	10 (66.6%)	4 (26.7%)	4 (66.7%)	0
<i>S10p</i>	1 (50%)	1 (50%)	10 (66.6%)	4 (26.7%)	5 (83.3%)	0
<i>Ddl, pgsA</i>	0	0	9 (60%)	4 (26.7%)	1 (16.7%)	0
<i>GdpD</i>	0	0	9 (60%)	4 (26.7%)	3 (50%)	1 (16.7%)
<i>Iso-tRNA</i>	0	0	9 (60%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
<i>vrarR, vraS</i>	0	0	9 (60%)	4 (26.7%)	0	0
<i>mgrA</i>	0	1 (50%)	9 (60%)	4 (26.7%)	4 (66.7%)	1 (16.7%)
<i>parE</i>	0	0	9 (60%)	4 (26.7%)	3	0

Resistance Genes	<i>S. aureus</i> (n = 2)		<i>S. chromogenes</i> (n = 15)		<i>S. haemolyticus</i> (n = 6)	
	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)
<i>TcaR</i>	0	0	9 (60%)	3 (20%)	2 (33.3%)	1 (16.7%)
<i>Alr</i>	1 (50%)	0	8 (53.3%)	4 (26.7%)	4 (66.7%)	0
<i>dxr</i>	0	0	8 (53.3%)	3 (20%)	0	0
<i>vraF</i>	0	0	8 (53.3%)	4 (26.7%)	0	0
<i>MprF</i>	0	0	7 (46.7%)	4 (26.7%)	3 (50%)	0
<i>sav1866</i>	1 (50%)	0	7 (46.7%)	4 (26.7%)	1 (16.7%)	0
<i>folA, Dfr</i>	0	0	6 (40%)	4 (26.7%)	1 (16.7%)	0
<i>blaZ</i>	0	0	1 (6.7%)	0	0	0
<i>ANT(4')-Ib, Erm(B), mepA, Tet(L)</i>	1 (50%)	0	0	0	0	0
<i>arlR, BceA, BceB</i>	0	0	0	0	2 (33.3%)	0
<i>BceR, BceS</i>	0	0	0	0	2 (33.3%)	1 (16.7%)
<i>dfrC</i>	0	0	0	0	1 (16.7%)	0
<i>FabK, FexA family</i>	0	0	0	1 (6.7%)	0	0
<i>Lnu(A)</i>	0	0	0	0	0	1 (16.7%)
<i>Lnu(D)</i>	0	0	0	1 (6.7%)	0	0
<i>mepR</i>	1 (50%)	1 (50%)	0	0	0	0
<i>RlmA(II)</i>	0	0	0	1 (6.7%)	0	0
<i>TcaA</i>	0	0	0	0	2 (33.3%)	0
<i>TcaB2</i>	1 (50%)	0	0	0	3 (50%)	1 (16.7%)

Resistance Genes	<i>S. aureus</i> (n = 2)		<i>S. chromogenes</i> (n = 15)		<i>S. haemolyticus</i> (n = 6)	
	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)	healthy cow n (%)	mastitis cow n (%)
<i>Tet(M)</i>	0	0	0	1 (6.7%)	0	0
<i>YkkCD</i>	0	0	0	0	5 (83.3%)	1 (16.7%)

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 (9). Thus, in this and other contexts, the presence of virulence factors as well as the genes responsible for mediating these factors, has an important role, 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 45 virulence genes. Other strains with high frequency of virulence genes were one *S. chromogenes* isolated from a cow with CM that had 40 virulence genes, the *S. aureus* isolated from another mastitic cow with 38 virulence genes, and three *S. haemolyticus* isolated from non-clinical fresh cows that had 13 virulence genes. The fresh cow infected with the *S. aureus* with 45 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 Pantone-Valentine leukocidin (PVL). PVL is associated with tissue necrosis and leukocyte destruction (44).

Among genes observed in strains from both clinical and non-clinical strains were the *msrA* and *sdrD*. The *msrA* gene encodes the mechanism which involves a macrolide efflux pump. The protein produced by this gene is able to export 14 macrolides and streptogramin B antibiotics from bacterial cells (45). In addition, this gene is responsible to produce methionine sulfoxide reductases in oxidative stress tolerance and was reported as an important virulence factor in *Staphylococcus aureus* (SINGH *et al.* 2015). The *sdrD* 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 blood and thus attenuates bacterial clearance (<https://www.uniprot.org/uniprot/O86488>). Both *msrA* and *sdrD* could difficult the antibiotic treatment as well the immune response, facilitating the infection onset and persistence.

