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Full Length Research Paper

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 *agr*I gene, 42 samples contained *agr*II gene, 19 samples contained *agr*III 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 $NaClO_4$ 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 GCG CAT TTC GTT 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 GTG CAC ATG C-3'), agr1 (5'-GTC ACA AGT ACT ATA AGC TGC GAT-3'), agr2 (5'-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3'), agr3 (5'-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3'), and agr4 (5'-CGA TAA TGC CGT 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; 26 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 X^2 statistical test.

RESULTS

A total of 360 raw milk samples from several major herds in the Chaharmahel 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 *agr*I gene, 42 specimens contained the *agr*II gene, 19 specimens contained the *agr*III gene and 15 samples contained the *agr IV* 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

Table 1. Frequency of presence of the *agr* genes in the *S. aureus* strains isolated from bovine mastitis milk in Iran.

Specimens	*agr*I	*agr*II	*agr*III	*agr*IV
86	10	42	19	15

Table 2. Number of virulence genes in bovine mastitis milk samples CMT 1+ to 3+ in Iran.

*agr*IV	*agr*III	*agr*II	*agr*I	Samples	Degree of CMT test
4	3	2	1	20	+
4	6	11	3	45	++
7	10	29	6	21	+++

+2, and 21 strains were relevant to mastitis +3. Type and the number of understudied virulence genes obtained from these mastitis cases are shown in Table 2.

DISCUSSION

S. aureus is one of the commonest causes of bovine mastitis which is of economic importance to the industry. *S. aureus* produces a large number of potential virulence factors. The staphylococcal accessory gene regulator (*agr*) is the most important locus responsible for the regulation of virulence factors (Robinson et al., 2005).

The *agr* locus of *S. aureus* recognized a quorum-sensing gene cluster, encodes a two-component signal transduction system that leads to down-regulation of surface proteins and up-regulation of secreted proteins during *in vitro* growth. A role for *agr* in virulence has been demonstrated by the attenuated virulence of *agr* mutants in different animal infection models (Mullarky et al., 2001; Robinson et al., 2005). The *agr* locus consists of the divergently transcribed P2 and P3 operons. The P2 operon consists of the genes *agrB*, *agrD*, *agrC*, and *agrA*. In essence, *agrB* activity leads to secretion of the auto inducing pheromone, *agrD,* which binds to and activates the histidine kinase receptor, *agrC*, which subsequently activates the response regulator, *agrA*. The P3 operon consists of the regulatory effector molecule of the *agr* system, RNAIII, and the gene encoding delta-hemolysin, *hld*. interestingly, amino acid changes within the *agrD* pheromone can cause inhibition of *agr* activity. Four allelic groups of *agr* have been characterized in *S. aureus* (numbered I to IV) that generally induce *agr* activity within a group and inhibit *agr* activity between groups. The inhibitory activity of these *agr* groups represents a form of bacterial interference that affects virulence gene expression (Robinson et al., 2005).

The study was conducted to determine the prevalence of the *agr* gene in *S. aureus* strains isolated from bovine mastitis milk in Chaharmahal va Bakhtiari and Isfahan provinces of Iran. Our results indicated that among the four types of *agr* gene, *agr*II 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 *agr*III 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 *agr*I group 8 specimens belonged to the *agr*I (27%) and *agr*I (13%) groups. 10 specimens were reported to be negative for *agr*I to *agr*III groups (Reinoso et al., 2008).

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

From the total of 42 indicated that samples containing the *agr*II 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 ($P \leq 0.05$). This result indicates that, the expression of the *agr* gene is effect on pathogencity 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 rote of involvement of these genes in pathogencity of the bovine mastitis.

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REFERENCES

Balaban N, Novick RP (1995a). Translation of RNAIII, the *Staphylococcus aureus agr* regulatory RNA molecule, can be activated by a 3'-end deletion. FEMS Microbiol. Lett. 133: 155-161.

