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RESEARCH ARTICLE

Investigation of Virulence Genes by PCR in *Stapylococcus aureus* Isolates Originated from Subclinical Bovine Mastitis in Turkey

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ABSTRACT

The aim of the present study was to characterize coagulase (*coa*) positive *Staphylococcus aureus* strains (n=92) isolated from bovine subclinical mastitis in Turkey by PCR amplification of clumping factor A (*clfA*) and protein A (*spa*) genes. All the *coa*-positive *S. aureus* isolates were determined to harbor the genes encoding the IgG binding region (*spa*-IgG) and the X region (*spa*-X) of *spa*. On the other hand, 84 (91.3%) isolates were positive for *clfA* gene. These three genes displayed size polymorphisms. It was concluded that *spa* gene polymorphisms for *S. aureus*, when used together with *coa*-PCR, can be proposed as good alternatives to conventional methods in typing *S. aureus* isolates of bovine origin which may provide valuable data for the development of effective control strategies against staphylococcal mastitis. The results of the present study showed that *S. aureus* isolates responsible for the mastitis cases in Turkey were genetically diverse.

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INTRODUCTION

Mastitis causes considerable economic losses to the dairy industry (Blosser, 1979). Although several bacterial pathogens can cause mastitis, *Staphylococcus aureus* (*S. aureus*) is the primary and probably the most lethal agent because it causes chronic and deep infection in the mammary glands that is extremely difficult to be cured (Miles *et al.*, 1992). It has been found responsible for more than 80% of subclinical bovine mastitis which may result in about 300 $ per year of economic losses per animal (De Graves and Fetrow, 1993; Wilson *et al.*, 1997). The main reservoir of *S. aureus* seems to be the infected quarter, and the transmission between cows usually occurs during milking (Matos *et al.*, 1991; Robertson *et al.*, 1994).

S. aureus has a capacity to produce a large number of potential virulence factors, including a variety of exotoxins and cell surface-associated proteins (Fitzgerald *et al.*, 2000; Foster, 2005; Kalorey *et al.*, 2007). This might be of significance for food hygiene especially in cases of subclinical mastitis due to *S. aureus* and might also contribute to an increased udder pathogenicity of the organisms. These factors may be of more importance than others in different diseases or at different stages of the pathogenesis of particular infections, as not all factors are produced by each strain (Annemuller *et al.*, 1999; Kalorey *et al.*, 2007). One of the major surface proteins is staphylococcal protein A (Spa), which bacterial cell wall product that binds immunoglobulin G and impairs opsonisation by serum complement and phagocytosis by polymorphonuclear leukocytes (Gao and Stewart, 2004). The decrease of protein A on the cell surface of *S. aureus* resulted in a greater number of free receptor sites for complement C3b and in an increase in phagocytosis (Gemmell and O'Dowd, 1983; Gao and Stewart, 2004). The gene encoding protein A (*spa*) is composed of some functionally distinct regions: IgG Fc binding region (*spa*-IgG), X region (*spa*-X) and at C terminus, a sequence required for cell wall attachment. The repetitive region X of the *spa* gene includes a variable number of 24-bp repeats. The number and sequence of individual repeats may differ among strains (Frenay *et al.*, 1996; Van Belkum *et al.*, 1997).

S. aureus is known to express numerous adhesions. Clumping factor A (*clfA*) is considered to be one of the most important adhesion factors of *S. aureus* to host cells. *clf*A is known to participate in the infection process by facilitating bacterial binding via soluble or immobilized fibrinogen. Because fibrinogen plays a significant role in platelet thrombus formation, it is likely that *clf*A may be involved in supporting bacterial-platelet interactions

(Mcdevitt *et al.*, 1994). This factor has been shown to inhibit phagocytosis in the absence of fibrinogen, and the inhibition was enhanced in the presence of fibrinogen. The ability of *Clf*A to inhibit phagocytosis by human PMNL may explain its importance in *S. aureus* virulence in a variety of animal models of infection (Higgins *et al.*, 2006).

At present little is known about the occurrence of these virulence genes among *S. aureus* isolates from cattle with bovine mastitis in Turkey. The aim of the present study was to characterize *coa*- positive *S. aureus* strains isolated from bovine subclinical mastitis in Turkey by Polymerase Chain Reaction (PCR) amplification of *clfA* and protein A (*spa*-IgG and *spa*-X) genes.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction

A total of 92 *coa*-positive *S. aureus* strains obtained from milk samples of 400 cows with subclinical mastitis which were collected from 50 small family farms (with the capacity of 5 to 10 cows) in Elazig and neighboring towns located in the east of Turkey between March 2002 and April 2003 were analyzed in this study (Karahan and Çetinkaya, 2007). Initially, the isolates were subcultured on Tryptic Soy Agar (TSA, Difco). Bacterial DNA was extracted using the modified method of Montanaro *et al*. (1999). A few colonies from pure cultures were transferred into a microcentrifuge tube containing 45 µl distilled water. Five microlitres of lysostaphin (Sigma, 100 µg/ml) was added and the mixture was incubated at 37 º C for 20 min. Then, 5 µl of proteinase K (Sigma, 100 µg/ml) and 150 µl of 0.1 M Tris/HCl, pH 7.5, were added to the suspension, which was incubated for a further 20 min. Finally, the suspension was heated at 95 º C for 10 min to inactive proteinase K.