The Venn diagram was constructed using the 15 most prevalent genes identified in strains isolated from fresh cows and animals with clinical mastitis (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). Twelve genes were concomitant in both categories of animals (Fig. 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.

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; however, the importance of biofilm formation during infection have been considered in this group of bacteria (46). The biofilm-producing CoNS were reported to be less susceptible to antibiotics than planktonic cells (47), which could be a factor increasing the persistence of certain species in this group. Other group of proteins involved with adhesion of bacteria to the host cells is the staphylococcal MSCRAMM (microbial surface components recognizing adhesive matrix molecules), 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 (12, 13).

Table 3 shows the prevalence of the resistance genes. It is important to highlight that many genes identified are constitutive and need a mutation to confer resistance. Resistance genes for tetracyclines, beta lactams, cloranphenicol, aminoglycosides, macrolides and others were observed as well as multidrug resistant coagulase negative staphylococci, which has been reported in previous studies (27, 48).

The bacterial resistance difficult mastitis treatment and is a problem for public health. The organisms can acquire resistance to antibiotics by diverse mechanisms. The antimicrobial resistance determinants can be classified into: acquisition of foreign DNA, when the bacteria acquire the DNA by transduction, transformation, and conjugation; mutations of preexisting genetic determinants which affect structural or regulatory genes; and mutations in acquired genes (49).

Beta-lactams, macrolides and lincosamides are antimicrobials used for prevention and treatment of mastitis (33). The resistance to lincosamides, streptogramins, macrolides, tetracycline, beta lactams and ciprofloxacin in bacterial strains isolated from bovine with mastitis were previously reported (7, 27, 50). The resistance to beta-lactams is a known public health problem worldwide (50, 51). Resistance in the *Staphylococcus* genus is explained by production of the beta-lactamase enzyme encoded by the gene *blaZ* and synthesis of penicillin-binding protein 2A (PBP2A) with a low affinity for binding to penicillin coded by the gene *mecA* (52, 53). The presence of *blaZ* gene is common (54), but just one strain presented this gene in our study and none had 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 (55) and the mechanisms which the bacteria resist to these antibiotics are enzymatic inactivation of active efflux and/or structural changes at the ribosomal target site (56). The strains genetically evaluated in the present study had the *Lnu(A)* and *Lnu(D)* genes, which are associated with the bacterial resistance to lincosamides (Table 3).

At least thirty-five different tetracycline resistance (*tet*) genes and three oxytetracycline resistant (*otr*) genes have been characterized and, in general, the tetracycline resistance occur by active efflux resulting

from the acquisition of these genes, or by a protein that protects bacterial ribosomes from the action of tetracyclines (57). In our sequencing results five genes that confer resistance to tetracycline were detected, *mepA*, *tet(L)*, *tet(M)*, *tet(38)* and S10p.

The *EF-G*, *EF-Tu*, *rpoB* and *rpoC* genes identified in this study, were present in all strains of *S. aureus*, both from healthy cows and cows with mastitis; for strains of *S. chromogenes*, the same genes were present in 73.3% of strains isolated from healthy cows and in 26.7% of strains isolated from cows with mastitis; and for strains of *S. haemolyticus*, they were present in 83.3% of strains isolated from healthy cows and in 16.7% of strains isolated from cows with mastitis.

The gene *EF-G* is related with resistance to fusidic acid. Fusidic acid binds to protein *EF-G* and the ribosome thereby inhibiting further bacterial protein synthesis. The alteration of the target protein *EF-G* and permeability of the bacterial envelope for the antibiotic are the resistance mechanisms suggested (58, 59). With regard to *S. aureus*, however, neither of these two mechanisms has yet been proven at the molecular level. A study have demonstrated that fusidic acid resistance in *S. aureus* results from point mutations within the chromosomal *fusA* gene encoding *EF-G* (60). *rpoB* and *rpoC* are genes that belong to *Staphylococcus* species and need a mutation to permit the resistance against the antibiotic (61). *EF-tu* is related with an elongation factor, *rpoB* with β subunit of bacterial RNA polymerase (62, 63); therefore, it is expected the presence of these genes in the strains studied. These genes could be a possible drug target or one mutation in them could develop a resistance to antibiotics.

