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Characterization of virulence and antibiotic profile and agr typing of *Staphylococcus aureus* from milk of subclinical mastitis bovine in State of Rio de Janeiro

[*Caracterização do perfil de virulência e resistência antimicrobiana e tipagem* agr *de* Staphylococcus aureus *oriundos do leite bovino de mastite subclinica no estado do Rio de Janeiro*]

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ABSTRACT

This study aims to detect the main virulence and antimicrobial resistance genes in *Stapylococcus aureus* from bovine mastitic milk as well as classifying them according to *agr* typing. A total of 55 strains from six dairy unities in the state of Rio de Janeiro were selected, of these 27.3% presented *fbn*A and 78,2% for *fbn*B genes, respectively. None of the strains tested were positive for *cap*5 gene, 3.6% were positive for *cap*8 gene. Additionally, 94.5% of strains had *hl*A gene and 89.1% had *hl*B gene while 67.3% of the strains had *ica*A gene and 87.3% had *ica*D gene. From these results it was possible to establish 12 different virulence profiles. Prevalence of *agr*II type was detected in 81.8% of the isolates. Concerning antimicrobial resistance evaluation, the studied strains were susceptible to all antibiotics tested except penicillin, 83.6% being resistant strains. None of the strains had *mec*A gene, however, 40% of the strains had *blaZ* gene. Associating virulence and resistance data made it possible to obtain 23 different profiles. This great diversity of strains shows wide array of bacterial strategies and the challenge of mastitis prevention in cattle. Despite antimicrobial susceptibility, these strains presented certain genes that allow its persistence in the herd.

Keywords: *agr* typing; antibiotics; bovine mastits; *Staphylococcus aureus*; virulence

RESUMO

O presente estudo teve como objetivo detectar os principais genes de virulência e resistência antimicrobiana em Staphylococcus aureus *oriundos de leite bovino mastítico e classificá-los de acordo com a tipagem do gene* agr. *Foram selecionados 55 isolados de seis unidades produtores no estado do Rio de Janeiro. Destas, o gene* fbnA *foi encontrado em 27,3% das cepas e 78,2% possuíam o gene* fbnB. *Em nenhuma cepa foi encontrado o gene* cap5 *e 3,6% possuíam o gene* cap8. *O gene* hlA *foi encontrado em 94,5% das cepas e 89,1% possuíam o gene* hlB. *O gene* icaA *foi encontrado em 67,3% das cepas e 87,3% possuíam o gene* icaD. *Com base nesses resultados, foi possível estabelecer 12 diferentes perfis de virulência. Prevalência do* agr *tipo II foi detectada em 81,8% dos isolados. Considerando-se a avaliação da resistência antimicrobiana, as cepas estudadas foram suscetíveis a todos os antibióticos exceto penicilina, sendo detectado um percentual de 83,6% de cepas resistentes. Nenhuma das cepas apresentou o gene* mecA, *contudo 40% das cepas apresentaram o gene* blaZ. *Vinte e três perfis diferentes foram estabelecidos por associação de dados de virulência e resistência. Essa grande diversidade de cepas mostra a ampla gama de estratégias bacterianas e o desafio da prevenção à mastite no gado bovino, considerando-se que, a despeito da suscetibilidade antimicrobiana, essas cepas apresentam genes que permitem sua persistência no rebanho.*

Palavras-chave: antibióticos, mastite bovina, Staphylococcus aureus, *tipagem* agr, *virulência*

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INTRODUCTION

Milk production is considered one of the main agricultural activities in Brazil (Rigolin-Sá *et al*., 2014). Mastitis is recognized as the main cause of economic losses in dairy industry due to reduced milk production in cows, increased expenditure due to treatment of cows and losses associated with withdrawal and disposal of affected milk during the infection period (Reshi *et al*., 2015). In addition, mastitis represents a potential risk to consumer health through transmission of zoonotic agents, possibility of triggering allergies, changes in the balance of intestinal microbiota and selection of resistant bacteria in digestive tract from the use of antibiotics (Cassol *et al*., 2010).

