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REGULAR ARTICLES

Frequency of enterotoxins, toxic shock syndrome toxin-1, and biofilm formation genes in *Staphylococcus aureus* isolates from cows with mastitis in the Northeast of Brazil

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Abstract
Staphylococcus aureus is among the microorganisms more frequently associated with subclinical bovine mastitis. *S. aureus* may produce several virulence factors. This study aimed at determining the frequency of virulence factors such as enterotoxins, toxic shock syndrome toxin 1, and *ica* adhesion genes. In addition, we assessed antimicrobial drug resistance in *S. aureus* isolated from clinical and subclinical cases of mastitis. A total of 88 cows with clinical or subclinical mastitis were sampled, resulting in 38 *S. aureus* isolates, from which 25 (65.78%) carried toxin genes, including *seb*, *sec*, *sed*, *tst*, and *icaD* adhesion gene. These *S. aureus* isolates belong to 21 ribotypes and three *S. aureus* strains belonged to the same ribotype producing *ica* adhesion gene. Approximately 90% of *S. aureus* strains obtained in our study demonstrated multiple resistance to different antimicrobial agents. The most efficacious antimicrobial agents against the isolates were gentamicin, amoxicillin, and norfloxacin. Gentamicin was the most efficacious agent inhibiting 78.95% of the *S. aureus* isolates. The least efficacious were penicillin, streptomycin, and ampicillin. Our results can help in understanding the relationship between virulence factors and subclinical mastitis caused by *S. aureus*. Further research about diversity of *S. aureus* isolates and genes responsible for the pathogenicity of subclinical mastitis is essential.

Keywords *Staphylococcus aureus* · Mastitis · Virulence factor

Introduction

Bovine mastitis is a major cause of economic losses in dairy cattle, and it is usually associated with low incidence of clinical cases and high incidence of subclinical infections, which poses a challenge for controlling this disease (De Vliegher et al. 2012; Thompson-Crispi et al. 2014). Some studies have reported that subclinical mastitis is more prevalent than the clinical disease form (Iyer et al. 2014; Viguier et al. 2009). Among several bacterial agents that can cause contagious mastitis, the genus *Staphylococcus* is one of the most important (Pyorala and Taponen 2009). The species *Staphylococcus aureus* is the pathogen more often associated with subclinical cases of mastitis (Persson et al. 2011). In addition, *S. aureus* has also relevance under a public health point of view, due to the high frequency of resistance to antimicrobial drugs, and the ability of this organism to produce toxins that is an important cause of food poisoning. Importantly, these toxins are resistant to high temperatures such as those employed during the pasteurization process (Schelin et al. 2011). Toxins genes and virulence factors are related to pathogenicity and resistance of *S. aureus* strains to various antimicrobial agents (Babra et al. 2013), interfering the effectiveness of therapy and control methods of this bacterium present in intramammary gland of dairy cows (Kirkan et al. 2005). The association between different virulence factors and the clinical disease manifestations is still barely known.

Among the remarkable range of virulence factors of this pathogen can be highlighted the toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SE) and *ica* adhesion

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gene (Melchior et al. 2007). Some of these factors may be of more importance than others in different stages of the pathogenesis of mastitis infections, as not all factors are produced by all strains of *S. aureus*. These factors can be associated to the severity of disease, chronic disease, and the difficulty of treatment (Babra et al. 2013).

Elucidating the presence of virulence factors that *S. aureus* can produce of accord with clinic form is valuable in developing control measures against this infectious disease. We investigated the possibility of differentiating *Staphylococcus* strains present in clinical and subclinical mastitis by ribotyping technique. We amplified the 16S and 23S rRNA genes that have been considered as the gold standard for identification and taxonomic classification of different bacteria species (Bouchet et al. 2008; Kolbert and Persing 1999). The genes encoding the enterotoxins Sea, Seb, Sec, Sed, and See were evaluated in this study.

In the Maranhão state, Northeast of Brazil, the molecular epidemiology of clinical and subclinical mastitis in bovine dairy herds is unknown, as there are no studies that show the epidemiological and antimicrobial resistance aspects of this disease. Therefore, the goal of this study was to characterize genotypes of *S. aureus* strains associated with mammary infections in this region, assessing the presence of genes related to toxin production, the *icaA* and *icaD* genes that are associated with biofilm formation, and the *tst* gene that is associated with the toxic shock syndrome as well as determine the profile of antimicrobial drug resistance of these isolates.

