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DETECTION OF DIFFERENT *STAPHYLOCOCCUS AUREUS* STRAINS IN BOVINE MILK FROM SUBCLINICAL MASTITIS USING PCR AND ROUTINE TECHNIQUES

Olney Vieira-da-Motta[1]; Márcio Manhães Folly[1]*; Cássia Camargo Hagen Sakyiama[2]

[1]Laboratório de Sanidade Animal, [2]Laboratório de Melhoramento Genético Vegetal. Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense-UENF, Campos dos Goytacazes, Rio de Janeiro, Brazil.

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ABSTRACT

Contamination of fresh milk with *Staphylococcus aureus* was assessed comparatively through routine phenotypic (coagulase tube test and coagulase slide test) and genotypic (PCR) screening of 128 *S. aureus* strains isolated from 555 milk samples. These samples were collected from 362 cows with subclinical mastitis, hosted in different dairy herds at various locations of the Northern and Northeastern rural areas of the State of Rio de Janeiro, 39.7% of which were CMT-positive. All *S. aureus* isolates tested positive for the presence of the coagulase gene by PCR and the isolates could be grouped into four distinct classes according to the size of the PCR product. The strains also yielded variable results when assayed with coagulase test. Taken together, these data indicate the existence of extensive polymorphism at the coagulase gene locus in the genus *Staphylococcus* and exemplifies the extent of molecular and phenotypic heterogeneity associated with the strains circulating in rural herds.

Key words: bovine mastitis, *Staphylococcus aureus*, coagulase, PCR

INTRODUCTION

Bacterial infections of the mammary gland result in inflammation of the infected organ, a condition known as mastitis. Mastitis can be either moderate (subclinical) or severe (clinical) in which alterations of both udders and milk are visible to the naked eye. Untreated mastitis is one of the main causes of continuous economical losses in the dairy industry (8, 9, 24). *Staphylococcus aureus* infections of the udder generally result in subclinical mastitis. However, infections can progress into clinical manifestation to include systemic signals, particularly at calving and postpartum periods (14, 24).

Successful technological advances towards rapid and sensitive methods for the differential diagnosis of microbes resulted in an increasing number of biochemical and enzymatic tests, most of which are based on genotypic analyses. Altogether, these tests can be used to discriminate between otherwise indistinguishible bacteria commonly found in contaminated milk and milk products (13). Although the coagulase tube test is the standard phenotipic routine test used to identify *S. aureus* in biological samples (5), several groups have implemented the molecular analysis of the coagulase gene as an accurate defined test (1, 11, 15). More recently, Mathews *et al.* (17), using arbitrary primers to amplify target genes by PCR, called attention to the risk of possible diagnostic errors in the identification and differentiation of coagulase-negative *S. aureus* through conventional phenotypic assays.

The present study reports results of the incidence of *S. aureus* in bovine milk samples from subclinical mastitis, collected in the North and Northeast regions of the State of Rio de Janeiro, Brazil. The study also reports differences in results using either routine techniques or molecular typing of the coagulase gene (*coa*) by primer specific PCR.

MATERIALS AND METHODS

Milk samples were collected from three hundred and sixty two cows, hosted in herds at 64 rural properties in 15 municipalities of the Northern and Northeastern areas in the State of Rio de Janeiro,

* Corresponding author. Mailing address: Lab. de Sanidade Animal, Universidade Estadual do Norte Fluminense, Av. Alberto Lamego, 2000, Horto, CEP 28015-620, Campos dos Goytacazes, RJ, Brazil. E-mail: folly@uenf.br

