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Survey of accessory gene regulator (*agr*) groups and TSST-1 encoding gene (*tst*) in *Staphylococcus aureus* isolated from ewes with mastitis in the northwest of Iran

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Summary

Accessory gene regulator (*agr* locus) is a global regulator of many virulence gene expressions in *Staphylococcus aureus*. Four interference classes related to genetic polymorphisms in the *agr* locus have so far been described. In the present study, the *agr* locus specificity groups were studied within a total of 43 *S. aureus* isolates which were isolated from ewes with mastitis in three regions in the Northwest of Iran. In addition, the isolates were examined for the presence of TSST-1 (toxic shock syndrome toxin-1) encoding gene (*tst*) using PCR. Identification of *agr* groups, using *agr* group-specific multiplex PCR, classified the majority of isolates into group I (51.16%) and to a lesser extent into *agr* group IV (44.19%). Only one isolate (2.23%) for each of the *agr* groups II and III was found. This study also indicated that 44.19% of isolates (n=19) possessed the *tst* gene and that 94.74% (n=18) belonged to *agr* group I. According to the results, certain *agr* groups comprised the great majority of sheep mastitis associated *S. aureus* isolates. Moreover, the high proportion of *S. aureus* isolates was *tst* positive, indicating an issue requiring consideration as it is relevant to food hygiene. The results also showed a variation in distribution of the *tst* gene among the different *agr* groups.

Key words: *Staphylococcus aureus*, Sheep mastitis, *agr* groups, *tst* gene, Iran

Introduction

Staphylococcus aureus has been reported to be one of the most common causal agents of mastitis in ewes (Goni *et al*., 2004; Mork *et al*., 2007) with relevant losses in the sheep industry worldwide (Mork *et al*., 2005). This microorganism produces a panoply of virulence factors that provide it the ability to colonize, invade, evade the immune response of and cause toxicity to the host (Cheung *et al*., 2004). Virulence expression in *S. aureus* is tightly regulated and the acuteness of infection associated to some strains depends on their ability to mobilize and express their virulence factors in a given context (e.g. infection site) as much as on their final gene content (Le Marechal *et al*., 2011). The most widely characterized of *S. aureus* regulatory loci is the accessory gene regulator (*agr*), with a polymorphism in the sequence of the autoinducing peptide (AIP) and its receptor, according to which clinical strains can be divided into four *agr* groups (I to IV) (Jarraud *et al*., 2002; Lina *et al*., 2003). These strains appear to compete with each other at the level of *agr* expression, as each AIP activates the *agr* response in isolates belonging to the same group, while inhibiting it in strains of other groups. Studies carried out by Gilot *et al*. (2002), and Gilot and Leeuwen (2004) indicated that specific genetic backgrounds of *S. aureus* strains, at least in the two-component regulatory factors, *agr* and *trap*, could be associated with particular hosts and diseases. Some correlation between *agr* groups and certain types of infections has been demonstrated, e.g. endocarditis is mainly caused by strains belonging to *agr*

group I and II, toxic shock syndrome (TSS) by strains belonging to *agr* group III (Jarraud *et al*., 2002), and recurrent furunculosis (RF) predominantly by strains belonging to *agr* group IV (Garbacz *et al*., 2011). The *agr* operon among several potentially associated factors is thought to positively regulate toxic shock syndrome toxin-1 (TSST-1) production (Wright and Holland, 2003), which is encoded by the *tst* gene. TSST-1 and staphylococcal enterotoxins are capable of acting as superantigens for cells of the bovine immune system and may potentially contribute to the pathological mechanisms of bovine mastitis caused by *S. aureus* strains producing these toxins (Yokomizo *et al*., 1995; Ferens *et al*., 1998). Due to the specificity of the host-pathogen interactions needed to produce mastitis, it has been postulated that the nature of the virulon and the regulation of its virulence-gene expression are determining factors when it comes to the ability of a strain to produce mastitis (Vautor *et al*., 2009; Piccinini *et al*., 2010).