Materials and methods

Farms and sampling

This study included 10 farms located at the São Luis Island (state of Maranhão, Brazil). Cattle were kept in a semi-extensive system on these farms so they stayed in pastures and received grain concentrate and a mineral supplement. Cows were milked manually once a day in the morning in the presence of their calves. Only on rare occasions washing and disinfection of the teats were performed prior to milking, and when performed, it was followed by wiping with a cotton tissue used for all cows. Pre- or post-dipping, strip cup test, or California Mastitis Test (CMT) were not performed on any of the farms.

During the course of this study, milking cows from all farms were subjected to the strip cup test and the CMT. These tests were used as a screening of clinical and subclinical cases of mastitis. Results were considered positive when milk samples from any of the quarters had clots, pus, blood, or abnormal discoloration, or a score ≥ 1 at the CMT. Samples from positive cows were collected for bacteriological culture, except from cows within the first 10 days of lactation or 30 days before the end of the lactation. Cows from four farms tested positive by the strip cup test and CMT were sampled. These positive farms were designated A, B, C, and D. A total of 88 cows were sampled. These samples were collected prior to milking and after wiping the teats with 70% alcohol with iodine, using sterile 100 mL vials. These samples were transported at approximately 4 °C to the microbiology laboratory at the Universidade Estadual do Maranhão (UEMA) for bacterial isolation and characterization.

Identification of *Staphylococcus aureus*

Milk samples were plated on Columbia agar (Acumedia, Baltimore, USA) with 5% ovine blood and Baird-Parker (Sigma-Aldrich, St Louis, USA), followed by incubation at 37 °C for 48 h. We selected three to five typical colonies from plates containing from 20 to 200 colonies for morphologic evaluation. Colonies of Gram-positive cocci were considered *Staphylococcus* sp. (Holmberg 1973).

These *Staphylococcus* spp. isolates were subjected to the catalase and coagulase tests. Isolates that were positive in these two tests were subjected to acetoin production assay by culturing in MRVP broth for differentiation between *Staphylococcus aureus*, *Staphylococcus hyicus*, *Staphylococcus delphini*, and *Staphylococcus intermedius*. Acetoin-producing isolates were tested for maltose and trehalose utilization for differentiation between *Staphylococcus aureus* and *Staphylococcus schleiferi* subspecies *coagulans* (Becker and Eiff 2011; Garcia 2010; Lancette and Tatini 2001). Isolates that were positive in all these tests were classified as *Staphylococcus aureus* (Garcia 2010, Lancette and Tatini 2001).

Antimicrobial susceptibility testing

The isolates were subjected to in vitro susceptibility using the disk diffusion test (Bauer et al. 1966), including the following antimicrobial drugs: amoxicillin (10 μg), lincomycin (2 μg), norfloxacin (10 μg), ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), oxacillin (1 μg), streptomycin (1 μg), enrofloxacin (5 μg), penicillin (10 μg), tetracycline (30 μg), and vancomycin (30 μg). Assessment of the inhibition halos was performed according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI Clinical and Laboratory Standards Institute 2003).

DNA extraction and genotypic characterization of *Staphylococcus aureus*

Isolates were grown in BHI broth at 37 °C for 24 h, and 1 mL of each culture was transferred to sterile 1.5 mL tubes, and genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (PROMEGA, Madison, WI, USA) according to the manufacturer's instructions.

S. aureus isolates were further characterized by PCR amplification of the *S. aureus*-specific *femA* gene (Johnson et al. 1995; Riyaz-Ul-Hassan et al. 2008). Each PCR reaction had a final volume of 25 μL, containing 2.5 μL of 10× reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of each primer (Table 1), 2.5 U of *Taq* DNA polymerase (Invitrogen, Brazil), and 5 μL of template DNA (20 ng/μL). Cycling parameters were an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 2 min, annealing at 57 °C for 2 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. The specific PCR product had 132 base pairs.

