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Virulence factors genes of *Staphylococcus* spp. isolated from caprine subclinical mastitis

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ABSTRACT

The aim of this study was to investigate genes involved in adhesion expression, biofilm formation, and enterotoxin production in isolates of *Staphylococcus* spp. from goats with subclinical mastitis and associate these results with the *staphylococcal* species. One hundred and twenty-four isolates were identified and polymerase chain reaction (PCR) was performed to detect the following genes: *cna*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *bap*, *sea*, *seb*, *sec*, *sed* and *see*. The most commonly *Staphylococcus* species included *S. epidermidis*, *S. lugdunensis*, *S. chromogenes*, *S. capitis ss capitis* and *S. intermedius*. With the exception of *fnbB*, the genes were detected in different frequencies of occurrence in 86.3% of the *Staphylococcus* spp. isolates. *Eno* (73.2%) and *bap* (94.8%) were more frequently detected in coagulase-negative staphylococci (CNS); *ebpS* (76%), *fib* (90.9%) and *fnbA* (87%) were the most frequent genes in coagulase-positive staphylococci (CPS). Regarding enterotoxins, genes *sed* (28.2%) and *see* (24.2%) had a higher frequency of occurrence; *sec* gene was more frequently detected in CPS (58.8%). There was no association between the presence of the genes and the *Staphylococcus* species. Different virulence factors genes can be detected in caprine subclinical mastitis caused by CNS and CPS. The knowledge of the occurrence of these virulence factors is important for the development of effective control and prevention measures of subclinical mastitis caused by CNS and CPS in goats.

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1. Introduction

Intramammary infections in dairy goats can cause economic losses due to decreased milk production as well as risks to public health [1,2]. Subclinical mastitis is mainly caused by coagulase-negative staphylococci (CNS), considered less pathogenic than *Staphylococcus aureus* (*S. aureus*), although they may cause persistent clinical infections in the mammary gland [1,3,4].

Bacterial adhesion to epithelial cells is generally the first stage in the infectious process, and helps to prevent the elimination of the bacterium from the gland during milking [5]. The adhesins that promote the interaction between Gram-positive bacteria and host cells are grouped in a family consisting of different proteins referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [6,7]. Studies demonstrated that MSCRAMM adhesin genes, which were previously detected only in *S. aureus*, can also be found in CNS [8–10].

Biofilm formation occurs through successive stages. First, bacteria attach themselves to a solid surface, where they multiply and groups of cells accumulate in multilayers, which results in the formation of a bacterial community [11,12]. The biofilm protects the bacteria against the host's immune response and against the action of antimicrobial agents [13]. The gene *bap* is essential for biofilm formation and was detected in *S. aureus* isolates from bovine mastitis [12].

Dairy cows with mastitis caused by *Staphylococcus* spp. are considered reservoirs of enterotoxins which can cause human health risks [4,14]. Many staphylococci species can produce these enterotoxins, but little is known about the enterotoxigenic

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potential of CNS [14].

In Brazil, the prevalence of mastitis in goats can reach values of 75% or even more, mainly subclinical mastitis [15]. Subclinical mastitis due to CNS and coagulase-positive staphylococci (CPS) in goats from different regions of Brazil have already been reported, with a higher frequency of occurrence of CNS [16–18]. Therefore, the implementation of control measures is needed to avoid mastitis.

Furthermore, the knowledge of the virulence factors of *Staphylococcus* spp. involved in subclinical mastitis is important for the elaboration of control and prevention measures of this disease. The aim of this study was to investigate the presence of genes associated with adhesion, biofilm formation and enterotoxin production in *Staphylococcus* spp. isolated from milk samples from goats with subclinical mastitis and associate these results with the *Staphylococcus* species.

2. Materials and methods

2.1. Sampling, identification and characterization of Staphylococcus spp

Milk samples were collected from goats in herds of São Paulo and Minas Gerais states, southeastern of Brazil, which are important regions for dairy goat production. The samples were subjected to the California mastitis test (CMT) to detect subclinical mastitis, according to the methodology described by [19]. In positive cases, milk samples were aseptically collected in sterile tubes and sent to the laboratory under refrigeration.