Although several bacterial pathogens can cause mastitis, *Staphylococcus aureus* is one of prevalent etiologic agents of this disease in dairy cattle worldwide. Furthermore, cases of mastitis are often subclinical and difficult to treat (Wang *et al*., 2015). Indiscriminate use of antimicrobials to combat mastitis has led to selection of resistant strains of *Staphylococcus* spp., undermining the efficacy of treatment. Beta-lactam antibiotics are routinely used to treat intramammary infections (Haveri *et al*., 2008). Limited success of antibiotic therapy may also be due to the ability of *S. aureus* to invade and survive within different cell types found in the mammary gland including phagocytes such as fibroblasts, osteoblasts, and various epithelial cell types (Bur *et al*., 2013).

In addition to resistance, pathogenicity of *Staphylococcus* associated with mastitis is an extremely important feature in the disease process that requires a better understanding. Ability of *S. aureus* to cause various infections and intoxication, results from the production of different virulence factors (Aung *et al*., 2011; Capurro *et al*., 2010). The capsule production increases microbial virulence of bacteria becoming resistant to phagocytosis and the serotypes 5 also 8 are prevalent in human and animal infections (Tuchscherr *et al*., 2005). The *S.aureus* infection can also be facilitated by fibronectin production which helps its adhesion to epithelial cells and glandular epithelium facilitating the dispersion in the host (El-Sayed *et al*., 2006). In addition, *slime* production is considered a virulence factor that inhibits the immune response of the host and facilitates the adhesion of the pathogen (Atkin *et al*., 2014). Finally, the α- and β-hemolysins are the most important virulent factors in the pathogenesis of bovine mastitis. They are pore-forming exotoxins that induce proinflammatory changes in mammalian cells, inactivate the immune system by their direct cytotoxic effect, and degrade tissues, providing bacteria with nutrients and facilitating spreading to new sites. The α and β hemolysin are encoded by *hl*A and *hl*B, respectively, and both genes are controlled by gene regulatory accessory *agr* (Bownik and Swicki, 2008).

The accessory gene regulator (*agr*) can regulate the expression of cell surface proteins and extracellular virulence factors (Moodley *et al*., 2006). *agr* (accessory gene regulator) locus is a quorum-sensing system that controls expression of a variety of genes involved in tissue colonization (e.g., surface proteins) and invasion (e.g., extracellular toxins). *agr* system is polymorphic and permits classification of *S. aureus* strains in four groups (Buzzola *et al*., 2007). Due to the considerable impact on milk production caused by the persistence of *S. aureus* in herds, the knowledge of the molecular profile of strains allows epidemiological studies of dispersion of this pathogen in rural properties, resulting in the elucidation of the mechanism of pathogenesis. Therefore, strategies and protocols prophylaxis and control of mastitis can be better assembled.

This study aims to characterize *S. aureus* isolated from cases of subclinical bovine mastitis in the State of Rio de Janeiro in order to establish virulence and resistance profiles as well as to classify *agr* typing.

MATERIALS AND METHODS

Six dairy cattle farms located in an important milk production region of Rio de Janeiro State, Brazil, were selected due to its high prevalence of subclinical mastitis through the California Mastitis Test (CMT) and Somatic Cell Count (SCC). A total of 512 milk samples were collected from October and November 2012. A total of 291 *Staphylococcus* spp. was isolated, among which 128 were *S. aureus*. After antimicrobial susceptibility test results, a representative strain from each of 55 antibiotype groups was randomly selected. The Veterinary Institute Animal Care and Use Committee

(protocol number, CEUA 3664040915) certified this study.

Bacterial total DNA extraction was performed according to the protocol established by LABACVET as follows: a 1.5mL overnight culture was prepared by inoculation of a single colony of *S. aureus* into broth and incubated at 37C. Broth was centrifuged (three times) and the cell pellet re-suspended in 600 µL of lysis solution (200mM TrisHCl, 25 mM EDTA, 25 mM NaCl, 1% SDS, pH8.0) and heated to 65°C for 30 min. DNA was extracted with chloroform: isoamyl alcohol 25:24:1 twice and precipitated by 2 volumes of ice-cold ethanol. DNA pellet obtained was washed with 70% ethanol and re-suspended in 30µL of TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0) and stored at -20°C until use.