Detection of the enterotoxin-encoding genes *sea*, *seb*, *sec*, *sed*, *see*, and *tst*

Presence of genes encoding the enterotoxins Sea, Seb, Sec, Sed, and See were detected by multiplex PCR using a previously described protocol (Becker et al. 1998). Multiplex PCR was performed in a final volume of 50 μL with 5 μL of 10× reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 3 mM $MgCl_2$, 160 μM of each dNTP, 20 pmol of each primer (Table 1), 1.2 U of *Taq* DNA polymerase (Invitrogen, Brazil), and 5 μL of genomic DNA (20 ng/μL). Cycling parameters were denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 2 min. *S. aureus* strains ATCC 13565, ATCC 14458, ATCC 19095, ATCC 23235, and ATCC 27664, carrying the genes *sea*, *seb*, *sec*, *sed*, and *see*, respectively, were obtained from the Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil) and used as positive controls.

The gene encoding TSST-1 (*tst)* was amplified by regular (monoplex) PCR using the primers described by Mehrotra et al. (2000). This gene was amplified in a 25-μL reaction with 2.5 μL of 10× reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of each primer (Table 1), 2.5 U of *Taq* DNA polymerase (Invitrogen, Brazil), and 5 μL of template DNA (20 ng/μL). Cycling parameters included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 2 min, annealing at 57 °C for 2 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min.

Detection of the *icaA* e *icaD* genes associated with biofilm formation

The *icaA* and *icaD* genes were amplified according to a previously described PCR protocol (Cramton et al. 1999). Reactions

Table 1 Primers and their sequences used in this study

Gene	Primer	Sequence (5′–3′)	Product (bp)*	Reference
sea	SEA-3	CCTTTGGAAACGGTTAAAACG	127	Becker et al. (1998)
	SEA-4	TCTGAACCTTCCCATCAAAAAC		
seb	SEB-1	TCGCATCAAACTGACAAACG	477	Becker et al. (1998)
	SEB-4	GCAGGTACTCTATAAGTGCCTGC		
sec	SEC-3	CTCAAGAACTAGACATAAAAGCTAGG	271	Becker et al. (1998)
	SEC-4	TCAAAATCGGATTAACATTATCC		
sed	SED-3	CTAGTTTGGTAATATCTCCTTTAAACG	319	Becker et al. (1998)
	SED-4	TTAATGCTATATCTTATAGGGTAAACATC		
see	SEE-3	CAGTACCTATAGATAAAGTTAAAACAAGC	178	Becker et al. (1998)
	SEE-2	TAACTTACCGTGGACCCTTC		
tst	TSST-1	ACCCCTGTTCCCTTATCATC	326	Mehrotra et al. (2000)
	TSST-2	TTTTCAGTATTTGTAACGCC		
femA	FEMA-1	AAAAAAGCACATAACAAGCG	132	Mehrotra et al. (2000)
	FEMA-2	GATAAAGAAGAAACCAGCAG		
icaA	ICAA-1	TCTCTTGCAGGAGCAATCAA	188	Cramton et al. (1999)
	ICAA-2	TCAGGCACTAACATCCAGCA		
icaD	ICAD-1	ATGGTCAAGCCCAGACAGAG	198	Cramton et al. (1999)
	ICAD-2	CGTGTTTTCAACATTTAATGCAA		
16-23S rRNA	rRNA1	TTGTACACACCGCCCGTCA	**	Cuny et al. (1996)
	rRNA2	GGTACCTTAGATGTTTCAGTT		

**bp* base pairs

***Variable size products

had a final volume of 20 μL, containing 5 μL of 5× reaction buffer (10 mM Tris–HCl, pH 8.5, 50 mM KCl), 2 μL of 25 mM $MgCl_2$, 1.6 μL of 10 mM of dNTPs, 10 pmol of each primer (Table 1), 5 U of *Taq* DNA polymerase (Promega), and 1 μL of template genomic DNA (100 ng/μL). Cycling parameters included an initial denaturation at 92 °C for 2 min, followed by 30 cycles of denaturation at 92 °C for 45 s, annealing at 56 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 7 min.

