Comparative analysis of \textit{agr} groups and virulence genes among subclinical and clinical mastitis \textit{Staphylococcus aureus} isolates from sheep flocks of the Northeast of Brazil

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Abstract

\textit{Staphylococcus aureus} is one of the most frequent mastitis causative agents in small ruminants. The expression of most virulence genes of \textit{S. aureus} is controlled by an accessory gene regulator (\textit{agr}) locus. This study aimed to ascertain the prevalence of the different \textit{agr} groups and to evaluate the occurrence of encoding genes for cytotoxin, adhesins and toxins with superantigen activity in \textit{S. aureus} isolates from milk of ewes with clinical and subclinical mastitis in sheep flocks raised for meat production. The \textit{agr} groups I and II were identified in both cases of clinical and subclinical mastitis. Neither the \textit{arg} groups III and IV nor negative \textit{agr} were found. The presence of \textit{cflA} gene was identified in 100\% of the isolates. The frequency of \textit{hla} and \textit{lukE-D} genes was high - 77.3 and 82.8\%, respectively, and all isolates from clinical mastitis presented these genes. The \textit{sec} gene, either associated to \textit{tst} gene or not, was identified only in isolates from subclinical mastitis. None of the following genes were identified: \textit{bbp}, \textit{ebpS}, \textit{cna}, \textit{fnbB}, \textit{icaA}, \textit{icaD}, \textit{bap}, \textit{hlg}, \textit{lukM-lukF-PV} and \textit{se-a-b-d-e}.

Key words: \textit{Staphylococcus aureus}, \textit{agr} groups, virulence genes, mastitis, sheep flock.

Introduction

Mastitis in sheep has a major economic impact for the farmer when compared to the effects on cow and goat. It can lead to loss of the mammary gland and even death of ewe and/or lamb (Menzies and Ramanon, 2001). The incidence of clinical mastitis in dairy sheep is usually lower than 5\% per year. In a low percentage of herds, the incidence is higher and may exceed 30-50\% of the animals, causing mortality or culling of up to 70\% of the herd (Bergonier \textit{et al.}, 2003). If untreated, it also constitutes a serious problem in ewes raised for meat production. The Santa Inês breed stands out because gains weight fast and it also reproduces quickly all through the year, representing thus an excellent matrix for breeding. However, Santa Inês breed presents characteristics that render a very efficient milk yield. In semi-intensive and intensive handling with a richer diet, there is greater predisposition to infection of the mammary gland because the surplus of milk is not consumed by the lamb (Sousa \textit{et al.}, 2005).

Staphylococci are one of the most frequent mastitis causative agents in small ruminants (Contreras \textit{et al.}, 2007). Even though the most common Staphylococci is coagulase-negative, the occurrence of \textit{Staphylococcus aureus} is concerning because this pathogen can cause gangrenous mastitis due to the production of specific toxins such as \textit{α}-toxin that produces necrosis in the alveoli (Santos \textit{et al.}, 2007). The expression of hemolysins is the main factor that contributes to bacterial infection and inhibition of the immune response of the host (Silva \textit{et al.}, 2005). Moreover, \textit{S. aureus} has the ability to produce many other virulence factors (Fitzgerald \textit{et al.}, 2000), such as bi-component leukotoxins and toxins with superantigen activity. The toxins of \textit{S. aureus} can cause vascular thrombosis, gangrene and, consequently, the affected gland gets gradually...
isolated from the surrounding tissue (Winter 2001). This pathogen may also produce specific adhesins that bind to a variety of host proteins, especially in the extracellular matrix, such as collagen, fibrinogen and fibronectin (Novick 2000). The interaction with the host tissue represents a critical role in the establishment of mastitis by *S. aureus* (Kerro Dego *et al.*, 2002).

The virulence factors of *S. aureus* are not expressed constantly, some are more important than others, according to different stages of the infection (Kalorey *et al.*, 2007). The expression of most virulence genes of *S. aureus* is controlled by an accessory gene regulator (*agr*) locus, which encodes a two-component signal transduction system that leads to down-regulation of surface proteins and up-regulation of secreted proteins during growth (Zhang *et al.*, 1998). Four allelic groups of the *agr* system have been identified in human isolates (Ji *et al.*, 1997). In *S. aureus* isolates from bovine mastitis, variations in the nucleotide sequences of *agr*B and *agr*D genes were identified (Takeuchi *et al.*, 2001), other than those described by Ji *et al.* (1997).

