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Full Length Research Paper

Detection of some virulence factors 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, *Staphylococcus 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 some of the virulence factors 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 a 360 raw milk samples, 86 samples contained 1250 bp fragment of the 23srRNA gene, 42 samples contained *coa* gene, 63 samples contained *clfA* gene, 69 samples contained IgG binding region gene, 22 samples contained X region coding gene protein A, 3 sample contained Toxic shock syndrome toxin gene (*tst*), 16 samples contained the exfoliative toxin A and B genes, 10 samples contained *agr*I gene, 42 samples contained *agr*II gene, 19 samples contained *agr*III gene and 15 samples contained *agr*IV gene.

Key words: Bovine mastitis, *Staphylococcus aureus*, virulence factors, polymerase chain reaction (PCR), Iran.

INTRODUCTION

Staphylococcus aureus is recognized worldwide as a frequent cause of subclinical intramammary infections in dairy cows. The main reservoir of *S. aureus* seems to be the infected quarter, and transmission between cows usually occurs during milking *S. aureus* produces a spectrum of extra cellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism. The staphylococcal enterotoxins (SEs) are recognized agents of the staphylococcal food poisoning syndrome and may be involved in other types of infections with sequelae of shock in humans and animals (Bergdoll, 1983; Marrack and Kappler, 1990).

Nine major antigenic types of SEs have been recognized and designated SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ (Bergdoll, 1983, Betley and Mekalanos, 1988; Monday and Bohach, 1999; Munson et al., 1998; Su and Wong, 1998; Zhang et al., 1998). All these toxins exhibit super antigenic activity by interacting with antigen-presenting cells and T lymphocytes without regard for the antigen specificity of the cells. This induces cellular proliferation and a high level of cytokine expression (Dinges et al., 2000). A distantly related protein, toxic

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Abbreviations: ***S. aureus*,** *Staphylococcus aureus*; ***coa*,** coagulase; ***clfA*,** clumping factor; ***tst*,** toxic shock syndrome toxin gene; ***agr*,** accessory gene regulator; ***eta*,** exfoliative toxin A; ***etb*,** exfoliative toxin B; **PCR,** polymerase chain reaction; **TSST-1,** toxic shock syndrome toxin 1.

shock syndrome toxin 1 (TSST-1), also produced by *S. aureus,* was the first toxin shown to be involved in toxic shock syndrome, in both menstrual and nonmenstrual cases (Bergdoll et al., 1981; Schlievert et al., 1981). However, no immunological identity and little amino acid homology between TSST-1 and the staphylococcal enterotoxins exist (Blomster-Hautamaa et al., 1986).

Some strains of *S. aureus* produce one or both of two immunologically distinct exfoliative toxins, exfoliative toxin A (ETA) or ETB (Lee et al., 1987; Marrack and Kappler, 1990). These toxins have been associated with impetiginous staphylococcal diseases referred to as staphylococcal scaled skin syndrome. At present little is known about the occurrence of these toxins among *S. aureus* isolates from cattle with bovine 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).

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., 1995; Novick et al., 1993; 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 tow region of Iran and to identify the various virulence factors.

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. The isolates were additionally investigated by PCR amplification of species specific parts of the gene encoding the 23S rRNA with the oligonucleotide primers shown in Table 1.

For PCR amplification, the reaction mixture (30 µl) contained 1 µl of primer F (10 pmol/µl), 1 µl of primer R (10 pmol/µl), 0.6 µl of deoxynucleoside triphosphate (10 mmol/liter; Fermentas), 3 µl of 10X PCR buffer (Fermentas), 1.8 µl of $MgCl_2$ (25 mmol/liter; Fermentas), 0.1 µl of *Taq* DNA polymerase (5 U/µl, Fermentas) and 20 µl of distilled water. Finally, 2.5 µl of DNA preparation was added to each 0.2 ml reaction tube. The tubes were subjected to thermal cycling (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) with the program shown in Table 1. For DNA preparation, 5 to 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. The presence of PCR products was determined by electrophoresis of 12 µl of the reaction product in a 2% agarose gel with Tris-acetate electrophoresis buffer (0.04 mol of Tris/liter, 1 mmol of EDTA/liter, pH 8) and a 100-bp DNA ladder (Fermentas) as a molecular marker (Akineden et al., 2001).

