Investigation of accessory gene regulator (agr) in Staphylococcus aureus isolated from clinical and subclinical bovine mastitis in Iran

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Mastitis is one of the common diseases of dairy cattle and an inflammatory response of the mammary glands tissue. Mastitis causes considerable loss to the dairy industry. Among several bacterial pathogens that can cause mastitis, S. aureus is probably the most lethal agent because it causes chronic and deep infection in the mammary glands that is extremely difficult to be cured. The present study was to detect agr group genes in the S. aureus isolated from 360 mastitis milk samples in Chaharmahel va Bakhtiari and Isfahan provinces of Iran via PCR by using specific primers. Among 360 milk samples, 86 samples contained 1250 bp fragment of the 23srRNA gene, 10 samples contained agrI gene, 42 samples contained agrII gene, 19 samples contained agrIII gene and 15 samples contained agrIV gene.

Key words: Bovine mastitis, S. aureus, agr group genes, PCR, Iran.

INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium that has remained a persistent pathogen, causing such infections as endocarditis, meningitis, and toxic shock syndrome in humans. S. aureus also is the leading cause of intramammary infections (mastitis), especially in dairy animals, from whose milk it is frequently isolated (Yancey, 1999). Neutrophils are the principle line of defense during the initial stages of mastitis, and the ability of these cells to phagocytize and kill invading bacteria is critically related to the establishment of new intramammary infections (Paape et al., 1979). Therefore, any bacterially derived component that may compromise neutrophil function would constitute an important virulence factor in the pathogenesis of S. aureus mastitis. Although a number of different virulence factors involved in the pathogenesis of S. aureus mastitis have been identified (Yancey, 1999), the differential expression of these factors as it relates to field strain prevalence of S. aureus genotypes has not been investigated. A better understanding of the epidemiology of S. aureus mastitis as it pertains to virulence will provide insight concerning important host-pathogen interactions during the pathogenesis of disease. Subtyping is an important tool for epidemiologic investigation of bacterial infections. In the past decade, numerous molecular techniques such as multilocus enzyme electrophoresis, phage typing, plasmid DNA restriction patterns, random amplified polymorphic DNA ribotyping, and coagulase genotyping have proved useful in identification and comparison of S. aureus isolates in epidemiological studies (Baumgartner et al., 1984; Matthews, 1993; Saulnier et al., 1993; Thomson-Carter et al., 1989; Wang et al., 1993).

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Abbreviations: S. aureus, Staphylococcus aureus; agr, accessory gene regulator.
However, very few studies have identified *S. aureus* isolates by the gene polymorphisms among important virulence-related genes. Among the virulence-related genes in *S. aureus*, we were particularly interested in the accessory gene regulator (agr), which has been shown to regulate the synthesis of many virulence factors during bacterial growth (Balaban and Novick, 1995b; Novick et al., 1993). The agr system coordinately down-regulates the production of cell wall-associated proteins and up-regulates secreted proteins at late to stationary growth phase in vitro (Ji et al., 1995; Novick et al., 1993; Novick et al., 1995; Recsei et al., 1986). The *agr* locus encodes a two-component signal-transducing system consisting of two divergent transcription units driven by promoters P2 and P3 (Ji et al., 1997). The P3 operon encodes the transcript for RNAIII, the effector of the agr response, while the P2 operon contains transcripts for four open reading frames designated *agrA*, *-B*, *-C*, and *-D* (Balaban and Novick, 1995a). *agrB* and *-D* generate an auto-inducing peptide that acts as an activating ligand for *agrC*. The present study was designed to investigate *S. aureus* isolates from cattle with bovine clinical and subclinical mastitis from different region of Iran, genotypically and by the identification of *agr* gene.

**MATERIALS AND METHODS**

Sample collection and identification

A total of 86 *S. aureus* isolates were collected from milk samples from 360 cows with mastitis from 10 different farms in tow region of Iran (140 samples from Chaharmahal va Bakhtiari and 220 specimens from Isfahan provinces). All of the isolates were identified by culture properties, by the detection of hemolysis (Skalka et al., 1979), and by the tube coagulase reaction.

Nucleic acid purification

For DNA preparation, 5 - 10 colonies of the bacteria were incubated in 100 µl of TE buffer (10 mmol of Tris- HCl/liter, 1 mmol of EDTA/liter, pH 8.0) containing 5 µl of lysostaphin (1.8 U/ µl; Sigma, Deisenhofen, Germany), for 1 h at 37°C and subsequently treated with proteinase K (14.0 mg/ml; Fermentas) for 120 min at 56°C. To inactivate the proteinase K, the suspension was heated for 10 min at 100°C and centrifuged at 10,000× g for 20 s. Ninety microliters of the supernatant was treated with 10 µl of 5 mol/liter NaClO4 and 50 µl of isopropanol (99.7%; Merck, Germany), mixed, placed on an ice block for 10 min, and centrifuged for 30 min at 13,000 rpm. The supernatant was discarded, 250 µl of ethanol (70%) was added, and the tube was again centrifuged for 5 min at 13,000 rpm. The supernatant was again discarded, and the pellet was dried in a desiccator for 5 min. After the addition of 50 µl of sterilized aqua dest, the tubes were cooled until they were used.