To our knowledge, no studies have been carried out to describe genetic variability of *agr* locus in *S. aureus* isolates associated with dairy sheep mastitis in Iran, and also little is known regarding the occurrence of *tst*-positive isolates of *S. aureus* in sheep. So, the purpose of this study was to gain an insight into the *agr* specificity groups and also to know the relationship between the unique *agr* types and the occurrence of the *tst* gene among the isolates of *S. aureus* obtained from mammary secretions from ewes in different geographical regions of Iran.

Materials and Methods

Bacterial isolates

A total of 43 *S. aureus* isolates were recovered from 311 milk samples aseptically collected from cases of sheep mastitis. The samples were taken from individual animals from nine flocks during a time span of June to October 2010 in three locations including Tabriz (n=28), Urmia (n=13), and Miandoab (n=2) within East and West Azerbaijan provinces, Iran. Of these isolates, 9 and 34 were relevant to clinical and subclinical mastitis ewe's milk, respectively. All of the isolates were initially identified based on the following scheme: colony morphology, Gram stain, catalase and coagulase tests. All isolates were further identified by amplification of the species-specific *nuc* gene from 4 µl of the purified nucleic acid solutions according to the procedures described previously (Saei, 2012). The sequence of oligonucleotides that was used for PCR amplification was as follows: 5-GCG ATT GAT GGT GAT ACG GTT -3 (primer 1) and 5- AGC CAA GCC TTG ACG AAC TAA AGC -3 (primer 2) (Brakstad *et al*., 1992). *Staphylococcus aureus* ATCC 29213 was used as positive control. For negative control template DNA was replaced with sterile water.

Detection of *agr* specificity groups by PCR

The *agr* specificity groups were identified by multiplex PCR according to Gilot *et al*. (2002), which involves a forward primer (pan-*agr*) common to all *agr* groups and four primers, each one specific to each *agr* group. Primer sequences and predicted size of the corresponding amplified product are shown in Table 1. *Staphylococcus aureus agr* reference strains RN6390 (*agr* group 1), RN6923 (*agr* group 2), RN8462 (*agr* group 3), and A880740 (*agr* group 4) were used as controls.

Detection of gene encoding TSST-1

All 43 *S. aureus* isolates were screened

Table 1: Primer sequences and predicted lengths of PCR amplification products

Primer	Sequence (5'-3')	Amplicon size (bp)	Reference
pan-*agr*	ATG CAC ATG GTG CAC ATG C		Gilot *et al*. (2002)
*agr*I	GTC ACA AGT ACT ATA AGC TGC GAT	441	
*agr*II	TAT TAC TAA TTG AAA AGT GGC CAT AGC	575	
*agr*III	GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	323	
*agr*IV	CGA TAA TGC CGT AAT ACC CG	659	

for the presence of gene encoding TSST-1 (*tst*). To detect the presence of the *tst* gene, PCR was performed as described by Johnson *et al*. (1991) using forward (5'-ATG GCA GCA TCA GCT TGA TA-3') and reverse (5'-TTT CCA ATA ACC ACC CGT TT-3') primers, which allow the amplification of a 350-bp DNA fragment of the *tst*-positive isolates. For the negative control, sterile water was added instead of nucleic acids. *Staphylococcus aureus* strain FRI913 was included as the positive control.

Results

PCR amplification revealed a 441-bp DNA fragment of the *agr* group I isolates, a 575-bp DNA fragment of the *agr* group II isolates, a 323-bp DNA fragment of the *agr* group III isolates, and a 659-bp DNA fragment of the *agr* group IV isolates (Fig. 1). From the 43 *S. aureus* isolates, 51.16% (n=22) were *agr* group I. The remaining 21 isolates fell into the *agr* groups IV (n=19; 44.19%), II (n=1; 2.32%) and III (n=1; 2.32%). According to the results, 7 out of 9 *S. aureus* isolates originated from clinical mastitis assigned to *agr* type I, and 2 remaining isolates assigned to *agr* type IV. *Staphylococcus aureus* isolates with the *agr* types II and III were not recovered from clinical cases. However, *agr* types I, II, III and IV were found in 15, 1, 1 and 17 isolates associated with cases of subclinical mastitis, respectively.

