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Virulence factors and antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis in Rio de Janeiro[1]

Shana M.O. Coelho[2], Elina Reinoso[3], Ingrid A. Pereira[2], Lidiane C. Soares[2], Mirta Demo[3], Cristina Bogni[3] and Miliane M.S. Souza[4*]

ABSTRACT.- Coelho S.M.O., Reinoso E., Pereira I.A., Soares L.C., Demo M., Bogni C & Souza M.M.S. 2009. **Virulence factors and antimicrobial resistance in *Staphylococcus aureus* isolated from bovine mastitis in Rio de Janeiro.** *Pesquisa Veterinária Brasileira 29(5):369-374.* Departamento de Microbiologia e Imunologia Veterinária, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ 23890-000, Brazil. E-mail: miliane@ufrrj.br

The study was conducted to characterize pheno-genotypically the virulence factors and resistance pattern of *Staphylococcus aureus* isolates from milk samples of cows with subclinical mastitis. All hemolytic isolates presented beta-hemolysin, and 38% of the non-hemolytic isolates were able to express hemolysins in the presence of a beta-hemolytic strain. The amplification of the *coa*-gene displayed four different size polymorphisms with about 400 bp, 600 bp, 700 bp and 900 bp. The *spaA* gene that encodes the IgG-binding region of protein A revealed sizes of 700 bp and 900 bp. The amplification of region X from *spaA* yielded a single amplicon for each isolate with the prevalent amplicon size being of 180 bp. Amplification of *sae* gene yielded an amplicon size of 920 bp in 71% of the isolates. Antibiotic resistance pattern revealed that 42% *S. aureus* were susceptible to all antimicrobials tested. Seven different antibiotic patterns were observed. Our results indicated that 47% and 25% of *S. aureus* strains exhibited resistance to penicillin and oxacillin respectively. All oxacillin-resistant isolates were *mecA*-positive.

INDEX TERMS: *Staphylococcus aureus,* antimicrobial resistance, virulence factors.

RESUMO.- [**Fatores de virulência e resistência antimicrobiana em *Staphylococcus aureus* isolados de mastite bovina no Rio de Janeiro.**] O presente estudo foi conduzido com o objetivo de caracterizar feno-genotipicamente os fatores de virulência e perfil de resistência aos antibióticos de *Staphylococcus aureus* isolados de amostras de leite de vacas com mastite clínica e subclínica. Em todos os isolados hemolíticos foi detectada a presença de beta hemolisina e 38% dos não-hemolíticos produziram hemolisinas na presença de cepa beta-hemolítica. A amplificação do gene *coa* apresentou quatro tipos polimórficos distintos com aproximadamente 400 bp, 600 bp, 700 bp e 900 bp. O gene *spaA* que codifica a região de ligação da proteína A à IgG apresentou bandas de 700 bp e 900 bp. A amplificação do gene que codifica a região X revelou um único amplicon para cada isolado sendo o tamanho prevalente o de 250pb. A amplificação do gene *sae* resultou em amplicons com 920 pb em 71% dos isolados. O teste de suscetibilidade antimicrobiana revelou que 42% dos *S. aureus* foram sensíveis a todos os antibióticos testados. Foram observados sete diferentes padrões de resistência. Os resultados indicaram que 47% e 25% dos isolados foram resistentes à penicilina e oxacilina, respectivamente. Todos os isolados resistentes à oxacilina foram positivos para o gene *mecA*.

TERMOS DE INDEXAÇÃO: *Staphylococcus aureus,* resistência antimicrobiana, fatores de virulência.

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[2] Curso de Pós-Graduação em Ciências Veterinárias, UFRRJ.

[3] Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nac. 36, Km 601, Código Postal X5804BYA Río Cuarto, Córdoba, Argentina.

[4] Departamento de Microbiologia e Imunologia Veterinária, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Seropédica, RJ 23890-000, Brazil.* Corresponding author: miliane@ufrrj.br

INTRODUCTION

Staphylococcus aureus is recognized as a major pathogen that also causes subclinical intramammary infections in dairy cows leading to severe economic losses in industry worldwide (Godden et al. 2002). In Brazil, several studies report the recovery of this agent from mastitic milk samples from cows (Schocken-Iturrino et al. 1996, Lange et al. 1999, Reis et al. 2003, Zafalon et al. 2007).This bacterium produces a variety of exoproteins that contribute to its ability to colonize mammary gland (Salasia et al. 2004), with five of them being different membrane-damaging toxins, four hemolysins (alpha-, beta-, gamma-, and delta-hemolysin) and leucocidin. Beta and alpha hemolysins are the most important in pathogenesis of the intramamarian infections (Park et al. 2004). The beta-toxin is a Mg^{2+}-dependent sphingomyelinase C, which degrades sphingomyelin in the outer phospholipid layer of the membrane (Linehan et al. 2003).