Brazil. The samples were collected in a two-year period (1995 to 1997). Farms, animals and samples were randomly selected. CMT of milk samples was performed using a commercially available test (FATEC, SP, Brazil). CMT-positive samples were transported to the laboratory on ice. All samples were incubated at 37ºC for 18 hours and then inoculated on selective Chapman (Merck, Germany) agar and 5% sheep blood agar plates. Hemolysis, morphology, and pigmentation were scored after 24 hours incubation at 37ºC. Growth on Chapman agar was scored after a 48 hours incubation at 37ºC. Colonies yielding Gram-positive cocci were subjected to biochemical tests for catalase, oxidase (DrySlide Oxidase Difco, USA), acetoin production (VP), anaerobic mannitol fermentation (Difco, USA). Coagulase test (5, 16) was performed as follows. Two drops of cultures in TSB (Tripticase Soy Broth, Merck, Germany) were added to tubes containing 0.5 ml of citrate rabbit plasma (Bacto Coagulase Plasma, Difco, USA) and clot formation was observed every two hours for a 24 hour period. *S. aureus* strain ATCC 25923 was used as a positive control and *S. schleiferi* (isolated by the authors) as a negative control. To investigate production of bound coagulase and protein A, the Bacto Staph Latex Test kit (Difco, USA) was used according to the manufacturer instructions. Production of DNAse was tested in agar DNAse, using HCl 1.5 M to visualize halo formation around the colonies.

Molecular typing of the *coa* gene was performed by the method of Goh *et al.* (11) modified by Aarestrup *et al.* (1). Cells from an overnight culture in TSB were washed once (4500 rpm, 5 minutes) with 500 µl of TE buffer (Tris-HCl 50 mM pH 8.3; 50 mM EDTA – Sigma; USA), lysed with 15 Uμl^{-1} Lysostaphin (Sigma, USA) followed by incubation at 37ºC for 60 minutes. Lysis was completed by adding 1 ml of lysis buffer [(0.45% Igepal, 0.45% Tween 20, and 60 µg Proteinase K (10 mg.ml^{-1}, all from Sigma, USA) in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 1% mM Tris-HCl pH 8.3)], then incubated at 56ºC for 1 hour, followed by 10 minutes at 95ºC to inactivate Proteinase K.

For amplification of the *coa* gene the following specific primers were used: 5'ACC ACA AGG TAC TGA ATC AAC G3' (COAG2) and 5'TGC TTT CGA TTG TTC GAT GC3' (COAG3) were used. The primers were purchased from Dr. Charles Lindsey (Escola Paulista de Medicina, Universidade Federal de São Paulo, SP, Brazil). The PCR profile reported by Aarestrup *et al.* (1) was used. Briefly, 1 µl of lysate was added to the PCR mixture containing 1 µM of each primer, 200 µM of each deoxynucleotide triphosphate (dNTP), 1 U of Taq Polymerase (Gibco, USA) and 3 µl of 10X PCR buffer, making a total volume of 40 µl. The reactions were carried out in a GeneAmp PCR System 9600 (Perkin Elmer Cetus, USA) microprocessor thermal cycler. Profile: 30 seconds at 95ºC, 2 minutes at 55ºC and 4 minutes at 72ºC, with a total of 40 cycles. PCR products were separated by horizontal electrophoresis on 1.4% agarose gels (Sigma, USA) in TAE running buffer (1 M Trizma base, 3 M Sodium Acetate, 0.5 M EDTA). DNA fragments were stained with Ethidium Bromide (25 µg ml^{-1} Sigma, USA) for 15 minutes and visualized in an UV transiluminator (Sigma, USA).

RESULTS

A total of 555 milk samples were collected from 362 cows with subclinical mastitis. Nearly 40% of the animals tested were positive in the CMT. From milk samples of CMT-positive cows, 128 strains of *S. aureus* were isolated. All isolates showed double hemolysis in blood agar, grew on Chapman selective agar, and were positive for DNAse production, mannitol fermentation, catalase and acetoin production (VP), but negative using the oxidase test.

Molecular typing of the *coa* gene of the 128 isolates, carried out by primer-specific PCR, on Lysostaphin lysates, revealed four fragment sizes (Fig. 1). Fifty-four strains (42%) were characterized by a distinctive band of approximately 964 bp (lane 4), 29.7% (38 strains) showed a band of 740 bp (lane 5), 19.5% (25 strains) a band of 870 bp (lane 3) and 8.6% (11 strains) a 612 bp band (lane 6).

Table 1 presents a comparison of results of the tests used to identify the *S. aureus* strains. While 100% of the isolates were positive for the presence of the *coa* gene, the least informative phenotypic test was the coagulase slide test, which exhibited a limited resolving power (2/15 strains). Isolates LSA25 and LSA38 were consistently negative in the tube test while positive in both the slide test and *coa* gene amplification. All other strains presented positive results in the PCR, tube test and slide test (results not shown). Isolates LSA212 and LSA214 were negative in both the tube and slide tests but positive using PCR.