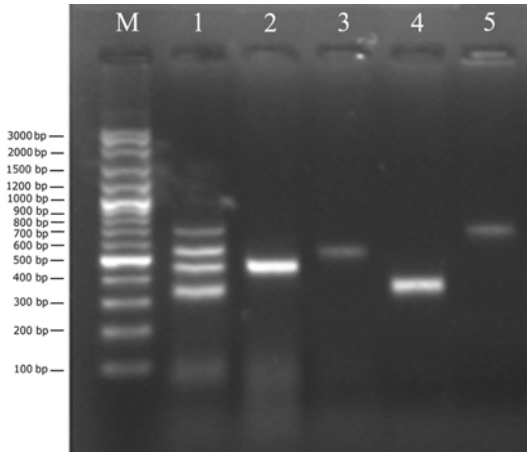

Fig. 1: *agr* group-specific multiplex PCR. Representation of the different *agr* types among tested *S. aureus* isolates: Lane 1: Chromosomal DNAs from standard strains were used for identification of four *agr* specificity groups using M-PCR, Lane 2: *agr* type I, Lane 3: *agr* type II, Lane 4: *agr* type III, Lane 5: *agr* type IV. Lane M: GeneRuler™ 100 bp DNA ladder plus marker

The PCR amplification of the TSST-1 encoding gene (*tst*) revealed an amplicon of the expected 350-bp DNA fragment (Fig. 2). As shown in Table 2, *tst* gene was detected in 19 (44.19%) of the 43 isolates.

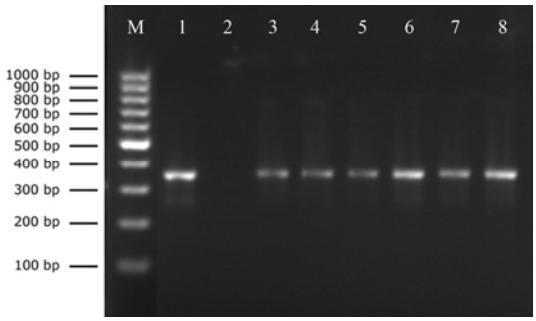

Fig. 2: Example of PCR results of *tst* gene (lanes 3-8). Lane 1: *S. aureus tst* positive control (350 bp), Lane 2: negative PCR control (reaction mixture minus DNA). Lane M: GeneRuler™ 100 bp DNA ladder (Fermentas)

A variation was also observed in the distribution of the *tst* gene among the different *agr* types. As shown, *tst* gene was detected in *S. aureus* isolates belonging to *agr* types I and IV. None of the isolates from *agr* types II and III carried the *tst* gene. Furthermore, comparison between dominant *agr* types I and IV revealed that 18 isolates (81.82%) of group I and a unique isolate of group IV possessed the *tst* gene.

Table 2: Distribution of *tst* gene among different *agr* types

agr type	No. of isolates	*tst*-positive
I	22	18
II	1	-
III	1	-
IV	19	1
Total	43	19

Discussion

The occurrence of *S. aureus* associated clinical and subclinical mastitis in different breeds of sheep has been investigated in various parts of the world (Al-Majali and

Jawabreh, 2003). The virulence factors of this bacterium are largely regulated by two-component regulatory systems, such as the *agr*, *saeRS*, *srrAB*, *arlSR*, and *lytRS* systems (Bronner *et al*., 2004). It seems that better understanding of the association between *agr* groups and specific infections may enable a therapeutic approach to case management. In principle, a non-pathogenic strain of *S. aureus* or other species of bacteria employing *agr* could be engineered to inhibit the regulatory systems of pathogenic strains using *agr*, thus down-regulating their production of virulence factors (Jabbari *et al*., 2012). It has been shown in a murine subcutaneous abscess model that the group II AIP attenuates the virulence of a group I strain, which suggests possible therapeutic value for the AIPs (Mayville *et al*., 1999). A variant AIP has also been developed that inhibits autologous as well as heterologous *agr* expression and is therefore a global inhibitor of the virulence response in *S. aureus* (Lyon *et al*., 2000). In the current study, investigation of the 43 *S. aureus* isolates from ovine mastitis for the *agr* specificity groups I to IV revealed the predominance of the *agr* group I (51.16%) and to a lesser extent *agr* type IV (44.19%). This observation is somewhat in agreement with Alves *et al*. (2009), who demonstrated the high prevalence (62.9%) of *agr* group I within small ruminants. Domination of *agr* group I could be explained by the intergroup inhibitory effect of the group I AIP, which excluded other *agr* types from infection or colonization site. Other speculation could be the unique characteristics of isolates belonging to *agr* group I, which enable the pathogen to overcome host defense mechanisms and to establish a successful IMI in ewes in comparison with other groups. It has been previously shown that *S. aureus* strains belonging to *agr* group I have significantly increased capacities to be internalized in bovine mammary epithelial cells and persist in higher numbers in murine mammary glands (Buzzola *et al*., 2007).