Staphylococcal protein A is a membrane-bound exoprotein characterized and well known for its ability to bind to the Fc region of immunoglobulins of most mammalian species (Alonso & Dagget 2000). This protein is codified by the *spaA* gene with a polymorphic (X) and a conserved region. The polymorphic region X consists of a variable number of repeated 24 pairs of bases being located in a codificant region of cellular wall C-terminal extremity (Koreen et al. 2004).

Coagulase protein has the ability to turn fibrinogen into fibrin threads by a mechanism different from natural clotting (Palma et al. 1999). Coagulase has also been shown to be a virulence factor in intrammmary infection. This protein is codified by the gene *coa* that possesses a conserved and a repeated polymorphic region that can be used to measure relatedness among *S. aureus* isolates. This region consists of repeated short sequences of 81 pairs of bases that are changeable in number and sequence and a fixed sequence of 330 pairs of base (Shopsin et al. 2000, Reinoso et al. 2004).

Sae operon is a two-component signal transduction system in *S. aureus* that regulates the expression of many virulence factors at the transcriptional level (Novik et al. 2003). This operon activates the expression of several virulence factors, including serine proteases, nuclease, coagulase (encoded by *coa*), alpha hemolysin (*hla*) and fibronectin-binding protein A (*fnbA)*. The absence of gene *sae* seems to lead á and â hemolisins, DNAse and coagulase reduction (Giraudo et al. 1999).

Besides virulence factors, the increased resistance of *S. aureus* isolated from mastitic cows to several antimicrobial agents has been reported (Gentilini et al. 2000) what impacts the effectiveness of therapy since control methods of this organism from dairy herds requires treatment of infected mammary glands with effective antimicrobial agents (Kirkan et al 2005). The determination of antimicrobial susceptibility of clinical isolates is required not only for therapy but also for monitoring the spread of resistant strains throughout the populations. ß-lactamic antibiotics are the most frequently used in intramammary infusion therapy. Bacterial resistance mechanisms to this class of antibiotics include production of ß-lactamases and low-affinity penicillin-binding protein 2a (PBP2a) determined by the presence of the chromosomal gene *mecA*. The latter, designated for methicillin resistance, precludes therapy with any of the currently available ß-lactam antibiotics, and may predict resistance to several classes of antibiotics (Moon et al. 2007). The isolation of *S. aureus* methicillin resistant (MRSA) from animals was first reported in 1972 following its detection in milk from mastitic cows (Devriese et al. 1972). Since then, reports have been published on MRSA infection in domestic animals, including dogs, cats, cattle, sheep, chickens, rabbits, and horses (Hartmann et al. 1997, Pak et al. 1999, Lee 2003, Goni et al. 2004). In recent years, the number of cases has shown an increasing trend (Rich & Roberts 2004, O'Mahony et al. 2005, Weese et al. 2005).

The aim of the present study was to characterize pheno and genotipically *Staphylococcus aureus* strains isolated from subclinical mastitis in Rio de Janeiro, Brazil. To reach these objectives, and increase the range of information available about the genetic diversity of *S. aureus* isolated from cows with mastitis, what would allow to formulate strategies to reduce the spread of infection by this bacterium, hemolytic properties, coagulase (*coa*) and protein A (*spa*) gene polymorphism, *S. aureus* exoprotein expression gene (*sae*), antibiotic resistance patterns and oxacillin resistance (*mecA*) were determined.

MATERIALS AND METHODS

Isolates. Milk samples were collected from 98 cows with subclinical mastitis. A total of 65 coagulase-positive *Staphylococcus* isolates was included in this investigation. Each one corresponding to a single animal.

Identification. The isolates were cultured in sheep blood agar and identified according to routine microbiological diagnostics, including cultural properties, catalase, coagulase, detection of hemolysis, maltose fermentation, acetoin production and nitrate reduction (Koneman 2008). The strains were further identified as *S. aureus* by PCR amplification of the 23S rDNA according Straub et al. (1999).