This study aimed to ascertain the prevalence of the different *agr* groups and to evaluate the occurrence of encoding genes for cytotoxin, adhesins and toxins with superantigen activity in *S. aureus* isolates from milk of ewes with clinical and subclinical mastitis in flocks of sheep raised for meat production in the Northeast of Brazil.

**Materials and Methods**

**Clinical examination and sample collection**

From August 2004 to October 2005, 31 herds located in 15 districts of State of Pernambuco (Northeast Brazil) were surveyed and 135 primiparous and multiparous ewes Santa Inês in different stages of lactation were sampled. 270 mammary glands were examined clinically following the recommendations of Diffay *et al.* (2002). The collection of milk samples and bacteriological culture was performed according to standard laboratory procedures of the National Mastitis Council (1999).

**Identification of *S. aureus* isolates**

*S. aureus* was isolated from sheep milk in 29.03% out of herds, located in 46.67% of the districts surveyed, accounting for 18 isolates - 6 from clinical mastitis samples and 12 from subclinical mastitis cases (Table 1). These isolates represent a population of *S. aureus* from 31 sheep flocks distributed in 15 different districts located in an area of 22,500 km$^2$.

Conventional methods that included Gram staining, colony morphology, catalase and coagulase tests were used (Quinn *et al.*, 1994). The colonies identified as *S. aureus* were confirmed by polymerase chain reaction (PCR) performed on the *nuc* gene using the primers F:5'TATGGTCTCTGAAGCAAGTG3' and R:5'GCCACGTCCATATTTATCAG3' that were designed based on sequences of genomic DNA of MRSA strain 252 (GenBank database accession number NC_002952).

**DNA extraction and amplification of *agr* and virulence genes**

The extraction of chromosomal DNA of isolates was performed using the technique of phenol-chloroform extraction adapted from Sambrook *et al.* (1989). The primers and PCR conditions used for amplification of the *hla*, *hlb*, *hlg*, *lukE-D*, *pvl*, *eta*, *etb*, *tst* and *sea-e* genes were described by Jarraud *et al.* (2002); for *bbp*, *cna*, *eno*, *ebp*, *fnbA*, *fnbB*, *fib*, *clfA*, *clfB* *icaA*, *icaD* and *bap* genes, by Tristan *et al.* (2003) and Vancaerwynst *et al.* (2004) and for *agr* groups by Gilot *et al.* (2002). The reactions were carried out in Veriti Thermal Cycler 6.5 (Applied Biosystems). PCR products were analyzed by electrophoresis through 1% agarose gels.

The ATCC 25923 strain was used as positive control for *hla*, *hlb*, *tst*, *sec*, *lukE-D*, *bbp*, *cna*, *eno*, *ebp* and *agr*III genes; N315 strain for the *icaA*, *icaD* and *agr*I genes; 10/8520 for *agr*I gene; Mu50 for *sea* gene; CC63 for *seb* gene; RN4220 for *sed* gene; T47 for *see* gene; ZM for *eta* gene; N5 for *etb* gene; MR108 for *pvl* gene; RN4420 for *hlb* gene and, for *clfA*, *clfB*, *fnbA*, *fnbB* and *fib* genes, some isolates of our collection that had their PCR products sequenced and compared with published sequences in GenBank (access numbers: Z18852, AJ224764, X95848,
lates in which the agr part

lates. The majority of these isolates was represented by one allele. (2002) identified 12 distinct ST-1728 and ST-1730, accounting for 50% of isolates. Its mastitis. adapt to the sheep host causing either clinical or subclinical mastitis. This fact makes it difficult to define the role of S. aureus of genes encoding for the virulence factors of S. aureus. The pathogen staphylococcal infections and to underscore the multifac- torsial aspect of virulence of this pathogen. The pathoge-

sis of S. aureus is complex and it probably involves the synthesis of surface-associated proteins along with the secretion of exotoxins, resulting in damaging effects on the host cells (Takeuchi et al., 2001).