Our findings also indicated that *agr* group IV occurs frequently among the *S. aureus* isolates associated with sheep mastitis. This finding differed from those reported previously (Alves *et al*., 2009; Vautor *et al*., 2009), indicating the presence of discrepancies in the frequency of any particular *agr* group among isolates from different areas. There seems to be a geographic distribution difference between *agr* groups (Yoon *et al*., 2007). *agr* types II and III, which have been reported previously from bovine mastitis cases (Takeuchi *et al*., 2001; Vautor *et al*., 2009), occurred only rarely among the *S. aureus* isolates from ovine used in this study. Based on these findings, it is tempting to think that the distribution of the *agr* groups differed between cattle and sheep, demonstrating the variation of *S. aureus agr* types that are able to cause IMI in small and large ruminants. However, these relationships need to be further studied, especially in the context of commonly used typing methods. Several studies using various molecular typing methods have shown that some *S. aureus* types preferentially colonize or infect a particular host species (Ben Zakour *et al*., 2008; van Elk *et al*., 2012).

As shown in the present study, 44.19% of the *S. aureus* isolates from dairy ewe's milk were positive for TSST-1 encoding gene (*tst*). Scherrer *et al*. (2004) found that 93.5% of the *S. aureus* strains were able to produce TSST-1 in bulk-tank milk samples of sheep. In a study carried out by Orden *et al*. (1992) the highest proportion of strains producing TSST-1 was obtained from sheep, twice as many as those from goats or cows. These results highlight possible public health consequences, for example through the consumption of raw milk and dairy products manufactured from it. Raw milk and cheeses made from raw milk are important vectors (De Buyser *et al*., 2001).

Results of the present study also revealed that almost all of the *tst*-positive isolates (18 out of 19; 94.7%) belonged to *agr* group I, confirming the results of other studies that *tst* gene may occurs in association with chromosomal backgrounds such as *agr* locus, which belongs to the core variable genome and is strongly linked with clonal lineages (Lindsay *et al*., 2006). Association of a particular *agr* type in clinical isolates harboring important virulence factors, such as toxic-shock

syndrome toxin (TSST-1) (Chini *et al*., 2006; Holtfreter *et al*., 2007) or exfoliatin toxin has already been observed (Jarraud *et al*., 2000). Bronner *et al*. (2004) also conclude that the virulence factors are differently distributed among strains and are also not always regulated in the same way in different strains. These results suggest non-uniformly distribution of the *tst* gene-carrying mobile genetic elements (MGEs) among *agr* types. It has been shown that horizontal movement of genetic elements encoding virulence factors is not random and may be restricted between certain genetic lineages (Moore and Lindsay, 2001).

In conclusion, PCR-based assays were used for the identification of *agr* specificity groups and the presence of TSST-1 encoding gene (*tst*) within *S. aureus* isolates was obtained from ovine mastitis. Findings of this study revealed the existence of representative strains of each *agr* type in *S. aureus* ovine isolates, from which *agr* groups I and IV were predominant. Our results also showed a high occurrence of *tst*-positive *S. aureus* isolates in ewe's mastitis milk, indicating an issue requiring consideration as it is relevant to food hygiene. Furthermore, results revealed the classification of almost all *tst*-positive isolates in *agr* group I, indicating the presence of *tst* gene may be *agr* related. Nevertheless, further studies are necessary to ascertain factors facilitating *S. aureus* infection of the ovine mammary gland, in order to improve strategies to reduce the occurrence of mastitis in sheep.

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