Genotypic characterization of *S. aureus* was performed by amplification of coagulase (*coa*) (Hookey *et al*., 1998) and species specific (*nuc*) (Ciftci *et al*., 2009) genes. The analysis of virulence factors comprised the detection of the following genes: *ica*A and *ica*D (Vasudevan *et al*., 2003) implicated in the production of slime; *fnb*A and *fnb*B that codifies fibronectin binding proteins; *cap*5 and *cap*8 related to the expression of capsule (El-Sayed *et al*., 2006) and the hemolysin genes *hl*A and *hl*B (Nilsson *et al*., 1999). ATCC 29213 *S. aureus* was used as quality control. PCR products were separated by electrophoresis on 1% agarose gels, which were revealed with SYBR Green (Invitrogen®) diluted dye (1:100), enabling the visualization and documentation of amplicons by the image capturing system L-PIX EX (Loccus Biotecnologia®).

Classification of *agr* system groups was based on the hyper variable domain of *agr* locus according to Shopsin *et al*. (2003). Duplex PCR was performed to type groups based on their products size. PCR products were separated by electrophoresis on 1% agarose gels which were stained with a 1:100 dilution of SYBR Green (Invitrogen®), enabling the visualization and documentation of amplicons by the image capturing system L-PIX EX (Loccus Biotecnologia®).

Phenotypic resistance detection were performed according to Clinical and Laboratory Standards Institute veterinary guidelines to the following antimicrobials: cefoxitin (30µg), oxacillin (10µg), penicillin (10IU), amoxicillin + clavulanic acid (30µg), and erythromycin (15µg) (CLSI VET01-A4, 2013). Standard strains *S. aureus* ATCC 43300 and *S. aureus* ATCC 29213 were used as quality control. For the genotipic characterization of beta-lactamic resistance, *mec*A (Murakami *et al*., 1991) and *bla*Z (Rosato *et al*., 2003) genes were amplified and PCR products were separated by electrophoresis on 1% agarose gels, stained with a 1:100 dilution of SYBR Green (Invitrogen®), enabling the visualization and documentation of amplicons by the image capturing system L-PIX EX (Loccus Biotecnologia®).

RESULTS

All strains were genotypically confirmed as *S. aureus.* They generated variable sized fragments compatible with those expected for *coa* gene and fragments of 279 bp compatible with presence of *nuc* gene confirming their identification as *S. aureus*.

Regarding virulence factors analysis, a total of 27.3% (15/55) were positive for *fbn*A gene and 78.2% (43/55) for *fbn*B gene, genes implicated in fibronectin production. The *cap*8 was detected in two strains whereas the unusual *cap5* gene was not found in any strain. Genes associated to hemolysin production were found in 94.5% (52/55-*hl*A) and 89.1% (49/55-*hl*B), respectively. The slime production associated *ica*A and *ica*D genes were detected in 67.3% (37/55) and 87.3% (48/55), respectively (Table 1).

Table 1. Prevalence of virulence associated genes in *S. aureus* associated with mastitis in cattle

Function	Genes	Prevalence
Hemolysin production	*hl*A	94.5% (52/55)
	*hl*B	89.1% (49/55)
Slime Production	*ica*A	67.5% (37/55)
	*ica*D	87.3% (48/55)
Fibronectin	*fbn*A	27.3% (15/55)
	*fbn*B	78.2% (43/55)
Capsule	*cap*5	0% (0/55)
	*cap*8	4.4% (2/55)

Genetic analysis of virulence yielded a total of 14 different profiles. Profile 11 was found to be the most prevalent , detected in 43.6% (24/55) of strains, followed by profile 12 observed in 12.7% (7/55) (Table 2).