Phenotypic virulence factors. The production of hemolysins was also determined by cultivation of bacteria on sheep blood agar plates and in parallel by the interference of the hemolysins with the β-toxin of a *S. aureus* reference strain as described by Salka et al. (1979).

Antibiotic susceptibility. It was determined by the standardized agar diffusion test on MüllerHinton agar (Merck) using the following disks (Sensifar-Cefar): ampicillin (10mg), gentamicina (10mg) penicillin (10 IU), oxacillin (1mg), vancomicyn (30mg) and ampicillin/sulbactam (10g). *S. aureus* ATCC 25923 was used as reference strain. Isolates were categorized as susceptible and resistant based upon interpretative criteria developed by the Clinical and Laboratory Standards Institute (CLSI) (2005).

Oxacillin susceptibility tests. It was included for detection of methicillin-resistant *S. aureus*. Resistance to methicillin was determined according to the test recommended by the CLSI, using an agar plate containing 6 mg/ml of oxacillin and Müller

Hinton agar supplemented with NaCl (4% w/v; 0.68 mol/L). Furthermore, oxacillin resistant strains were confirmed by PCR amplification of *mecA* gene (Coelho et al. 2007).

DNA extraction for PCR analysis. *Staphylococcus aureus* DNA was extracted from overnight cultures in 10mL of brain heart infusion broth with slight modifications of the method reported by Senna et al. (2002). Briefly, bacterial cells were collected by centrifugation for 30 s at 14,000 rpm, washed in 1mL of TE buffer (10m*M* Tris HCl, pH 8.0; 1m*M* EDTA; 100m*M* NaCl), and recentrifuged. The pellet was resuspended in 400μL of TE buffer including 5μL of lysostaphin (stock concentration 1μg/mL; Sigma-Aldrich) and incubated for 30min at 37°C. Lysis was completed by the addition of 20 μL of 10% SDS solution and incubation for 15min at room temperature. DNA was then extracted with phenol-chloroform-isoamylalchohol 25:24:1 and chloroform-isoamylalchohol 24:1, respectively. The DNA was purified by ethanol precipitation and dissolved in a buffer containing10mM TrisClH(pH7.6) and 0.1mM EDTA (Reinoso, 2004).

PCR analysis. Polymerase chain reaction analysis of the *coa, sae, spaA, rDNA and mecA* genes were carried out using the primers and respective amplifications program described in Table 1. Reaction was performed in a final volume of 20μL of mixture containing PCR buffer (10m*M* TrisHCl, pH 9.0; 50m*M* KCl, and 0.1% Triton X-100), 3.5m*M* $MgCl_2$, 250μ*M* of each of the deoxynucleoside triphosphates, 3.0μ*M* each gene-specific primers, 2.5 U of *Taq* DNA Polymerase (Promega, Madison, WI) and 5μL of template. The products of 10μL were analyzed by electrophoresis through a 1.0% agarose gel and pictures taken as described by Reinoso (2004).

RESULTS AND DISCUSSION

Among the 65 staphylococcal isolates from milk samples, 21 were identified as *Staphylococcus aureus* according to the results of phenotypical assays. These strains were confirmed by PCR amplification of the 23s DNA specific to *S. aureus.*

Nine isolates were hemolytic, with seven of them producing both alpha- and beta-hemolysis and two producing only beta-hemolysis. Interaction between alpha and beta toxins increases the adherence to bovine mammary epithelial cells and the proliferation of *S. aureus* (Cifrian et al. 1996). Furthermore, the capacity of the strains isolated from subclinical mastitis to produce both hemolysins indicates that these toxins might be necessary for the establishment of *Staphylococcus* strains in mammary glands as previously described by Cifrian et al. (1996) and Bownik & Siwicki (2008).

Beta toxin hydrolyses the sphingomyelin present in the exoplasmic leaflet of the plasma membrane of most mammalian mammary glands cells, resulting in increased permeability with progressive loss of cell surface charge (Graves et al. 2007). Also, hydrolysis of sphingomyelin may render the cells more susceptible to the action of alpha toxin. In this study, beta hemolysin was present in all nine hemolytic isolates suggesting that this hemolysin may have an important role in mastitis pathogenesis (Park et al. 2004).

Twelve isolates were non-hemolytic. Five of these non-hemolytic isolates were able to express hemolysins in the presence of a beta-hemolytic strain. This phenomenon can probably be explained by the production of a delta-hemolysin, whose expression depends on the presence of a beta-hemolytic isolate. Hemolytic synergism seems to be independent of hemolysin production, nevertheless, its action is considered as an enhancer to the colonization ability of *Staphylococcus* (Ali-Vehmas et al. 2001).