PCR amplification of the variable region of the *agr* operon

PCR amplification of the 1070 bp variable region of the *agr* operon was performed with primers B1(5-TAT GCT CCT GCA GCA ACT AA-3) and C2 (5-CTT GCC CAT TTC GTT GA-3) described by van Leeuwen et al. (van Leeuwen et al., 2000). The variable *agr* region was amplified from 2 µl of the purified nucleic acid solution in a 100 µl reaction mixture containing 2.5 U of *Taq* DNA polymerase (Fermentas), 200 µM deoxynucleotide triphosphates (dNTPs) (Fermentas), 0.5 µM primer B1, 0.5 µM primer C2, 2 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, and 10 mM Tris HCl (pH 9.0). Amplifications were carried out in a thermocycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) through the following temperature program: 1 cycle of 4 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 74°C; and finally 1 cycle at 74°C for 3 min (Gilot et al., 2002).

**Agr** group-specific multiplex PCR

The *agr* sequences were amplified from 2 µl of the purified nucleic acid solutions in a 25 µl reaction mixture containing 1.25 U of *Taq* DNA polymerase (Fermentas), 200 µM dNTPs (Fermentas), 5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris HCl (pH 9.0), and a 0.3 μM concentration of each of the following primers: Pan (5’-ATG CAC ATG GTC ATG CAG-3’), agr1 (5’-GTC AGT ACT ATA AGC TGC GAT-3’), agr2 (5’-TAT TAC TAA TTA GAA AGT GCC CAT AGC-3’), agr3 (5’-GTA ATG TAA TAG CTG GTA TAA TAC TCA GCA-3’), and agr4 (5’-GCA TAA TGC CTT AAT ACC CG-3’). These primers allow the amplification of a 441-bp DNA fragment of the *agr* group 1 strains, of a 575-bp DNA fragment of the *agr* group 2 strains, of a 323-bp DNA fragment of the *agr* group 3 strains, and of a 659-bp DNA fragment of the *agr* group 4 strains. Amplifications were carried out in a thermocycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) through the following temperature program: 1 cycle of 5 min at 94°C; 28 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C; and finally 1 cycle of 72°C for 10 min (Gilot et al., 2002). Amplification products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and visualized by transillumination under UV.

Statistical analyses

The analysis of all data was calculated by using SPSS software, version 16 and X² statistical test.

RESULTS

A total of 360 raw milk samples from several major herds in the Chaharmahal va Bakhtiari (n = 140) and Isfahan (n = 220) provinces of Iran were used in this study. 86 specimens (23.88%) of 360 milk samples in microbiological studies were identified to infected with *S. aureus*.

The PCR assay was able to detect, *S. aureus* DNA from 86 samples of milk by using primers mentioned in materials and methods. The existence of 1250 bp fragment in samples that showed positive PCR assay. All of the samples contained 1250 bp DNA fragment bands.

Of the 86 samples 10 specimens contained the *agrI* gene, 42 specimens contained the *agrII* gene, 19 specimens contained the *agrIII* gene and 15 samples contained the *agrIV* gene. The results are shown in Table 1.

*S. aureus* strains isolated in this study was relevant to the clinical and sub clinical mastitis cases which in the CMT test had show a positive reaction. From the total number of 86 strains, 20 strains were relevant to mastitis +1 (in the CMT test), 45 strains were relevant to mastitis...
mastitis milk in Chaharmahal va Bakhtiari and Isfahan province of Iran. Our results indicated that among the four types of agr gene, agrII with frequency 48.83% was the most frequently present type among the isolated Staphylococcus strains (Table 1).

Expression of agr gene in the pathogenic staphylococci is very crucial in bacterial colonization. Regarding the agr gene being polymorphic, it is probable that expression of this gene could be effect in ability of S. aureus for transfer from one host to another. Involvement of the agr genetic locus to regulation expression of virulence gene, incited researchers to use the agr system for identifying different Staphylococcus spp. (Robinson et al., 2005).

Many researches has been done in different countries to determine the genotype of the agr gene, for example. In the research conducted by Shopsin et al. from 196 S. aureus strains isolated from children and 64 S. aureus isolated from adults, polymorphism of the agr gene was studied. In this study it was found out that the Staphylococcus spp. caring the agr gene are capable to colonization (Shopsin et al., 2003).

In another study performed in 2008 by Reinoso et al. from 45 Staphylococcus strains isolated from various sources such as human infections and mastitis were studied from of different virulence factors. In human samples 8 specimens (36%) belonged to the agrII group and from 14 human samples belonged to the groups I (14%) and II (27%). Among the samples isolated from the bovine mastitis, 7 specimens (47%) belonged to the agrII group 8 specimens belonged to the agrI (27%) and agrII groups. 10 specimens were reported to be negative for agrI to agrII groups (Reinoso et al., 2008).

In the present study most of the S. aureus strains contained the agrII gene which corresponds, with results obtained by Reinoso et al. (Reinoso et al., 2008).

From the total of 42 indicated that samples containing the agrII gene, 29 strains were 3+ CMT test. In the statistical analysis by Chi-square test was statistically significant relationship between the presence of agr gene and degree of bovine mastitis in CMT test (P ≤ 0.05). This result indicates that, the expression of the agr gene is effect on pathogenicity of S. aureus in developing mastitis. As a whole, different results reported from various studies indicate that many factors such as geographical situation and main source of bacteria involved in developing mastitis can play a role in type and percentage of presence of virulence genes in the S. aureus strains.

Results of this study can be an introduction for more complete studies on distribution of agr genes in strains of Staphylococcus isolated from mastitis cases and the role of involvement of these genes in pathogenicity of the bovine mastitis.

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