Through the analysis of the virulence genes implicated in the pathogenesis of mastitis it was possible to observe that they are widely distributed among studied strains confirming their high potential for causing this disease. Most strains 81.8% (45/55) were classified as *agr* type II and 18.2% (10/55) strains could not be classified in any *agr* type. Also, the prevalent profile 11 was found in strains from all analyzed farms demonstrating its ample dissemination in the studied region. Most strains (22/24) belonging to this profile were typified as *agr* group II; however, two strains were not typeable.

Table 2. Distribution of virulence gene profiles among the studied *Staphylococcus aureus* strains

Profiles	Genes	Farms	*agr* Typing	Prevalence
1	*hl*A, *hl*B, *ica*A *and ica*D	E	II	3,6 (2/55)
2	*fbn*A, *fbn*B, *hl*A, *hl*B, *ica*A and *ica*D	D	II	9,1 (5/55)
3	*fbn*B, *hl*A**,** *hl*B**,** *ica*A and *ica*D	D; E	NT; II	5,6 (3/55)
4	*fbn*B, *hl*A, *hl*B and *ica*D	D	NT; II	3,6 (2/55)
5	*fbn*B and *ica*D	D	II	1,8 (1/55)
6	*fbn*A, *fbn*B, *hl*A, *hl*B and *ica*D	C	II	1,8 (1/55)
7	*fbn*A, *fbn*B, *hl*A and *ica*D	E	II	1,8 (1/55)
8	*fbn*A, *fbn*B and *ica*A	D	II	1,8 (1/55)
9	*fbn*B	A	NT	1,8 (1/55)
10	*fbn*A, *cap8*, *hl*A, *hl*B and *ica*A	C; F	II	3,6 (2/55)
11	*fbn*A, *hl*A, *hl*B, *ica*A and *ica*D	A; B; C; D; E; F	NT; II	43,6 (24/55)
12	*fbn*A, *hl*A, *hl*B and *ica*D	C; D; E	NT; II	12,7 (7/55)
13	*hl*A and *hl*B	A; B	NT; II	5,6 (3/55)
14	*fbn*A, *hl*A and *ica*D	D; E	NT; II	3,6 (2/55)

Regarding antimicrobial resistance assays all strains were susceptible to the tested antibiotics with exception of penicillin (83.6% - 46/55). None isolate tested positive to *mec*A gene and 40% (22/55) were positive for *blaZ* gene.

Through clustering data virulence and resistance, it was possible to obtain 23 different profiles. Profile 12 was the prevalent profile (23.6% - 13/55) and it was distributed in 3 different farms (Table 3).

DISCUSSION

Mastitis caused by *S. aureus* is the result of the production of a large array of virulence factors that may contribute to its pathogenesis in different ways. Virulence factors of *S. aureus* allow the bacteria to attach, colonize and invade the host. In this study, the presence of virulence genes related to several steps of bovine mastitis pathogenesis was investigated.

The ability of *S. aureus* to produce biofilm is considered important as a virulence determinant in pathogenesis of mastitis. Biofilm helps in adhesion and colonization of organism in the epithelium of mammary gland and also increases antibiotic resistance. Involvement of biofilm infections has led to increased interest in characterizing genes involved in biofilm formation. In this study, most *S. aureus* strains presented the genetic ability to produce biofilm since the slime production associated *ica*A and *ica*D genes were detected in 67.3% (37/55) and 87.3% (48/55), respectively. In Belgium, Ote *et al.* (2011) detected 86.9% *ica*A and 95% *ica*B positive *S. aureus* strains in a 229 bovine mastitis sampling corroborating the present data of high prevalence of these genes in dairy environment. Presence of at least one of genes has been detected in most isolates of *S. aureus* bovine mastitis demonstrating its importance as virulence factors in the pathogenesis of bovine mastitis (Atkin *et al*., 2014).