The 21 *S. aureus* strains were additionally investigated for the presence of *coa*, *spa* and *sae* genes. All these genes displayed polymorphisms.

The amplification of the *coa*-gene displayed four different size polymorphisms with approximately 400 bp for one (4%) strain, 600 bp for 12 (57%) strains, 700 bp for 2 (9%) strains and 900 bp for 6 (28%) strains and based in these data the calculated number of repeats according to Hookey et al. (1998) were 1, 4, 5 and 7 repeats, respectively. These results are in agreement with the findings of Cabral et al. (2004) suggesting that an amplicon of about 600 bp are predominant in bovine strains collected from Brazil. It is important to note that in the present study, some strains presented more than one amplicon what could be explained by the presence of more than one allelic form of the coagulase gene (Goh et al. 1992, Aslantas et al. 2007). In an

Table 1. Sequences of oligonucleotides primers with corresponding programs

Gene	Primers (5´ - 3´)	Program[a]	References
23S rDNA	acg gag tta caa agg acg ac agc tca gcc tta acg agt ac	1	Straub et al., 1999
spaA (X region)	caa gca cca aaa gag gaa ggc ttg ttg ttg tct tcc tc	2	Reinoso, 2001
spaA (IgG binding region)	cac ctg ctg caa atg ctg cg ggc ttg ttg ttg tct tcc tc	2	Reinoso, 2001
coa	ata gag atg ctg gta cag g gct tcc gat tgt tcg atg c	3	Reinoso, 2001
sae	tgc tgc tag ttt ctt tgg agc att gat gag aag gat gcc ca	4	Giraudo et al. 1999
mecA	aaa atc gat ggt aaa ggt tgg c agt tct gca gta ccg gat ttg c	6	Coelho et al. 2007

[a] **1** = 3 7x (94°C - 40s, 64°C - 60s, 72°C - 75s); **2** = 30 x (94°C - 60s, 60°C - 60s, 72°C - 60s); **3** = 30 x (4°C - 60s, 58°C - 60s, 72°C - 60s), **4** = 29 x (93ºC 1min, 55ºC 1min, 72º C 1:30 min); 72ºC 10 min; **5** = 30 x (94°C - 60s, 60°C - 60s, 72ºC - 60s).

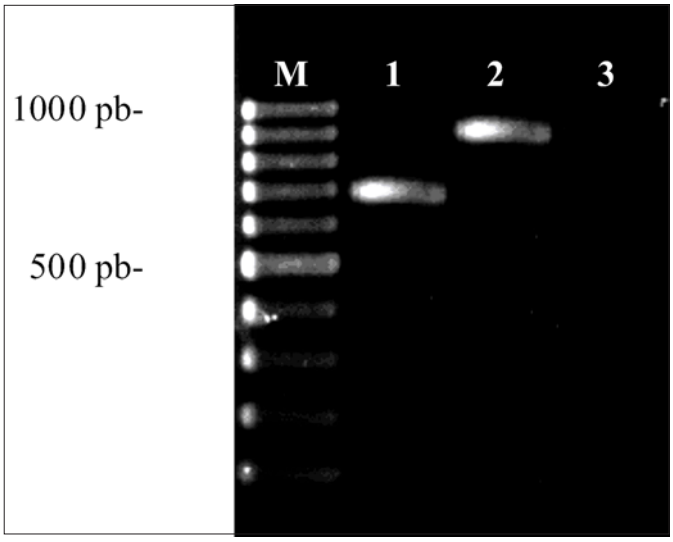

Fig.1. Agarose (1,5%) gel electrophoresis of *spaA Staphylococcus aureus* PCR products. M = 100 bp molecular weight standard, 1 = positive strain (700pb), 2 = positive strain (900pb), 3 = control reaction.

earlier study, performed in the south of Brazil, seven *coa* PCR types were observed, and 2 accounted for more than 50% of the isolates (Lange et al. 1999). Thus, in some Brazilian regions most cases of mastitis may be caused by *S. aureus* strains with the same *coa* genotype.

