

Coagulase gene polymorphism of *Staphylococcus aureus* isolated from clinical and sub-clinical bovine mastitis in Isfahan and Chaharmahal va Bakhtiari provinces of Iran

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Received: 9 January 2010 / Accepted: 26 May 2010 / Published online: 16 June 2010
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Abstract Mastitis is a common disease in dairy cattle and is an inflammatory response of the breast tissue to bacterial attack to this tissue. Mastitis causes considerable loss to the dairy industry, among the 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 cure. Several virulence factors including coagulase gene are produced by *S. aureus* and may contribute to its pathogenicity. This study was conducted to investigate the coagulase gene polymorphism of *S. aureus* isolated from clinical and sub-clinical bovine mastitis milk samples in Isfahan and Chaharmahal va Bakhtiari provinces of Iran.

Amplification of the coagulase gene from 86 *S. aureus* strains isolates by specific primers showed 31 specimens contained 970 bp fragment, and 11 strains contained 730 bp fragment relevant to *coa* gene (coagulase) in PCR. After enzymatic digestion with *AluI*, 31 specimens contained three bands: 320, 490, and 160 bp (genotype I) and 11 specimens contained two bands: 490 and 240 bp (genotype VIII) in the RFLP.

Keywords *Staphylococcus aureus* · Bovine mastitis · PCR · Polymorphism · Coagulase gene · Iran

Introduction

Bovine mastitis is a major disease that affects the dairy industry, and *Staphylococcus aureus* is one of the most frequently isolated pathogens from both sub-clinical and chronic infections (Watts 1988). Currently used comprehensive mastitis control protocols have decreased the incidence, but *S. aureus* still remains one of the most significant organisms associated with clinical and sub-clinical bovine mastitis, not only in the USA but worldwide. A better understanding of the epidemiology of *S. aureus* is needed for the improvement of current mastitis control protocols. In the past decade, numerous molecular techniques have been developed and used for the identification and comparison of *S. aureus* isolates in epidemiological studies. Among these methods, coagulase gene typing has proven to be a simple and effective means to identify coagulase-positive *S. aureus* isolates from both human and animal sources (Aarestrup et al. 1995; Goh et al. 1992; Hookey et al. 1998; Kobayashi et al. 1995; Lawrence et al. 1996; Nada et al. 1996; Schwarzkopf and Karch 1994; Su et al. 1999; Tenover et al. 1994).

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In Iran, little information is available on the genetic diversity of *S. aureus* isolated from cows with clinical and sub-clinical bovine mastitis. In this study, *coa* gene polymorphism was used for typing and differentiation of *S. aureus* strains isolated from bovine mastitic milk samples from two different provinces in Iran.

Materials and methods

Bacterial isolates A total of 86 *S. aureus* isolates from Chaharmahal va Bakhtiari ($n=30$) and Isfahan ($n=56$) were isolated from 360 clinical and sub-clinical bovine mastitic milk samples (140 samples from Chaharmahal va Bakhtiari and 220 samples from Isfahan). Milk samples were inoculated onto blood agar base (Merck) supplemented with 5% defibrinated sheep blood. Isolates were identified by conventional methods, including Gram staining, colony morphology, hemolysis, tests for catalase, clumping factor, DNase, acetoin, and anaerobic fermentation of mannitol.

Extraction and purification of DNA Bacterial DNA extraction was carried out according to Rodrigues da Silva and da Silva (2005). Bacterial cell lysate was prepared from 0.5 ml of overnight trypticase soy broth cultures. After centrifugation at $12,000\times g$ for 10 min, bacterial pellets were washed with 500 μ l of Tris–hydrochloride–ethylene diamine tetra acetic acid (TE) buffer (10 mM Tris–HCl, pH 7.5, and 1 mM EDTA) and centrifuged again. The pellets were re-suspended in 200 μ l of TE buffer (pH 7.5) with 15 U of lysostaphin (2 mg, Sigma) per milliliter and incubated at 37°C for 1 h. Next, 15 μ l of proteinase K (20 mg/ml; Fermentas) was added, and the suspension was incubated at 56°C for 1 h. The suspension was then heated at 95°C for 15 min to inactivate the proteinase K. An equal volume of phenol–chloroform was added and the mixture centrifuged at $12,000\times g$ for 10 min. The upper phase was carefully transferred into another Eppendorf tube and mixed with two volumes of 95% ethanol and stored overnight at –20°C. The mixture was then centrifuged at $12,000\times g$ for 5 min. The DNA pellet was washed with ice-cold 70% ethanol, re-centrifuged, and dried by tube inversion. The DNA was suspended in 100 μ l of sterile TE, quantified in a spectrophotometer (at 260 nm), and kept frozen at –20°C.