Table 3. Distribution of virulence and resistance genes and antibiotype among the studied *Staphylococcus aureus* strains

Virulence and resistance genes and antibiotype profile	Profile	Prevalence
*fbn*A, *fbn*B, *hl*A, *hl*B, *ica*A, *ica*D,PEN (R),AMC (S), CFO (S), OXA (S) and ERI(S)	1	9%(5/55)
*fbn*A, *fbn*B, *hl*A, *hl*B, *ica*D, PEN (S), AMC (S), CFO (S), OXA (S), ERI(S) and *blaZ*	2	1,8%(1/55)
*fbn*A, *fbn*B, *hl*A, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S) and ERI(S)	3	1,8%(1/55)
*fbn*A, *fbn*B, *ica*A, PEN (R), AMC (S), CFO (S), OXA (S) and ERI(S)	4	1,8%(1/55)
*fbn*B, *hl*A, *hl*B, *icaA, ica*D, PEN (S), AMC (S), CFO (S), OXA (S) and ERI(S)	5	1,8%(1/55)
*fbn*B, *hl*A, *hl*B, *ica*A, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S) and ERI(S)	6	1,8%(1/55)
*fbn*B, *hl*A, *hl*B, *ica*A, *ica*D. PEN (R), AMC (S), CFO (S), OXA (S), ERI(S) and *blaZ*	7	1,8%(1/55)
*fbn*B, *hl*A, *hl*B, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S) and ERI(S)	8	3,6%(2/55)
*fbn*B, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S), ERI(S) and *blaZ*	9	1,8%(1/55)
*fbn*A, *cap8*, *hl*A, *hl*B, *ica*A, PEN (R), AMC (S),CFO (S), OXA (S), ERI(S) and *blaZ*	10	5,4%(3/55)
*fbn*A, *hl*A, *hl*B, *ica*A, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S), ERI(S) and *blaZ*	11	10,9%(6/55)
*fbn*A, *hl*A. *hl*B, *icaA, ica*D, PEN (R), AMC (S), CFO (S), OXA (S) and ERI(S)	12	23,6%(13/55)
*fbn*A, *hl*A. *hl*B. *ica*A, *ica*D, PEN (S), AMC (S) CFO (S), OXA (S), ERI(S) and *blaZ*	13	7,2%(4/55)
*fbn*A, *hl*A, *hl*B, *ica*A, *ica*D, PEN (S), AMC (S), CFO (S), OXA (S) and ERI(S)	14	1,8%(1/55)
*fbn*A, *hl*A, *hl*B, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S), ERI(S) and *blaZ*	15	5,4%(3/55)
*fbn*A, *hl*A, *hl*B, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S) and ERI(S)	16	5,4%(3/55)
*fbn*A, *hl*A, *hl*B, *ica*D,PEN (S), AMC (S), CFO (S), OXA (S) and ERI(S)	17	1,8%(1/55)
*fbn*A, *hl*A, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S) and ERI(S)	18	1,8%(1/55)
*fbn*A, *hl*A, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S), ERI(S) and *blaZ*	19	1,8%(1/55)
*hl*A, *hl*B, *ica*A, *ica*D, PEN (R), AMC (S), CFO (S), OXA (S) and ERI(S)	20	3,6%(2/55)
*hl*A, *hl*B, PEN (S), AMC (S), CFO (S), OXA (S), ERI(S) and *blaZ*	21	1,8%(1/55)
*hl*A, *hl*B, PEN (R), AMC (S), CFO (S), OXA (S) and ERI(S)	22	1,8%(1/55)
*hl*A, *hl*B, PEN (R), AMC (S), CFO (S), OXA (S), ERI(S) and *blaZ*	23	1,8%(1/55)

*S: susceptible and R: resistant

The genes responsible for production of capsular polysaccharide were also investigated. The *cap*8 gene was only detected in two strains whereas the unusual *cap5* gene was not found. Occurrence of *cap*5 or *cap*8 genes varies in each geographic region. According to Tuchscherr *et al.* (2005), bacteria that do not express capsule induce chronic mastitis in mice, suggesting that the absence of capsule synthesis may help the bacteria to persist in the mammary glands. This idea was highly supported by the fact that the studied strains were obtained from cows presenting subclinical mastitis only detected by CMT and CCS.