Ribotyping analysis of *Staphylococcus aureus* isolates

S. aureus isolates were compared by PCR-ribotyping as previously described by Cuny et al. (1996) with modifications.

Table 2 Antimicrobial resistance profile of *S. aureus* isolates from milk of cows with clinical or subclinical mastitis

S. aureus isolates	Clinical signs*	Antibiotic resistant	GEN	AMX	AMP	TET	ERY	LCM	ENR	NOR	PEN	CHL	OXA	VAN	STR
1	SC	5	S	R	R	R	S	R	I	S	R	S	I	S	S
2	SC	13	R	R	R	R	R	R	R	R	R	R	R	R	R
3	SC	10	I	S	R	R	R	R	R	R	R	R	R	S	R
4	SC	6	R	S	S	I	I	S	S	S	R	R	R	R	R
5	SC	6	S	S	S	R	S	S	S	S	S	R	S	R	I
6	SC	3	S	R	R	S	R	R	I	I	R	I	R	R	S
7	SC	7	S	S	S	I	I	I	S	S	R	S	R	S	I
8	C	2	R	R	R	R	R	R	R	R	R	R	S	R	R
9	SC	12	S	S	S	S	I	I	S	S	R	R	S	S	I
10	SC	2	S	S	S	S	S	S	S	S	S	S	S	S	S
11	SC	0	S	R	R	S	I	R	I	I	R	R	R	S	S
12	SC	6	S	R	R	S	R	S	S	S	R	R	S	S	R
13	C	6	I	S	R	R	R	R	R	R	R	R	R	S	I
14	SC	9	S	R	S	I	I	S	R	S	R	S	S	R	R
15	SC	5	S	S	R	S	I	I	S	S	R	S	S	S	S
16	SC	2	S	R	R	R	I	R	S	S	R	S	R	I	I
17	SC	6	S	S	S	I	I	S	S	S	R	R	R	R	S
18	SC	4	S	S	S	S	S	S	S	S	S	S	S	I	S
19	SC	0	S	S	S	R	I	R	S	R	R	S	S	S	I
20	SC	4	S	S	R	I	I	I	I	S	S	S	S	S	R
21	SC	2	S	R	R	R	R	R	R	I	R	S	R	I	R
22	SC	9	S	S	I	I	S	I	S	S	R	S	S	S	R
23	SC	2	S	R	R	R	R	R	S	I	R	I	R	I	I
24	C	7	S	S	S	S	S	S	R	R	S	R	S	S	R
25	SC	4	S	R	R	S	I	R	S	I	R	S	R	R	S
26	SC	0	S	S	S	S	S	S	S	S	S	S	S	S	S
27	SC	8	S	S	S	R	R	R	R	R	R	S	R	S	R
28	SC	10	I	R	R	R	R	R	R	R	R	S	R	I	R
29	C	3	S	I	S	R	S	I	S	S	R	S	R	I	S
30	C	4	S	I	S	S	R	I	S	S	R	S	R	S	R
31	SC	5	I	S	S	S	R	R	R	S	R	S	S	S	R
32	SC	5	S	S	S	S	S	S	R	S	R	I	R	R	R
33	SC	5	S	S	R	R	S	S	R	S	R	S	S	S	R
34	SC	5	S	S	R	I	S	S	R	R	R	R	S	S	S
35	SC	6	S	S	R	R	S	S	R	S	R	S	R	S	R
36	SC	3	S	R	S	S	S	S	R	S	S	S	S	R	S
37	SC	0	S	S	S	S	S	S	S	S	S	S	S	S	S
38	C	5	I	S	R	R	S	R	R	S	R	S	S	S	I

*Clinical signs: *SC* subclinical, *C* clinical

Briefly, amplification reactions were composed of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 25 mM $MgCl_2$, 10 μM of each nucleotide, 10 pM of each primer (rRNA1 and rRNA2; Table 1), and 5 U/μL of *Taq* polymerase in a final volume of 20 μL. Amplification parameters were initial denaturation at 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 4 min. PCR products were analyzed by 2% agarose gel electrophoresis.

A dendrogram was generated using the Bionumerics software (version 6.5) based on band-based similarity index (Dice coefficient) with equal weighting for each typing system. The discrimination index was calculated using the formula recommended by Hunter and Gaston (1988). All isolates that presented similarity coefficient equal to or more than 0.94 (94%) were considered as having the same ribotype.

Results

Subclinical mastitis was more frequently diagnosed than clinical mastitis. Subclinical mastitis was diagnosed in 55.68% among the 88 cows, which had 18.46% of their mammary quarters affected (in a total of 352 quarters). Only 1.70% of the mammary quarters had clinical mastitis.