A PCR amplification was performed for the genes encoding staphylococcal proteins and toxins, A PCR amplification was performed for the genes encoding staphylococcal coagulase (*coa*), clumping factor (*clfA*), protein A (*spa*), TSST-1 (*tst*), ETA (*eta*), ETB (*etb*), *agr* operon and *agr* region (*agr* 1,2,3,4). The sequences of the oligonucleotide primers, the thermocycler programs, and the references are summarized in Table 1. Amplification products were electrophoresed in a 1-1.5% agarose gel containing ethidium bromide and visualized by trans illumination under UV.

RESULT

A total of 360 raw cow milk samples from 10 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 micro-biological studies were identified to infect 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

Table 1. Oligonucleotide primers and PCR programs for amplification of the genes encoding staphylococcal 23SrRNA and staphylococcal proteins including various toxins.

Gene	Primer Sequence (5'-3')	PCR program[a]	Reference	Size of Product (bp)
23SrRNA	Staur4 ACG GAG TTA CAA AGG ACG AC Staur6 AGC TCA GCC TTA ACG AGT AC	1	Straub et al., 1999	1250
coa	Coa-1 CGA GAC CAA GAT TCA ACA AG Coa-2 AAA GAA AAC CAC TCA CAT CA	2	Aslantas et al., 2007	970,730
clfA	ClfA-1 GGC TTC AGT GCT TGT AGG ClfA-2 TTT TCA GGG TCA ATA TAA GC	3	Stephan et al., 2001	980
spa (X region)	spa-III CAA GCA CCA AAA GAG GAA spa-IV CAC CAG GTT TAA CGA CAT	4	Fre´nay et al., 1996	320
spa (IgG binding region)	spa-1 CAC CTG CTG CAA ATG CTG CG spa-2 GGC TTG TTG TTG TCT TCC TC	2	Seki et al., 1998	920
tst	TSST-1 ATG GCA GCA TCA GCT TGA TA TSST-2 TTT CCA ATA ACC ACC CGT TT	5	Johnson et al., 1991	350
eta	ETA-1 CTA GTG CAT TTG TTA TTC AA ETA-2 TGC ATT GAC ACC ATA GTA CT	5	Johnson et al., 1991	119
etb	ETB-1 ACG GCT ATA TAC ATT CAA TT ETB-2 TCC ATC GAT AAT ATA CCT AA	5	Johnson et al., 1991	200
agr operon	B1 TAT GCT CCT GCA GCA ACT AA C2 CTT GCG CAT TTC GTT GTT GA	6	van Leeuwen et al., 2000	1070
*agr*I	Pan ATG CAC ATG GTG CAC ATG C agr1 GTC ACA AGT ACT ATA AGC TGC GAT	7	Gilot et al., 2002	441
*agr*II	Pan ATG CAC ATG GTG CAC ATG C agr2 TAT TAC TAA TTG AAA AGT GGC CAT AGC	7	Gilot et al., 2002	575
*agr*III	Pan ATG CAC ATG GTG CAC ATG C agr3 GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	7	Gilot et al., 2002	323
*agr*IV	Pan ATG CAC ATG GTG CAC ATG C agr4 CGA TAA TGC CGT AAT ACC CG	7	Gilot et al., 2002	659

a 1, 37 times (94°C, 40 s; 64°C, 1 min; 72°C, 75 s); 2, 30 times (94°C, 1 min; 58°C, 1 min; 72°C, 1 min); 3, 35 times (94°C, 1 min; 57°C, 1 min; 72°C, 1 min); 4, 30 times (94°C, 1 min; 60°C, 1 min; 72°C, 1 min); 5, 30 times (94°C, 2 min; 55°C, 2 min; 72°C, 1 min); 6, 40 times (94°C, 1 min; 50°C,1 min; 74°C,2 min); 7, 26 times (94°C, 30 s; 55°C, 30 s; 72°C, 1 min)

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 42 specimens contained the *coa* gene, 63 specimens contained the *clfA* gene, 69 specimens contained the *spa* gene (x-region), 22 specimens contained *spa* gene (IgG Binding region), 3 sample contained the *tst* gene, 16 samples contained the *etA* and *etB* genes, 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 2 which shows frequency of presence of the virulence genes in the *S. aureus* strains isolated from bovine mastitis milk in Iran.

S. aureus strains isolated in this study was relevant to the clinical and subclinical 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 +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 3.

DISCUSSION

S. aureus has been recognized as a pathogen in human and animal infections. Mastitis causes considerable loss to the dairy industry of which *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. Epidemiologic studies indicates that *S. aureus* strains agents of mastitis produce a group of virulence factors and it is believed that there is a relationship between severity of mastitis and the virulence factors produced by *S. aureus* (Akineden et al., 2001).