In our study, we did not carry out a quarter-level assessment of somatic cell count (SCC), which could indicate the presence of subclinical mastitis in non-clinical fresh cows. Epidemiological studies have agreed that cows with SCC > 200.000 cells/mL are likely to have mastitis (64, 65). At the quarter level, SCC of 100,000 cells/mL have been used as the limit to differentiate infected mammary quarters from uninfected (66). Although the lack of SCC assessment may be a limitation of the present study, our objective was comparing the genetic features of staphylococci strains isolated from quarters with clinical mastitis with those from non-clinical fresh cows. Furthermore, the milk sample collections from fresh cows were carried out in a moving rotary parlor after automatic removal of milking units from the cows. This restricted the period for sample collection, and therefore, we prioritized an adequate collection of milk samples for microbiological culture to avoid contamination.

In our study, we also performed a phylogenetic analysis of the isolates (Fig. 2) and four clades were detected: two clades of *S. chromogenes*, one of *S. haemolyticus* and one of *S. aureus*. Naushad et al. (2016) observed five clades in their study, in which they build a tree with non-aureus staphylococci species isolated from bovine intramammary infection, and there were consistent interspecies relationships within clades in WGS phylogenetic reconstructions (31).

CoNS or non-aureus staphylococci are heterogeneous and is common conflicting phylogenies in these species mainly just one gene is used (31, 67). It is important to consider that the use of WGS sequences to build a phylogenetic tree offer great accuracy in reconstructing evolutionary relationships for identification and elucidation of evolutionary histories of bacterial organisms (31). *S. aureus* strains in

our study were more related with *S. haemolyticus* in phylogenetic tree, but the *S. chromogenes* strains presented a higher number of virulence and resistance genes, being more virulent than *S. haemolyticus*.

Conclusion

The phylogenetic tree showed the relation among the species and four clades were observed. Three strains, two isolated from milk from mastitic cows and one from fresh cows, presented more than 13 virulence genes and they were identified as multidrug resistant strains. Herein it was not possible to relate the genes found in the strains with mastitis. The isolated strain of the cow diagnosed with mastitis that eventually died was identified as CoNS. As already described in the literature and also mentioned in this study, CoNS are more resistant to antibiotics than *S. aureus*, which may explain their presence in greater numbers in this study, both in clinical and non-clinical cows. The presence of virulence and resistance genes in all clinical conditions of the cow may facilitate studies on drug and vaccine targets in the future.

Methods

Origin and isolation of strains

The *Staphylococcus spp.* strains evaluated in this study belonged to a bacteria collection and were isolated from twenty-two fresh and non-clinical cows in addition to seven cows with clinical mastitis. The strains were isolated from milk samples collected in a large commercial dairy farm located in Scipio, New York. The farm milked approximately 4,100 Holstein cows 3 times daily in a 100-stall rotary milking parlor. The animals were housed in freestall barns, with concrete stalls covered with mattresses and bedded with manure solids. 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 four protocols for treatment of clinical mastitis caused by Gram-positive pathogens (Tomazi et al., 2020). In the latter 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 colonies color and morphology observed in the plates. Pink and orange colonies were selected for species confirmation and microbiological procedures.

For 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 laboratorial procedures 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, 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. Subsequently, one isolated colony was taken from the last inoculated plate and suspended in 15 mL of BD Bacto™ Brain Heart Infusion (BHI) broth (BD Biosciences, Sparks, MD). After an overnight incubation at 37°C in a shaker, the samples were centrifuged for 30 minutes (4,200 rpm at 4°C). The supernatant was discarded and the pellet was resuspended in 5 mL of BHI broth with 25% of glycerol. Finally, 2 mL of resuspended bacteria was transferred to cryotubes and stored at -80°C until further analysis.

Fresh cows with isolation of *Staphylococcus* spp. were monitored for 3 months after milk sample collection for identification of eventual clinical mastitis cases according reports extracted from the Dairy Comp 305 (DC305) management software (Ag Valley Agricultural Software, Tulare, CA).