We found 38 (43.18%) *S. aureus* isolates by amplification of a 132 bp fragment of the *femA* gene that is a specific molecular marker for *S. aureus* (Johnson et al. 1995; Riyaz-Ul-Hassan et al. 2008) in these 88 cows, 32 in cows with subclinical mastitis and six in cows with clinical mastitis. These 38 *S. aureus* isolates were tested for resistance to 13 antibiotics and the antimicrobial resistance profiles are presented on Table 2. The most efficacious antimicrobial agents against the isolates were gentamicin, amoxicillin, and norfloxacin. Gentamicin was the most efficacious agent inhibiting 78.95% of the *S. aureus* isolates. The least efficacious were penicillin, streptomycin, and ampicillin (Fig. 1).

Table 3 demonstrates that 25 out of the 38 *S. aureus* isolates (65.78%) carried at least one of the toxin genes evaluated in this study. Table 4 demonstrates the frequency of each of the toxin genes. The *icaD* gene was detected in 14 isolates (36.84%), followed by *tst* (26.31%), *seb* (7.89%), *sec*, and *sed* (5.26%). *sea*, *see*, and *icaA* genes were not detected in this study. Therefore, the biofilm-associated gene, *icaD*, and the gene that encodes the TSST-1 toxin were detected with higher frequencies. Somatic cell counts (SCC) are demonstrated in Table 5. SCC were measured in raw milk samples from farms that were positive by CMT. Twenty-three isolates belonged to farm B and 12 to farm C. In other words, 35 out of the 38 *S. aureus* isolates belonged to the two farms with highest SCC.

PCR-ribotyping analysis of *S. aureus* isolates revealed 21 different genotypes and a discriminatory capacity of 0.94 ($D = 0.94$). The most frequent ribotypes were 20, 16, and 3, which included 6, 5, and 4 isolates, respectively. The remaining 23 isolates were evenly distributed in 18 groups of smaller size (Fig. 2). Interestingly, three *S. aureus* strains belonging to ribotype 15 carried the virulence gene *icaD*. Figure 2 also indicates the ribotypes isolated from clinical or subclinical cases of mastitis.

Discussion

In this study we reported the occurrence of important virulence factors in *S. aureus* strains isolated from cows with

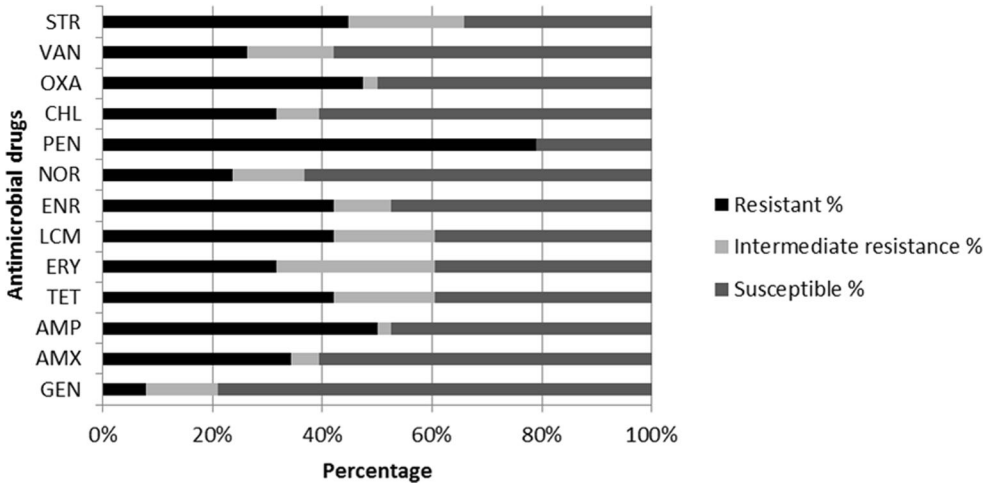


Fig. 1 Percentage of antimicrobial susceptibility of 38 *S. aureus* strains isolated from milk samples from cows with subclinical and clinical mastitis. EST (streptomycin), VAN (vancomycin), OXA (oxacillin), CLO (chloramphenicol), PEN (penicillin), NOR (norfloxacin), ENR (enrofloxacin), LIN (lincomycin), ERI (erythromycin), TET (tetracycline), AMP (ampicillin), AMO (amoxicillin), and GEN (gentamicin)

Table 3 Antimicrobial resistance and virulence genes in *S. aureus* isolates from milk of cows with clinical or subclinical mastitis