Balaban N, Novick RP (1995b). Autocrine regulation of toxin synthesis by *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA. 92: 1619-1623.

Baumgartner A, Nicolet J, Eggimann M (1984). Plasmid profiles of *Staphylococcus aureus* causing bovine mastitis. J. Appl. Bacteriol. 56:159–163.

Gilot P, Lina G, Cochard T, Poutrel B (2002). Analysis of the genetic variability of genes encoding the RNA III-activating components Agr and TRAP in a population of *Staphylococcus aureus* strains isolated from cows with mastitis. J. Clin. Microbiol. 40: 4060-4067.

Ji G, Beavis R, Novick RP (1997). Bacterial interference caused by autoinducing peptide variants. Science 276: 2027–2030.

Ji G, Beavis RC, Novick RP (1995). Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc. Natl. Acad. Sci. USA. 92: 12055-12059.

Matthews RC (1993). PCR fingerprinting microbes by random amplification of polymorphic DNA. J. Med. Microbiol. 39: 161-162.

Mullarky IK, Su C, Frieze N, Park YH, Sordillo LM (2001). *Staphylococcus aureus agr* genotypes with enterotoxin production capabilities can resist neutrophil bactericidal activity. Infect. Immun. 69: 45-51.

Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, Vandenesch F, Moghazeh S (1995). The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol. Gen. Genet. 248: 446-458.

Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth G, Moghazeh S (1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory molecule. EMBO J. 12: 3967-3975.

Paape MJ, Wergin WP, Guidry AJ, Pearson RE (1979). Leukocytes-second line of defense against invading mastitis pathogens. J. Dairy Sci. 62: 135-153.

Recsei P, Kreiswirth B, O'Reilly M, Schlievert P, Gruss A, Novick RP (1986). Regulation of exoprotein gene expression in *Staphylococcus aureus* by agar. Mol. Gen. Genet. 202: 58-61.

Reinoso EB, El-Sayed A, Lämmler C, Bogni C, Zschöck M (2008). Genotyping of *Staphylococcus aureus* isolated from humans, bovine subclinical mastitis and food samples in Argentina. Microbiol. Res. 163: 314-322. Robinson DA, Monk AB, Cooper JE, Feil EJ, Enright MC (2005). Evolutionary genetics of the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*. J. Bacteriol. 187: 8312-21.

Saulnier P, Bourneix C, Prevost G, Andremont A (1993). Random amplified polymorphic DNA assay is less discriminant than pulsed-field gel electrophoresis for typing strains of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. 31: 982-985.

Shopsin B, Mathema B, Alcabes P, Said-Salim B, Lina G, Matsuka A, Martinez J, Kreiswirth BN (2003). Prevalence of *agr* specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. J. Clin. Microbiol. 41: 456-459.

Skalka B, Smola J, Pillich J (1979). A simple method of detecting staphylococcal hemolysins. Zentralbl. Bakteriol. Orig. A. 245: 283-286.

Thomson-Carter FM, Carter PE, Pennington TH (1989). Differentiation of staphylococcal species and strains by ribosomal RNA gene restriction patterns. J. Gen. Microbiol. 135: 2093-2097.

van Leeuwen W, van Nieuwenhuizen W, Gijzen C, Verbrugh H, van Belkum A (2000). Population studies of methicillin-resistant and –sensitive *Staphylococcus aureus* strains reveal a lack of variability in the *agrD* gene, encoding a staphylococcal autoinducer peptide. J. Bacteriol. 182: 5721-5729.

Wang G, Whittam TS, Berg CM, Berg DE (1993). RAPD (arbitrary primer) PCR is more sensitive than multilocus enzyme electrophoresis for distinguishing related bacterial strains. Nucleic Acids Res. 21: 5930–5933.

Yancey RJ (1999). Vaccines and diagnostic methods for bovine mastitis:fact and fiction. Adv. Vet. Med. 41: 257-273.