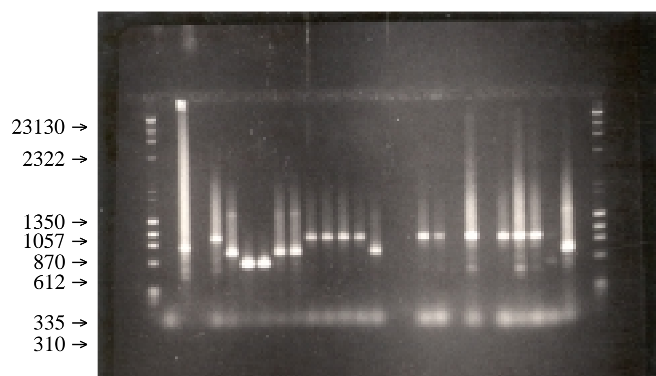

Figure 1. PCR products of selected *S. aureus* strains visualized on agarose gel electrophoresis. Lane 2 negative control *(Staphylococcus schleiferi);* lane 3 (870 bp), lane 4 (964 bp), lane 5 (740 bp) and lane 6 (612 bp) are examples of the fragment sizes seen in *S. aureus*; lane 21 positive control (*S. aureus* ATCC 25923); lane 22 distilled water control; lane 1 and lane 23 MW marker drigest III (λDNA-*Hind* III ϕX-174 RF DNA-*Hae* III Digest).

DISCUSSION

Mastitis can be classified as clinical or subclinical. Clinical mastitis is characterized by systemic signals such as anorexia, depression, toxemia, fever, and local signals like hardness of the udder at palpation, edema**,** increased temperature, hyperemia, and pain. Changes in the mammary secretion can also be seen; usually milk clots can become creamy (pus) and progressive infection can lead to aqueous secretions (20). These symptoms are not observed in subclinical mastitis, but changes in the udder can be detected in the field by qualitative tests (21).

The CMT results for the animals under study showed that 39.7% of them were positive, indicating that the prevalence of subclinical mastitis in the rural areas studied is higher than previously reported by other authors, in other areas of Brazil. Ribeiro *et al.* (19), working with 144 mammary quarters of infected cows in Minas Gerais State, found that 15.6% of them were CMT-positive. Nader Filho *et al.* (18) found 11.9% of infected cows in São Paulo State and Ferreiro *et al.* (10), in Rio Grande do Sul State, 25.5% CMT-positive. Our results are similar to those found by Harrop *et al.* (12) and De Freitas and Magalhães (6), in Pernambuco and Rio de Janeiro with 38.15% and 37.65% CMT positives, respectively.

Routine bacteriological tests used in the identification of *S. aureus*, like mannitol fermentation, DNAse production, VP, etc., are not enough for definitive characterization (7, 16). Nevertheless, coagulase production is still the most traditional test used to identify *S. aureus* (23). The role of coagulase in intramammary infection (IMI) remains uncertain, but it is known that most *S. aureus* strains isolated from such infections do, in fact, coagulate bovine plasma. However, the relation between *in vitro* activity and the actual role of coagulase during IMI has not been established (25). A comparative analysis of 15 strains (Table 1) was thus carried out to re-appraise the discriminatory power of the coagulase tube test, the slide test and PCR. The data showed a marked variation for the slide test. Two out of four strains negative to the tube test (3.1%) scored positive by both the slide test (bound coagulase) and PCR. These results suggest that these four strains, although possessing the *coa* gene, do not express free coagulase *in vitro*. Similar discrepancies were reported by Schmmitz *et al.* (22) while analyzing the enterotoxin C gene in *S. aureus* strains of human origin. They observed that certain *S. aureus* strains expressed undetectable levels of enterotoxin C, while tested positive for the SEC gene as assayed by PCR. As noted by the authors, for epidemiological reasons, toxin gene-positive staphylococci may be considered as toxin producers since *in vivo* toxin production (i.e., during infection) can not be ruled out. Thirteen (10.1%) strains were negative by the slide test and positive by PCR, underlying the discrimination power of the latter (Table 1). Baumgartner *et al.* (2) working with plasmids profiles, found that 11.3% of the strains were negative using the slide test. Tenover *et al.* (26) compared traditional and molecular techniques to identify different strains of *S. aureus* from human origin, and that the DNA-based techniques and immunoblotting were the most effective in strain grouping.