S. aureus isolates	Clinical signs*	Antibiotic resistant	Virulence genes**				
			icaD	*seb*	*sec*	*sed*	*tst*
1	SC	5	−	−	+	−	−
2	SC	13	+	−	−	−	−
3	SC	10	−	−	−	−	−
4	SC	6	−	−	−	−	−
5	SC	6	−	−	−	−	−
6	SC	3	−	−	−	−	−
7	SC	7	−	−	−	−	−
8	C	2	+	−	−	−	−
9	SC	12	−	+	−	−	−
10	SC	2	−	−	−	−	−
11	SC	0	−	−	−	−	+
12	SC	6	+	+	−	−	+
13	C	6	+	−	−	−	−
14	SC	9	+	−	−	−	−
15	SC	5	−	−	−	−	−
16	SC	2	−	−	−	−	+
17	SC	6	+	−	−	−	−
18	SC	4	+	−	−	−	−
19	SC	0	−	−	−	−	−
20	SC	4	−	−	−	−	−
21	SC	2	+	−	−	−	−
22	SC	9	+	−	−	−	−
23	SC	2	+	−	−	−	−
24	C	7	−	+	−	−	+
25	SC	4	−	−	−	−	−
26	SC	0	−	−	−	−	+
27	SC	8	−	−	−	−	−
28	SC	10	+	−	−	−	−
29	C	3	−	−	−	−	−
30	C	4	−	−	−	+	+
31	SC	5	−	−	−	−	−
32	SC	5	+	−	−	−	−
33	SC	5	+	−	−	−	−
34	SC	5	−	−	+	−	+
35	SC	6	−	−	−	−	+
36	SC	3	−	−	−	−	+
37	SC	0	−	−	−	−	+
38	C	5	+	−	−	+	−

*Clinical signs: *SC* subclinical, *C* clinical

***sea*, *see*, and *icaA* gene sequences were not detected in any of the isolates

subclinical mastitis, such as the TSST-1, which is responsible for causing food poisoning in people, and the *ica* adhesion genes of the major virulence factors related to the pathogenesis of mastitis (Cramton et al. 1999). Little is known about the pathogenesis of subclinical mastitis. Thus, our data can help in prevention and control subclinical mastitis, decreasing the risk of spreading among cows in a given dairy herd and consequently decreasing financial losses as well as decreasing the risks for public health.

Table 4 Frequency of the *sea*, *seb*, *sec*, *sed*, *see*, *tst*, and *icaD* genes in 38 *S. aureus* isolates from dairy cows with clinical or subclinical mastitis

Gene	Number of *S. aureus* strains	%
sea	0	0
seb	3	7.89
sec	2	5.26
sed	2	5.26
see	0	0
tst	10	26.31
icaA	0	0
icaD	14	36.84