Polymerase chain reaction (PCR) amplification PCR was performed in a 50- μ l reaction mixture containing 2 μ l of template DNA (approximately 500 ng/ μ l), 5 μ l of $10\times$ PCR buffer (750 mM Tris–HCl (pH 8.8), 200 mM $(\text{NH}_4)_2\text{SO}_4$, and 0.1% Tween 20), 200 μ M of each of the four deoxynucleotide triphosphates, 1 U of Taq DNA polymerase (Roch Applied Science), and 50 pmol of each primer (COAG2: CGA GAC CAA GAT TCA ACA AG; COAG3-

AAA GAA AAC CAC TCA CAT CA). The PCR reaction was performed in a thermocycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) using the following cyclic conditions: initial denaturation at 95°C for 2 min, 30 cycles of 30 s each with denaturation at 95°C, 2 min annealing at 58°C, 4 min extension at 72°C, and a final 7 min extension at 72°C (Goh et al. 1992).

Restriction enzyme digestion The PCR products were digested with *AluI* (Fermentas) for restriction analysis. For this aim, 12.5 μ l of PCR products was mixed with 10 U of enzyme and 10×1.5 μ l restriction buffer and then incubated at 37°C overnight (Aslantas et al. 2007).

Agarose gel electrophoresis The PCR products and the digested fragments were separated in 1% and 3% agarose gel (Fermentas), respectively, with 10 mg/ml aqueous solution of ethidium bromide (Fermentas) and then were photographed under ultraviolet illumination. The 100-bp marker (Fermentas) was used as a size standard for the calculation of the sizes of the *coa* and *AluI*-generated *coa* fragments.

Results

A total of 360 clinical and sub-clinical bovine mastitic milk samples from several major herds in the Chaharmahal va Bakhtiari and Isfahan provinces were used in this study. Eighty-six specimens (23.88%) from these samples were identified as infected with *S. aureus* in microbiological studies.

The PCR assay was able to detect *S. aureus* DNA from 86 samples of milk by using primers mentioned in the **Materials and methods** above. Eighty-six specimens

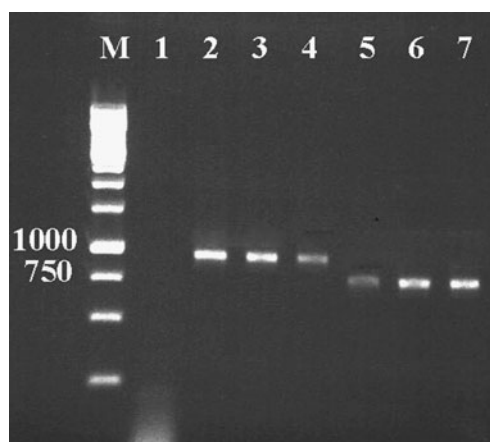


Fig. 1 Agarose gel electrophoresis of *coa* gene. Line M 1 kb DNA ladder, line 1 negative control, lines 2–4 positive samples with 970-bp fragment; lines 5–7 positive samples with 730-bp fragment

Table 1 Relationship between severity of mastitis (from the viewpoint of CMT test) and presence of the *coa* gene in the *Staphylococcus aureus* strain isolated from the cases of bovine mastitis

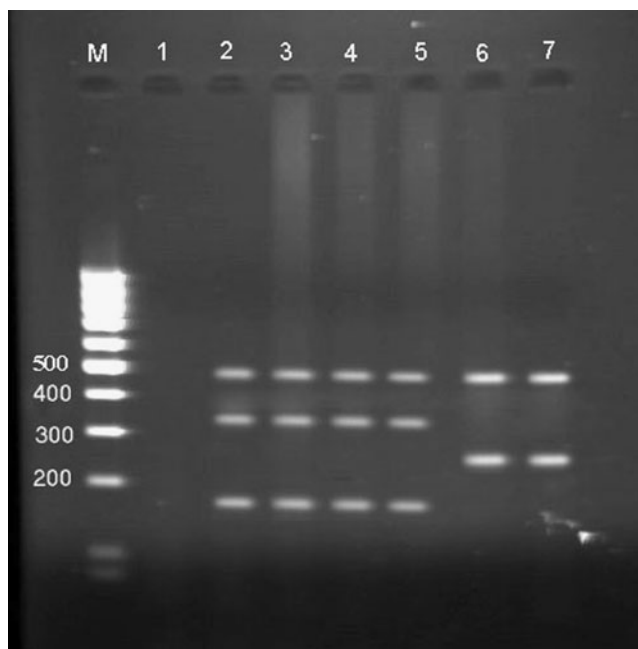
Specimens	<i>coa</i>	Degree of CMT
20	7	CMT+
45	12	CMT++
21	23	CMT+++

contained 1,250-bp DNA fragment of the 23srRNA for *S. aureus*.