In our previous study, MLST analysis of these isolates from sheep milk showed the occurrence of four STs (ST-750, ST-1728, ST-1729 and ST-1730) associated both with clinical and subclinical mastitis cases, being the last three recently reported in http://saureus.mlst.net. ST-750 and ST-1728 isolates were the most prevalent - 38.9% and 50%, respectively - occurring in all the districts surveyed (Almeida et al., 2011). In the present study, we observed that the agr group I was identified in S. aureus isolates be- longed to ST-750 and ST-1729, whereas the agr group II was identified in ST-1728 and ST-1730 (Table 2). The occurrence of agr group I have already been ob- served in S. aureus isolates of other animal species. Gilot et al. (2002) identified 12 distinct agr alleles in an epidemiologically unrelated collection of bovine mastitis iso- lates. The majority of these isolates was represented by one particular agr allele from agr group I, suggesting the occurrence of either host-adapted or tissue-adapted S. aureus isolates in which the agr restriction type (allele) may play a significant role. Our results indicate that S. aureus isolates with agr group I are also postively able to infect and to adapt to the sheep host causing either clinical or subclinical mastitis.

The agr group II was the most prevalent in this study (ST-1728 and ST-1730), accounting for 50% of isolates. Its distribution ranged from isolates that presented greater combination of virulence factor genes - including the association of sec and tst genes - to isolates carrying only one gene for adhesin and none for exotoxins. Similarly, the agr group I, representing the other 50% (ST-750 and ST-1729), was detected both in isolates carrying multiple virulence genes and in isolates carrying few of these genes. This puts in doubt the specificity of the relations of these agr groups exclusively with combinations of multiple genes encoding virulence factors, but suggests that the alleles I and II have an important role in the ability of S. aureus isolates to invade and survive in different cell types of sheep host. Buzzola et al. (2007) reported that bovine S. aureus isolates with agr group I showed increased abilities to invade MAC-T cells. Conversely, isolates of agr groups II, III and IV were internalized less efficiently, suggesting that these isolates may be more susceptible to attack by the host immune response because they tend to remain in larger amounts in the extracellular environment.

Neither the agr groups III and IV, nor negative agr were found among the S. aureus isolates from ewes in this study, results that differ from a recent survey (Vautor et al., 2008), which identified the agr group III in a predominant clone found only in sheep and goats.

Staphylococcus aureus isolates from clinical and subclinical mastitis belonging to the same ST showed no differences in genetic background related to adhesins and proteins associated with biofilm formation. The presence of clfA gene (receptors for fibronectin) in 100% of the isolates of this study suggests its involvement in the colonization process of the mammary gland, regardless of the clinical picture of mastitis subsequently developed. The presence and expression of this gene may promote the adherence of S. aureus to the tissues of the mammary gland (Que et al., 2001), but it was unable to correlate the presence of clfA gene with the clinical manifestation of the disease, since it was identified in isolates of the same ST both in clinical and subclinical mastitis.

None of the following genes were identified in the isolates: bhp (receptor for bone sialoprotein), ebpS (elastin-binding protein), cna (collagen-binding protein), fnbB (fibrinogen-binding protein), fib (fibrinogen-binding pro-

Table 2 - Distribution of agr groups and virulence genes among S. aureus isolates from ewes with clinical and subclinical mastitis in Pernambuco, Brazil.

| Origin of isolate | Sequence type (ST) - MLST | agr group | Adhesins and proteins related to biofilm formation | Citotoxins and toxins with superantigen activity | Number/total of isolates |
|-------------------|---------------------------|-----------|-----------------------------------------------------|-----------------------------------------------|------------------------|
| Clinical mastitis | ST750                     | I         | clfA                                                 | hla, lukE-D and tst                           | 2/ 11 1               |
| Subclinical mastitis | ST750                  | I         | clfA                                                 | hla, hlb, lukE-D and sec                       | 5/ 27 5               |
| Clinical mastitis | ST1729*                  | I         | clfA                                                 | hla and lukE-D                                | 1/ 5 5                |
| Subclinical mastitis | ST1729*                 | I         | clfA                                                 | lukE-D                                       | 1/ 5 5                |
| Clinical mastitis | ST1728*, ST1730*         | II        | clfA and clfB                                        | hla and lukE-D                               | 3/ 16 6               |
| Subclinical mastitis | ST1728*                 | II        | clfA and clfB                                        | hla, lukE-D, tst and sec                      | 3/ 16 6               |
| Subclinical mastitis | ST1728*                 | II        | clfA                                                 |                                             | 3/ 16 6               |

* Novel STs recently reported in http://saureus.mlst.net.
tein), eno (laminin-binding protein), icaA, icaD and bap (proteins related to biofilm formation).