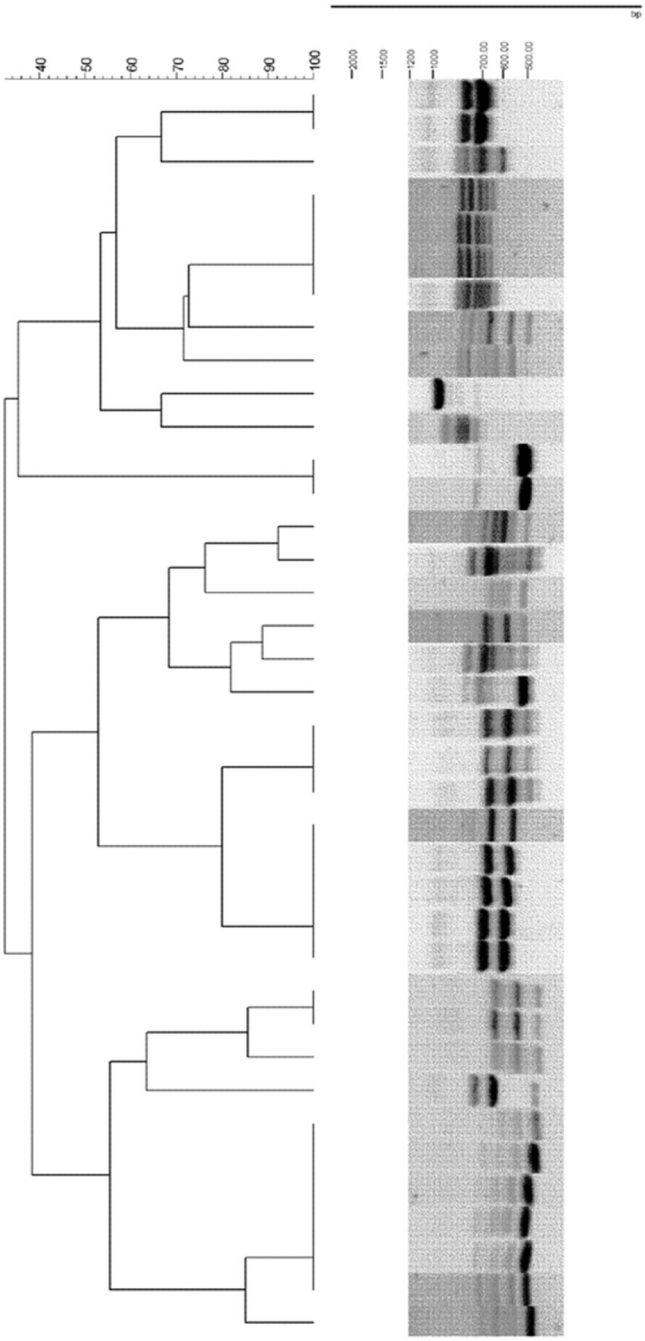
S. aureus cause important economic losses and represent a potential risk to public health due to its capacity to produce enterotoxin (Vicosa et al. 2010). *S. aureus* produces extracellular toxins such as staphylococcal enterotoxins (SE) and toxic shock syndrome toxin-1 (TSST-1). We detected 38 *S. aureus* strains; 25 strains (65.78%) were found to harbor toxin genes as enterotoxin genes *seb* (7.89%), *sec* and *sed* (5.26% each), and *tst* gene (26.31%), besides *ica* adhesion genes, *icaD* (36.84%). Five strains possessed multiple toxin genes. Some authors related that SE and *tst* genes are encoded in genomic islands, such as pathogenicity islands, so some toxin genes may travel together, but still the reason for this occurrence is not known (Omoe et al. 2005).

S. aureus strains expressing SE genes were found with a lower frequency in this study; interestingly, all *seb* and not *sec* producers were TSST-1 positives differing of some studies that related *S. aureus* strains produce *sec* and TSST-1, simultaneously (Akineden et al. 2001; El-Ghodban et al. 2006). Although Zschock et al. (2004) relate that production of TSST-1 is associated with clinical cases of mastitis by *S. aureus*, Kuroishi et al. (2003) have demonstrated that *sec* is of higher importance more than TSST-1 in the pathology of staphylococcal mastitis. We diagnosed a larger number of subclinical mastitis cases, which would explain the lower

Table 5 Average values for somatic cell counts in raw milk from positive samples by CMT

Farm	×1000 cells/mL*
A	413.3 ± 478.8
B	1359.7 ± 439.5
C	1815.9 ± 3418.7
D	955.4 ± 584.8

*Mean ± standard deviation



Isolate	Ribotype	Local	Mastitis
19	I	São Luís	Subclinical
26	I	São Luís	Subclinical
22	II	São Luís	Subclinical
8	III	Raposa	Subclinical
11	III	São Luís	Subclinical
12	III	São Luís	Clinical
27	III	São Luís	Subclinical
2	IV	Raposa	Subclinical
47	V	Raposa	Clinical
20	VI	São Luís	Subclinical
40	VII	Raposa	Subclinical
23	VIII	São Luís	Subclinical
42	VIII	Raposa	Subclinical
1	IX	São Luís	Subclinical
32	X	São Luís	Clinical
41	XI	Raposa	Subclinical
9	XII	São Luís	Subclinical
28	XIII	São Luís	Subclinical
21	XIV	São Luís	Clinical
29	XV	São Luís	Subclinical
30	XV	São Luís	Subclinical
31	XV	São Luís	Subclinical
4	XVI	São Luís	Subclinical
13	XVI	São Luís	Subclinical
14	XVI	São Luís	Subclinical
24	XVI	São Luís	Subclinical
25	XVI	São Luís	Subclinical
34	XVII	São Luís	Subclinical
36	XVII	São Luís	Subclinical
35	XVIII	São Luís	Subclinical
33	XIX	São Luís	Subclinical
37	XX	Raposa	Clinical
38	XX	Raposa	Clinical
39	XX	Raposa	Subclinical
43	XX	Raposa	Subclinical
44	XX	Raposa	Subclinical
46	XX	Raposa	Subclinical
5	XXI	São Luís	Subclinical