Identification of strains by 16S rDNA gene sequencing

The DNA was extracted from each bacterial isolate using DNAasy PowerFood Microbial Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The extracted DNA was quantified by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). After, a PCR for the 16S ribosomal DNA gene was performed using 10 pmol of each fD1 forward and rP2 reverse primers (68), Econo-Taq Plus Green 1× Master Mix (Lucigen, Middleton, WI), 280 to 350 ng of template DNA, and ultrapure distilled water was added to complete the volume to 100 µL. The parameters used for amplification were: 94°C for 5 min, 57°C for 2 min, and 72°C for 2 min followed by 29 cycles of 94°C for 2 min, 57°C for 30 s, and 72°C for 2 min, with a final extension of 72°C for 10 min (WOOD *et al.*, 1998). The presence of PCR products was confirmed by agarose gel electrophoresis (1.2% wt/vol) with 0.5 µg/mL ethidium bromide. The PCR products were purified using Gel/PCR Fragments Extraction Kit (IBI Scientific, Peosta, IA) following the manufacturer's instructions. Purified samples were submitted to the Institute of Biotechnology at Cornell University (Ithaca, NY) for Sanger sequencing using 8 pmol of primer fD1 and 300 ng of PCR products. DNA sequences in FASTA format were aligned with sequences stored in GenBank using the BLAST algorithm (69) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). This identification was made previously for confirmation of genus and species before whole genome sequencing.

Whole genome sequencing (WGS)

Samples were diluted by adding ultrapure water (Invitrogen, Waltham, MA) until a concentration of 0.2 ng/µl, measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA). After normalization, the samples were used as an input to the Nextera® XT DNA Library Prep Kit (Illumina Inc., USA). The library preparation was done according to the manufacturer's protocol (Nextera® DNA Library Prep Reference Guide).

Tagmentation of samples was done using 1 ng of template, the PCR amplification was carried out using a unique combination of barcode primers provided by manufacture (kit Nextera® XT Index Kit, Set A,

Illumina Inc., USA). The libraries purification was performed using Mag-Bind TotalPure NGS (Omega BioTek - Norcross, GA) bead purification and normalized according to Library Normalization beads/additives step. For sequencing, equal volumes of normalized libraries were combined, diluted in hybridization buffer and heat denatured according to MiSeq System Denature and Dilute Libraries Guide (Illumina Inc., San Diego, CA). Finally, we performed pair-end sequencing 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 original reads was evaluated using FASTQC. The potential contamination of sequences was checked by Kraken2. Sequencing reads were submitted to the comprehensive genome analysis service using the Pathosystems Resource Integration Center (PATRIC) (70). Reads were assembled using SPAdes (71) and the genomes were annotated using the Rast tool kit found in the PATRIC (PATRIC 3.2.96), as part of the all-bacteria Bioinformatics Resource Center available online (72).

The identification of samples after WGS was carried out by the online software available at the Center of Genomic Epidemiology website (<https://cge.cbs.dtu.dk/services/KmerFinder/>). In addition, the resistance genes were submitted to The Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/analyze/rgi>) and to Resfinder of the Center of Genomic Epidemiology (<https://cge.cbs.dtu.dk/services/ResFinder/>). The data were deposited in the PATRIC software. The quality and parameters of the strains' genome sequences can be seen in Table 1. The PATRIC software performed the alignments of the sequences.

The phylogenetic tree was built using The Phylogenetic Tree Building Service of the PATRIC software (<https://www.patricbrc.org>) using the assembled sequences. The codon tree method utilizes PATRIC PGFams as homology groups and analyzes aligned proteins and coding DNA from single-copy genes using the program RAXML.

Declarations

Ethics approval and consent to participate

This study was carried out in strict accordance with the recommendations of The Animal Welfare Act of 1985 (P.L. 99–198). The research protocol was reviewed

and approved by the Institutional Animal Care and Use Committee of Cornell University (protocol number 2017–0073).

Consent for publication

Not applicable for that section

Availability of data and materials

The datasets generated and analyzed in the current study are available at www.patricbrc.org and directly from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Nathália C.C. Silva and Marjory X. Rodrigues performed bacteria isolation and DNA sequencing; Ana C.C.H.Tomazi and Tiago Tomazi collected samples and farm data; Bruna L. Crippa revised and corrected the manuscript; Rodrigo C. Bicalho and Nathalia C.C. Silva conceived the study and performed data analysis. All authors reviewed the manuscript.