Table 1. Comparison of results for coagulase tests and PCR assay of selected strains of *S. aureus* isolated from mastitic cows.

Strain	Coagulase test		
	tube test	slide latex test	PCR
LSA 25	Negative	Positive	Positive
LSA 38	Negative	Positive	Positive
LSA 87	Positive	Negative	Positive
LSA 94	Positive	Negative	Positive
LSA 106	Positive	Negative	Positive
LSA 113	Positive	Negative	Positive
LSA 123	Positive	Negative	Positive
LSA 126	Positive	Negative	Positive
LSA 154	Positive	Negative	Positive
LSA 160	Positive	Negative	Positive
LSA 165	Positive	Negative	Positive
LSA 207	Positive	Negative	Positive
LSA 212	Negative	Negative	Positive
LSA 214	Negative	Negative	Positive
LSA 233	Positive	Negative	Positive

* LSA = Laboratório de Sanidade Animal (Animal Health Laboratory)

Table 2. Product size clones obtained in PCR amplification of the *coa* gene of *S. aureus* of different sources.

Author/Reference	PCR product size class (bp)	Source
Aarestrup *et al.* (1995)/ (1)	Not shown	Bovine
Goh *et al*. (1992)/ (11)	480-730 730 730-980 710-810 355-810 355-610 460-915 500 390-810 760 610	Human
Schwarszkopf *et al.* (1994)/(23)	654 735 816	Human
Vieira-da-Motta *et al.* (this paper)	612 740 870 964	Bovine

In our hands, PCR amplification of the *coa* gene, using the same primer sequences previously described as *S. aureus* specific (1), yielded four product size classes (Table 2). Goh *et al.* (11) and Schwarszkopf *et al.* (23), using human isolates, reported the occurrence of ten and three PCR product size classes, respectively. Aarestrup *et al.* (1), working with bovine isolates, further analyzed the extent of the polymorphism associated with the coagulase gene PCR products by restriction enzyme fragment length polymorphisms, and reported fifteen different electrophoretic patterns. In spite of differences in experimental protocols used in these laboratories, it is clear that there is extensive polymorphism associated with the *coa* gene in circulating strains of either human or bovine origin. Whether the reported PCR product size classes may represent distinct *S. aureus* strains awaits further molecular analysis, particularly at the sequence level. The biological significance of this heterogeneity is also unclear. The question of whether or not such heterogeneity can be manifested as varying coagulase activities also remains. However, it may well be related to differences in virulence/pathogenicity of the strains (3, 11). Previous molecular population genetic analysis of *S. aureus* strains recovered from cows (15) or hospitalized patients (4) showed an alarming heterogeneity of circulating strains, even within a given herd or hospital. Regardless a phenotypic association, we believe that the heterogeneity observed for the *coa* gene has a potential discriminatory power for future epidemiological studies of veterinary and medical importance.

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RESUMO

Detecção de diferentes cepas de *Staphylococcus aureus* de mastite bovina subclínica através da técnica de PCR e técnicas tradicionais

Quinhentas e cinqüenta e cinco amostras de leite, provenientes de 362 vacas com mastite subclínica em diferentes propriedades rurais do Estado do Rio de Janeiro, Brasil, de 1995 a 1997, foram submetidas ao teste "Califórnia Mastitis Test" (CMT). 39,7% das amostras foram positivas, das quais foram isoladas 128 cepas de *Staphylococcus aureus.* Todas as cepas isoladas foram positivas para o gene da coagulase utilizando a técnica de PCR, todavia, resultados de coagulase através das técnicas em tubo e "coagulase slide test" foram variáveis. Após a amplificação do gen de coagulase através da técnica de PCR utilizando iniciadores específicos para o referido gen, fragmentos com diferentes pesos moleculares foram vistos através de análise em gel de agarose, sugerindo a ocorrência de polimorfismo genético. O estudo também sugere a ocorrência de diferentes cepas da bactéria atuando nos rebanhos leiteiros causando mastite bovina.

Palavras-chave: mastite bovina, *Staphylococcus aureus*, coagulase, PCR

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