Fig. 2 Dendrogram of ribotyping patterns of *S. aureus* strains from cow's milk established using BioNumerics software. Percentage similarity was determined using Dice coefficient and clustering was performed by unweighted pair group method with arithmetic mean (UPGMA)

incidence of isolates producing *sec* genes. We observed a presence of 10 *S. aureus* isolates producing TSST-1 and only two isolates with *sec* gene. There is a number limited of data on the distribution of *tst* gene in subclinical mastitis by *S. aureus* isolates. TSST-1 and enterotoxins are pyrogenic toxin superantigens that cause intense effects on the host, so it is essential to verify the presence of TSST-1 and SE produced by *S. aureus* in food sources (Farahmand-Azar et al. 2013). These toxins are considered like super antigenic toxins those are able to induce immunosuppression in dairy cows, which promotes the persistence of bacteria in host and contributes to chronic mastitis (Omoe et al. 2003). However, the role of virulence factors in bovine subclinical mastitis and the importance of toxin produced by *S. aureus* for udder pathogenesis are still poorly known (Schuberth et al. 2001).

The gene more prevalent in this study was *ica* adhesion gene, the percentage of isolates producing *icaD* was 36.84%. Studies showed that *S. aureus* strains had no ability to form biofilm unless they produce *icaD* gene (Namvar et al. 2013). The presence of biofilm genes is associated to

pathogenicity because it can contribute to the evasion of the host immune defenses and to the difficulty of bacteria eradication. Some authors have related that *Staphylococcus* biofilm is one of the major causes of chronic mastitis (Melchior et al. 2007). In addition, studies have associated the biofilm production by bacteria to higher resistant to antimicrobials (Raza et al. 2013; Stewart et al. 2009). Approximately 90% of *S. aureus* strains obtained in our study demonstrated multiple resistances to different antimicrobial agents. The indiscriminate use of antimicrobial in animal feed is implicated in the increase of *S. aureus* strains resistant to antimicrobial agents, increasing rates of human infection through contaminated food. The emergence of methicillin-resistant *S. aureus* (MRSA) infection in dairy animals is of great concern for livestock and public health. The resistance of *S. aureus* is becoming more complicated by changes in multi-drug resistance mechanism (Wang et al. 2015).

S. aureus is one of the most common bacterium from subclinical mastitis (Barkema et al. 2006). Subclinical mastitis do not have obvious clinical symptoms such as fever and swelling of the udder, as is observed in clinical mastitis. The subclinical mastitis diagnosis is usually based in the California mastitis test (CMT) and somatic cell count (SCC). There is an important relationship between SCC and the milk production: the cell count increases as production decreases due to the occurrence of mastitis (Viguier et al. 2009). These facts may hinder the diagnosis of subclinical mastitis causing persistence and dissemination of the disease in dairy herds. So, additional information about genetic and phenotypic variation of *S. aureus* isolates involved in subclinical mastitis is of great importance to prevent and control effectively.

In this study, we showed that most of the analyzed isolates (65.78%) from cows with mastitis presented one or more virulence factors that can be related to antimicrobial resistance and mainly to subclinical mastitis cases. In addition, three *S. aureus* strains of subclinical mastitis belonging to a same ribotype produced *ica* adhesion gene. This ribotype can be associated to resistant bacteria strains. Molecular studies on bovine *S. aureus* strains shown that there is a large number of strains involved in bovine mastitis etiology worldwide (Aarestrup et al. 1997; Kalorey et al. 2007). Our results can help in the understanding of the relationship between virulence factors and subclinical mastitis caused by different *S. aureus* strains. However, further investigations about diversity of *S. aureus* isolates and genes responsible for the pathogenicity of subclinical mastitis are essential.

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Compliance with ethical standards

The manuscript does not contain clinical studies or patient data.

This experimental protocol has been approved by the Ethics Committee on Animal Use of the Universidade Estadual do Maranhão (CEEA-UEMA, Protocol 039/2011).

Conflict of interest The authors declare that they have no conflict of interest.

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