Protein A is a component of *S. aureus* cell wall and is covalently bound to the peptidoglycan. The PCR amplification of the gene encoding the IgG-binding region of protein A revealed bands of 700 bp and 900 bp (Fig.1). The amplification of region X from *spaA* yielded a single amplicon for each isolate with the prevalent amplicon size being of 250 bp for 10 strains (38%), 280 bp for 9 strains (14%) and 180 bp for 8 strains (47%). Frénay et al. (1996) affirmed that *spaA* gene above 260bp tends to be more related to epidemic than sporadic strains. The variability and stability of this gene indicate that sequence analysis of *spaA* gene could be use as an alternative system to the molecular typing of *S. aureus* isolates.

Amplification of *sae* gene yielded an amplicon size of 920 bp for 15 (71%) of the investigated *S. aureus*. According to the literature, this result could be explained by two possibilities, (1) either the gene *sae* is not present in all studied lineages (Reinoso, 2001) or (2) gene *sae* is polymorphic and is not always amplified by primers usage (Steinhuber et al. 2003, Goerke et al. 2005). From all *sae*-positive isolates, ten showed no hemolytic profile and only four were alpha-beta hemolytic. Virulence gene expression in *S. aureus* is controlled by regulator genes like *agr* and *sae*. However, little information is available about the distribution of *sae* gene in *S. aureus* strains isolated from bovine and to our knowledge this is the first report that characterizes *sae* gene of *S. aureus* strains isolated from Brazil. Table 2 displayed these results plus the hemolytic properties, *coa* and *spaA* genes of the isolates.

Antibiotic resistance determination revealed that 9 isolates (42%) were susceptible to all antimicrobials tested. Seven different antibiotic patterns were observed (Table 3). Twelve *S. aureus* isolates (57%) were resistant to one or more than one antibiotic. Eight (38%) isolates were resistant to three or more antibiotics. The predominant resistance pattern ampicillin/penicillin resistance was observed in 9 isolates (42%), either alone or in combination

Table 2. Pheno and genotypic characteristics of *Staphylococcus aureus* strains

Strain	Hemolysis	*spaA* gene (bp)		No. of repeats	*coa* gene (bp)	No. of repeats	*sae* gene
		IgG binding Region	X Region				
SB 123	NH[a]	700	180	7	600	4	+
SB 124	NH	900	250	10	900	7	+
SB 125	NH	700	250	10	600	4	-
SB 126	NH	900	180	7	600	4	+
SB 127	NH	700	180	7	600	4	-
SB 128	b	700	250	10	900	7	+
SB 129	NH	700	250	10	600	4	ND[b]
SB 130	NH	700	250	10	600	4	+
SB 131	NH	700	250	10	900	7	+
SB 132	NH	700	180	7	600	4	+
SB 133	β and α	700	250	10	900	7	+
SB 134	β and α	700	280	11	900	7	+
SB 135	β and α	700	250	10	900	7	-
SB 136	β and α	700	180	7	600	4	+
SB 137	β and α	700	180	7	600	4	+
SB 138	NH	700	250	10	700	5	+
SB 139	β and α	700	180	7	400	1	-
SB 140	NH	700	180	7	700	5	+
SB 141	NH	700	280	11	600	4	+
SB 142	NH	700	280	11	600	4	+
SB 143	β and α	700	250	10	600	4	-

[a] NH = non-hemolytic, [b] ND = non-determined

Table 3. Antibiotic resistance patterns of *Staphylococcus aureus* strains

Pattern	Resistance phenotype[a]	Number of strains
1	Amp, Pen, Oxa, Van, Gen	4
2	Amp, Pen, Oxa, Gen	2
3	Amp, Pen,Gen	2
4	Amp, Pen	1
5	Pen, Gen	1
6	Gen	2
7	-	9

[a] Amp = ampicillin, Gen = gentamicin, Pen = penicillin, Oxa = oxacillin, Van = vancomicin.

with resistances to other antimicrobials and 47% of *S. aureus* strains exhibited resistance to penicillin and this widespread of resistance could be a consequence of the widespread application of ß-lactamic antibiotics frequently used in intramammary infections in Brazil agreeing with the WHO report (2002) that suggest that overuse and misuse of antibacterial agent could be responsible as the major selective force leading to the development of bacterial resistance. Also, 6 strains (25%) resistant to oxacillin and 4 strains (17%) resistant to vancomicin were detected with the oxacillin resistance confirmed by *mecA* detection through PCR assay (Lee et al. 2004).

CONCLUSIONS

To our knowledge, this is the first report of *sae* gene in *Staphylococcus aureus* strains isolated from animal origin in Brazil.

The study of biological and molecular characteristics of these isolates demonstrated the presence of resistance to several antimicrobial agents and production of different virulence factors related to the pathogenesis of this agent.

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