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Figures

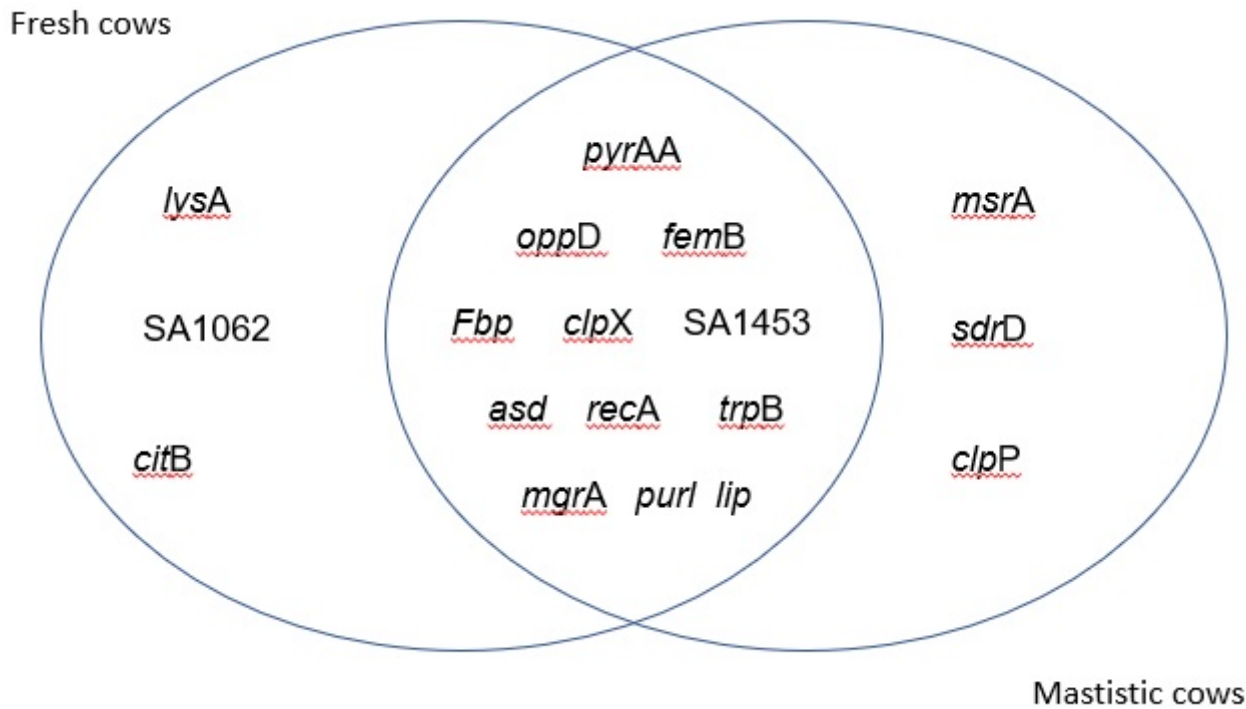


Figure 1

Correlation of the most prevalent virulence genes in mastitis and fresh cows. Venn diagram illustrating the most prevalent virulence genes across all *Staphylococcus* spp. isolates from milk of healthy cows and cows affected with mastitis.

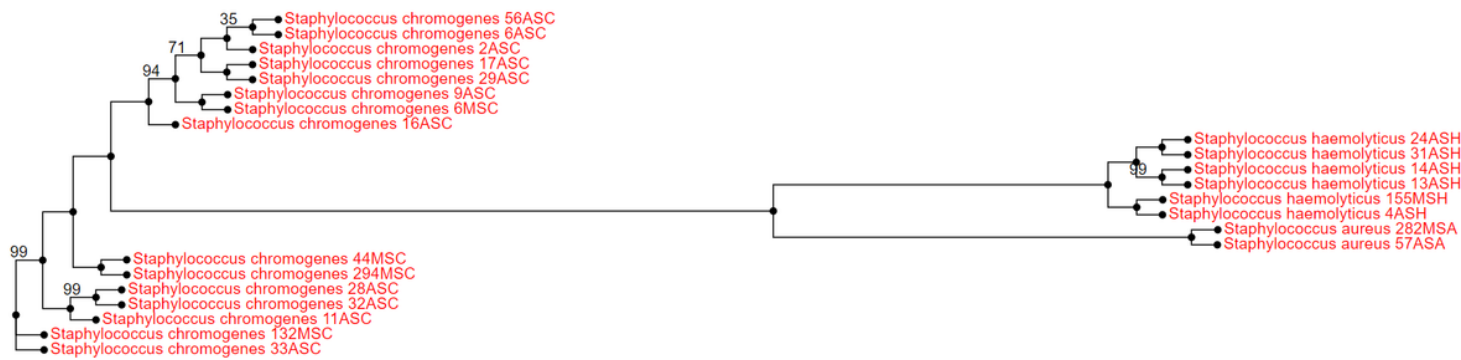


Figure 2

Phylogenetic tree with all negative and positive coagulase strains isolated from milk of dairy cows with healthy mammary gland and mastitis, being the letter “A” after number strains isolated from fresh cows and letter “M” after number strains isolated from mastitis cows.

Supplementary Files

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