Milk samples were plated onto Petri dishes containing sheep blood agar (5%) and incubated at 37 °C for 24 hours [20]. The identification of *Staphylococcus* spp. isolates was performed using biochemical tests, [21] followed by classification as established by [22]. The isolates were submitted to Gram stain, catalase and coagulase tests. The genus *Staphylococcus* was differentiated from the genus *Micrococcus* using the test for sensitivity to bacitracin. Other biochemical tests were performed: fermentation of lactose, maltose, mannitol, sucrose and trehalose; urease and acetoin production and resistance to novobiocin and polymyxin B [22,23]. The RapID Staph system (Thermo Fisher Scientific®) was used for confirmation of results.

2.2. DNA extraction

The chromosomal DNA of *Staphylococcus* spp. isolates was obtained from bacterial colonies according to [24], preceded by addition of proteinase K (20 mg/mL) and lysozyme (100 mg/mL). The supernatant obtained was stored at −20 °C prior to analysis.

2.3. Polymerase chain reaction (PCR)

Six pairs of primers were used for amplification of genes encoding adhesins, one gene related to biofilm production, and five genes encoding enterotoxins (Table 1).

Six PCR reactions were performed for each isolate – PCR-1, PCR-2, PCR-3, PCR-4, PCR-5 and PCR-6. In PCR-1, the genes *cna*, *eno* and *ebpS* were amplified, whereas in PCR-2, the genes *fnbB* and *fib* were amplified; both reactions were performed according to the protocol described by [6]. The amplifications of the genes *fnbA* (PCR-3) and *bap* (PCR-4) were performed according to [8,25], respectively. The genes *sea*, *seb* and *sec* were amplified in PCR-5, and the genes *sed* and *see* were amplified in PCR-6; both reactions were performed according to the method described by [26]. Isolates from the collection of the Bacteriology and Mycology Laboratory of the Department of Preventive Veterinary Medicine and Animal Health of the Faculty of Veterinary Medicine (University of São Paulo) were used as positive controls.

Detection of the amplified products was performed using electrophoresis on a 1.5% agarose gel stained with 0.05 mg/mL ethidium bromide and visualized under ultraviolet light.

2.4. Statistical analysis

The evaluations of the frequencies of the *Staphylococcus* species and genes encoding adhesins, biofilms and enterotoxins, as well as the associations between these genes and the staphylococcal species, were performed using the multiple comparison test between proportions with the R software and according to the methods of

Table 1
Primers sequences used to amplify adhesion-, biofilm formation- and enterotoxin-genes in *Staphylococcus* spp. isolates.

Putative pathogenic determinants	Gene	Nucleotide sequence of primers (5′–3′)	Amplicon size (bp)	PCR number
Collagen binding protein	*cna-1*	GTCAAGCAGTTATTAACACCAGAC	423	1
	cna-2	AATCAGTAATTGCACTTTGTCCACTG		
Laminin binding protein	*eno-1*	ACGTGCAGCAGCTGACT	302	1
	eno-2	CAACAGCATCTTCAGTACCTTC		
Elastin binding protein	*ebpS-1*	CATCCAGAACCAATCGAAGAC	186	1
	ebpS-2	CTTAACAGTTACATCATCATGTTTATCTTTTG		
Fibronecting binding proteins A	*fnbA-1*	GCGGAGATCAAAGACAA	1280	3
	fnbA-2	CCATCTATAGCTGTGTGG		
Fibronecting binding proteins B	*fnbB-1*	GTAACAGCTAATGGTCGAATTGATACT	524	2
	fnbB-2	CAAGTTCGATAGGAGTACTATGTTC		
Fibrinogen binding protein	*fib-1*	CTACAACTACAATTGCCGTCAACAG	404	2
	fib-2	GCTCTTGTAAGACCATTTTTCTTCAC		
Biofilm associated protein	*bap-1*	CCCTATATCGAAAGGTGTAGAATTG	971	4
	bap-2	GCTGTTGAAGTTAATACTGTACCTGC		
Enterotoxin A	*sea-1*	GCAGGGAACAGCTTTAGGC	521	5
	sea-2	GTTCTGTAGAAGTATGAAACACG		
Enterotoxin B	*seb-1*	ACATGTAATTTTGATATTCGCACTG	667	5
	seb-2	TGCAGGCATCATGTCATACCA		
Enterotoxin C	*sec-1*	CTTGTATGTATGGAGGAATAACA	284	5
	sec-2	TGCAGGCATCATATCATACCA		
Enterotoxin D	*sed-1*	GTGGTGAAATAGATAGGACTGC	385	6
	sed-2	ATATGAAGGTGCTCTGTGG		
Enterotoxin E	*see-1*	TACCAATTAACTTGTGGATAGA	171	6
	see-2	CTCTTTGCACCTTACCGC		

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