The fibronectin binding proteins (FBN) A and B of *S. aureus* are multifunctional MSCRAMMs which recognize fibronectin, fibrinogen and elastin. FBN promotes internalization of *S. aureus* into epithelial and endothelial cells which are not normally phagocytic. Moreover, the promote evasion of immune responses and antibiotics. FBNA and FBNB are encoded by two closely linked but separately transcribed genes, *fbn*A and *fbn*B (Burke *et al*., 2010). Most of the strains in this study presented the *fbn*B gene. These results are similar to those found by Kot *et al*. (2016) and are supported by reports that the gene *fbn*B is more closely related with *S. aureus* isolates from subclinical mastitis and the absence of this gene may affect the ability to invade host cells. The adhesion to fibronectin is an important step in establishment of pathogenesis of the bovine mastitis (Kot *et al*.,2016).

The α- and β-haemolysins produced by *S. aureus* are pore-forming exotoxins that induce proinflammatory changes in mammalian cells, inactivate the immune system by their direct cytotoxic effect, and degrade tissues, providing bacteria with nutrients and facilitating spreading to new sites (Haveri *et al*., 2007). The α-haemolysin, encoded by *hla* gene, has been suggested to be involved in peracute, gangrenous bovine mastitis. In this study, a high prevalence of both haemolysin genes, *hla* and *hlb* was observed, pointing to the bacterial potential for acute infection. Most of *S. aureus* from bovine mastitis produce α- and β-haemolysins. The high frequency of haemolysins genes show that these genes play an important role in pathogenesis of bovine mastitis. Previous studies report that hemolysin production may be unnecessary to cause mastitis, once strains that tested negative

for both genes were detected in cattle affected by mastitis (Haveri *et al*., 2007).

Besides those virulence genes, this study also determined the *agr* typing, being the *agr*II type the most prevalent one. Melchior *et al*. (2009) suggested a better adaptation of *agr*II than *agr*I strains to dairy environment based on the higher prevalence of type II in bovine milk isolates.

The evaluation of antimicrobial resistance yielded a high prevalence of penicillin resistant strains confirming the common sensing that penicillin is rarely considered an option in treating *Staphylococcus* spp. infections. The indiscriminate use of penicillin to control and prevent infectious diseases in cattle without adequate control , leading to a series of consequences such as toxic effects, allergy problems and development of resistant strains Silva *et al.,* 2012). In this study, 83.6% (46/ 55) of the tested isolates were resistant to penicillin, similar to data reported from Silva *et al.* (2012), who found 95% of penicillin resistance in *S. aureus* from subclinical mastitis in Pernambuco, Brazil. Moreover, penicillin resistance of *S. aureus* has been associated with chronic mastitis due to the low cure rate of mastitis caused by *S. aureus* resistant to penicillins which makes these animals to be reservoirs of penicillin-resistant *S. aureus* causing the spread to other animals (Haveri *et al*., 2007). Silva *et al.* (2012) emphasizes the zoonotic risk of the presence of *S. aureus* strains resistant to penicillin from bovine mastitis due to the potential risk of transmission to humans of resistance in order to limit or prevent use for treatment.

Despite the high penicillin resistance detected, none of the strains tested positive for *mec*A gene,.and only sixteen strains (34.7%) presented *bla*Z gene in a scope of 46 strains. Detection of the *mec*A gene in isolates of bovine origin with oxacillin resistance phenotype is problematic. Melo *et al*. (2014) detected point mutations in the annealing region of primer, which resulted in design of new primers for the detection of *mec* gene in isolated bovine (*mec* bovine). Garcia-Alvarez *et al*. (2011) also detected mutations in the gene *mec*, and described a new allele called *mec*C, this gene has been found in humans and animals, but until the present date, this allele was not detected in the Americas. These data point to the need of studies about the underlying mechanism of the observed penicillin resistance.

CONCLUSION

The spread of *S. aureus* in dairy herds is of concern not only because of its ability to cause the mammary gland infection due to its virulence potential but also considering how difficult it is to create effective preventive measures. This study has concluded that penicillin is not an antimicrobial choice for *S. aureus* infection treatment and it must be banished from dairy environment.

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