The absence of cna gene, which encodes the collagen binding protein, is consistent with the reports of Smeltzer et al. (1997) that S. aureus isolates from animals do not usually have this gene. No isolates presented the fnbB gene, but this does not exclude the possibility of presence of variants of this gene (Sung et al., 2008) involved with cases of acute gangrenous mastitis. Vautor et al. (2009) reported that fnbB gene, which can bind to host proteins such as fibrinogen, fibronectin and elastin was missing in the S. aureus strain responsible for a case of acute gangrenous mastitis and was also less common in high virulence isolates, being associated with a smaller spread of infections (Vautor et al., 2008).

Our results showed greater diversity in the combination of exotoxin genes. Besides presenting adhesin genes, 55.2% also presented genes for cytotoxins and toxins with superantigen activity. Combinations of the staphylococcal exotoxins, as well as the amount of their secretion may define the pathogenic potential of the bacteria. The pore-forming exotoxins induce pre-inflammatory changes in mammalian cells, inactivating the immune system and degrading tissues, thus providing the bacteria with nutrients facilitating their dispersal in other sites (Projan and Novick, 1997). Some authors have suggested the involvement of α-toxin with gangrenous mastitis in cattle (Anderson 1983), but relations between this toxin and severe manifestations of the disease are still under scrutiny. In sheep, data correlating the presence of hla gene in S. aureus and the occurrence of clinical mastitis are scarce. The frequency of hla and lukE-D genes among the isolates of this study was high - 77.3 and 82.8%, respectively, and all S. aureus isolates from clinical mastitis presented the combination of the genes encoding for α-toxin and LUKE-D leukocidin.

The hlb gene, which encodes β-hemolysin, was identified only in isolates from subclinical mastitis, accounting for 27.5% of total isolates. The presence and possible expression of this gene may explain the relation between S. aureus isolates carrying hlb gene and the occurrence of chronic cases, because this gene can promote the escape of bacteria from the host immune system and assist in its process of obtaining nutrients (Huseby et al., 2007), helping the survival of the pathogen.

The presence of sec gene (enterotoxin C) was identified in 44.1% of isolates, all from subclinical mastitis (ST-750 and ST-1728). These results are consistent with the prevalence of this gene in S. aureus isolates from the same animal species observed by Orden et al. (1992). The tst gene (toxic shock syndrome toxin) was found in 27.7% of isolates (16.6% associated with sec gene). This association was unique to isolates from subclinical mastitis (ST-1728), but the presence of tst gene was also identified in isolates from cases of clinical mastitis (ST-750). The frequency of the combination of sec and tst genes was lower (16.6%) than the 74% found by Orden et al. (1992) in sheep. Almost all genes for toxins with superantigen activity are related to pathogenicity islands or other mobile genetic elements, some coexisting in the same isolate. In cattle, a putative pathogenicity island encoding multiple superantigens, the SaPIbov, was identified in the genome of a bovine isolate (Fitzgerald et al., 2001).

The production of toxic shock syndrome toxin (TSST-1) in staphylococcal isolates from different anatomical sites of healthy sheep and the detection of antibodies to this toxin in milk and whey were studied by Valle et al. (1991), suggesting a frequent contact these animals with isolates producing of the TSST-1. In S. aureus isolates from cattle, the genes for toxins with superantigen activity have been linked to persistent intramammary infections (Haveri et al., 2008). These toxins may contribute to spread of bacteria within a host, and even between hosts, since they are secreted during periods of high bacterial density (Katsuda et al., 2005). Our tests showed that S. aureus isolates from clinical and subclinical mastitis - 11.1% and 16.6% respectively - carried the tst gene. Takeuchi et al. (2001) suggested that the agr locus variations of S. aureus bovine isolates may be related to the low production of α-toxin and TSST-1 among these isolates. However, little is known about this in S. aureus isolates from sheep.

The hlg (gamma-hemolysin), lukM-lukF-PV (Panton-Valentine bi-component leukotoxin - PV), eta-b (Exfoliative toxins A-B) and sea-b-d-e (A, B, D, E enterotoxins) genes were not identified in any of the isolates.

Conclusions

We identified the distribution of the agr groups I and II in S. aureus isolates from ewes both with clinical and subclinical mastitis raised for meat production suggesting that these alleles are involved in overcoming host defenses and to establish an intramammary infection in sheep. Our data support a significant role of the clfA gene in the establishment of mastitis by S. aureus, such as the associations of the hla/lukE-D genes and tst/sec genes on spread of infection in clinical and subclinical mastitis, respectively.

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