Table 2. Frequency of presence of the virulence genes in the *S. aureus* strains isolated from bovine mastitis milk in Iran.

Specimens	coa	clfa	x-region	IgG binding region	*tst*	*etA*	*etB*	*agr*I	*agr*II	*agr*III	*agr*IV
86	42	63	69	63	3	16	16	10	42	19	15

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

*agr*IV	*agr*III	*agr*II	*agr*I	*coa*	*c1fA*	x-region	IgG binding region	*tst*	*etA*	*etB*	Samples	Degree of CMT test
4	3	2	1	7	10	11	3	0	2	2	20	+
4	3	2	1	12	21	19	4	0	4	4	45	++
7	10	29	6	23	32	39	15	3	10	10	21	+++

The present study was to detect some of the virulence factors in the *S. aureus* isolated from 360 mastitis raw cow's milk samples in Chaharmahel va Bakhtiari and Isfahan provinces via PCR by using specific primers.

In the present study, 86 *S. aureus* strains isolated from subclinical bovine mastitis cases were identified and further characterized by PCR amplification of various virulence genes. 42 strains (48.83%) contained the *coa* gene. Most of the strains were isolated from the CMT 3+ mastitis cases.

These results correspond significantly with similar results obtained by Karahan and Cetinkaya (2007) and Akineden et al. (2001). In their study from the 200 *S. aureus* strains isolated from the subclinical bovine mastitis, 161 samples (80.6%) contained the *coa* gene. These results show that this is a direct relation ship between presence of the *coa* gene in *S. aureus* and bovine mastitis. Zecconi et al. (2006) reported it was revealed that the entrotoxins A and J are considered as a risk factor in developing subclinical bovine mastitis.

In the research conducted by Turkyilmaz and Kaya (2006) in Turkey, some of the virulence factors in *S. aureus* isolated from bovine mastitis case, dog's external ear infection and chicken infections were studied and it was revealed that coagulase negative strains of *S. aureus*, are more dangerous than positive coagulase strains in developing infection.

Presence of the *clfA* gene and the gene encoding the X-region of the protein A are considered as the *Staphylococcus* spp. virulence genes in development and severity of mastitis (Akineden et al., 2001, Sharma et al., 2000).

This study indicates that these genes are the most frequent genes isolated from the pathogenic *S. aureus* strains and 73.25 and 80.23% of the strains, respectively, contained these genes. On the other hand, this study shows that 50.79 and 56.52% of the *S. aureus* strains respectively contained the *clfA* and X-region genes relevant to 3+ mastitis cases indicating existence of a statistically significant relationship between these genes in the *Staphylococcus* strains and the developed mastitis.

Another point is presence of other genes agents of virulence including *etA* and *etB* (exfoliative toxins A and B) in strains isolated fro the cases of CMT 3+ mastitis (Table 3). Even regarding the *tst* gene, the 3 positive case from the total 86 isolated bacteria is relevant to 3+ positive indicating involvement of this gene in developing super acute mastitis along with toxemia symptoms in cow. The staphylococcal accessory gene regulator (*agr*) is the most important locus responsible for the regulation of virulence factors (Robinson et al., 2005). Our results in this study indicated that among the four types of *agr* gene, *agr*I with frequency 48.83 % was the most frequently present type among the isolated *Staphylococcus* strains (Table 1). 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. (2003) 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.

In another study performed in 2008 by Reinoso et al. (2008) 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*II (13%) groups. 10 specimens were reported to be negative for *agr*I to *agr*III groups. In the present study, most of the *S. aureus* strains contained the *agr*II gene which corresponds, with results obtained by Reinoso et al. (2008).

From the total of 42 indicated that samples containing the *agr*II gene, 29 strains were 3+ CMT test. This results indicats that, the expression of the *agr* gene is effect on pathogencity of *S. aureus* in developing mastitis.

As a whole, results obtained of this research can be onset for more complete experimental study of the genes encoding virulence factors in developing bovine mastitis, cloning of virulence genes in the prokaryotic system and use of the recombinant protein is efficient in control procedures and management of this economic problem in dairy cattle. Another point is existence of some differences in results of this study composed with those obtained else where. This finding can indicates interference of many factors such as geographical situation and origin of the bacteria involved in developing positive on type and percentage of virulence genes in strains of *S. aureus* that through more perfect studies regarding isolating *Staphylococcus* spp.. from various sources of infection in bovine and various forms of *Staphylococcus* diseases this problem can be over come.

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