From the total of 86 bacterial strains isolated in this study, 42 of them contained the *coa* gene. The results are shown in Fig. 1:

S. aureus strains isolated in this study are relevant to the clinical and sub-clinical mastitis which in the California mastitis test (CMT) test had shown positive reactions. From the total of 86 strains isolated in this study, 20 specimens were 1+ mastitis (in CMT test); 45 specimens were 2+ mastitis, and 21 specimens were 3+ mastitis. The number of *coa* gene of *S. aureus* are shown in Table 1.

Restriction fragment length polymorphism (RFLP) was used to determine the polymorphism of the coagulase gene from the 42 strains of *S. aureus* isolated in this study. Thirty-one strains contained 970 bp, and 11 strains contained 730-bp fragment relevant to *coa* gene in the PCR. After enzymatic digestion with *AluI* in the PCR,

**Fig. 2** Agarose gel electrophoresis, RFLP of the *coa* gene *S. aureus* strains isolated from bovine mastitis milk. Line M 100 bp DNA ladder, line 1 negative control, lines 2–5 genotype I *coa* gene, lines 6–7 genotype VIII *coa* gene

products indicated that 31 strains contained three bands of 490, 320, and 160 bp and 11 strains contained two bands of 490 and 240 bp. The results are shown in Fig. 2.

Discussion

The production of coagulase is an important feature used worldwide for the identification of *S. aureus*. The 3' end of the *coa* gene contains a series of 81-bp tandem repeats, which differ among *S. aureus* isolates, both in their number and in the location of *AluI* restriction sites (Goh et al. 1992). Classification based on the *coa* gene of *S. aureus* isolated has been considered a simple and accurate method for molecular typing (Goh et al. 1992; Rodrigues da Silva and da Silva 2005). Raimundo et al. (1999) reported that this technique could be used in epidemiological investigations of *S. aureus* isolates from bovine mastitis because of its high reproducibility and good discriminatory power, it is the easiest with which to analyze *coa* gene polymorphism among a large number of bacterial isolates, and it generates multiple distinct polymorphism patterns (Raimundo et al. 1999).

Among the 86 *S. aureus* strains, 31 strains contained 970 bp fragment, and 11 strains contained 730 bp fragments relevant to the *coa* gene. Following enzymatic digestion with the *AluI*, five different models were observed in the RFLP test such that 31 strains of genotype I contained three separate bands of 490, 320, and 160 bp, and 11 strains relevant to genotype VIII contained two bands of 490 and 240 bp (Fig. 2). These results indicate a considerable heterogeneity in the *coa* gene of the strains isolated in the Chaharmahal va Bakhtiari and Isfahan provinces, and as shown by Su et al. and Aarestrup et al., in each country, a special genotype is dominant (Aarestrup et al. 1995; Su et al. 2000).

Results of similar studies correspond with ours. In two studies performed by Su et al. (1999) and Aarestrup et al. (1995), 83.3% of the isolated strains in the Hatay region were relevant to type I, and 65.4% were relevant to type VIII of coagulase.

A study by Aslantas et al. (2007) from Turkey indicated greater variability of RFLP models of *coa* gene of the *S. aureus* strains isolated from bovine mastitis cases.

This variation is relevant to the bacterial pool, hard management, and environmental conditions in each geographical region. In this study, polymorphism model of the *coa* gene, as individual bands measuring 730 to 970 bp, was observed (Aslantas et al. 2007; Goh et al. 1992; Rodrigues da Silva and da Silva 2005). In samples from Brazil, a double bond from the reproduced products of *coa* gene in the strains isolated from the bovine mastitis cases has been reported (Rodrigues da Silva and da Silva 2005).

In the study performed by Karahan and Cetinkaya (2007) in Turkey, of the 200 strains of *S. aureus* isolated from 700 milk samples of cows bovine mastitis milk samples, 16 samples contained *coa* gene, which were positive following RFLP with the *AluI* and *HinGI* enzymes; 23 and 22 bands were observed in the electrophoresis model, respectively.

Saei et al. (2009) in 58 *S. aureus* strains isolated from 370 bovine mastitis milk samples reported 490 to 850 bp fragment from PCR product after digestion with *HaeIII* enzyme, 39.66% of the strains were relevant to genotype I, and 24.14% of strains were relevant to genotype VIII coagulase.

As a whole, results obtained from this study indicate that cases of mastitis in cattle husbandries in the Chaharmahal va Bakhtiari and Isfahan provinces in Iran are caused by certain strains of *S. aureus* mostly carrying a coagulase genotype and/or 1 or 2 genotypes of the *coa* gene is predominant in them. This information might help better control and managing of cases of mastitis caused by *S. aureus*.

Acknowledgements We thank Dr. A. Sharifzadeh, Dr. M. Moradi, Dr. S. Seidali, and Dr. O. Ghorbani for their cooperation. This work was supported by the Islamic Azad University, Shahr-e-kord Branch in Iran.

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