PCR amplification of clumping factor (*clf*A) gene

A pair of primers specific to *clfA* gene of *S. aureus* described previously by Stephan *et al*. (2001) was used in the PCR. The PCR was performed in a Techne TC-512 gradient thermalcycler (Techne, UK) in a total reaction volume of 50 µl containing 5 µl of 10 x PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween - 20), 5 µl of 25 mM MgCl, 5 µl of 250 µM of each deoxynucleotide triphosphate, 0.25 µl of 1.25 U Taq DNA polymerase (MBI, Fermentas), 2.5 µl of 20 pmol of each primer, 5 µl of 25 ng of template DNA and 25 µl of sterile distilled water. Following the initial denaturing step at 94ºC for 1 min, a total of 35 cycles were performed; denaturing at 94ºC for 1 min, annealing at 57 ºC for 1 min and extension at 72ºC for 1 min. A last step of extension at 72ºC for 5 min was applied. A 100 bp DNA ladder (MBI Fermantes SM 321) was used as molecular weight marker to evaluate the size of bands. Reference *S. aureus* strains (ATCC25923-Department of Microbiology, Faculty of Veterinary Medicine, Firat University, Elazig) were included as positive controls and distilled water was used as negative control in all the assays. The amplified products were detected by ethidium bromide (0.5 µg/ml) staining after electrophoresis at 80 V for 2 h in 1.5% agarose gels.

PCR amplification of protein A (*spa*) gene.

A pair of primers specific to IgG region of spa gene (*spa*-IgG) of *S. aureus* described previously by Seki *et al*. (1998) was used in the PCR. The PCR mixture was prepared as described above. Following the initial denaturing step at 94ºC for 5 min, a total of 30 cycles were performed; denaturing at 94ºC for 1 min, annealing at 58ºC for 1 min and extension at 72ºC for 1 min. A last step of extension at 72ºC for 5 min was applied.

A pair of primers specific to X region of *spa* gene (*spa*-X) of *S. aureus* described previously by Frenay *et al*. (1994) was used in the PCR. The PCR mixture was prepared as described above. Following the initial denaturing step at 94ºC for 5 min, a total of 30 cycles were performed; denaturing at 94ºC for 1 min, annealing at 60ºC for 1 min and extension at 72ºC for 1 min. A last step of extension at 72ºC for 5 min was applied.

RESULTS

It was determined that 91.3% (84/92) of *coa* positive *S. aureus* isolates of bovine subclinical mastitis origin carried *clfA* gene. Sixty-eight (81.0%) of the isolates which were positive in terms of *clfA* gene was determined to form amplicons at the molecular length of approximately 1000 bp, whereas the remaining 16 isolates (19.0%) produced amplicons at the size of approximately 900 bp (Fig. 1).

Also, two different domains *spa* gene which is regarded as significant as *coa* for *S. aureus* were examined in the present study and all the isolates were determined to carry both the domains. In the PCR amplification of *spa*-IgG, most of the isolates (n=67, 72.8%) produced amplicons at the molecular length of approximately 920 bp, while the remaining 25 isolates (27.2%) formed bands at the size of approximately 750 bp (Fig. 2).

PCR products at the molecular lengths of approximately 110, 140, 170, 190, 220, 240, 270, 290 and 320 bp with 3, 4, 5, 6, 7, 8, 9, 10 and 11 repeat units were obtained in the PCR amplification of *spa*-X in the present study (Fig. 3). The isolates forming products with 10 repeat units at the size of 290 bp were determined as predominant (n=18, 19.6%). On the other hand, eight, six, five and three repeat units at the molecular lengths of 240, 190, 170 and 110 bp, respectively, were obtained from less than 10 isolates (Table 1).

Table 1: PCR amplification results of X domain of *spa* gene in *coa*-positive *S. aureus* isolates of bovine subclinical mastitis origin in eastern Turkey.

Number of Isolates (%)	Size of the amplicons (bp)	Repeat units
18 (19.6)	290	10
17 (18.4)	140	4
16 (17.4)	320	11
14 (15.2)	270	9
12 (13.0)	220	7
8 (8.7)	240	8
3 (3.3)	190	6
3 (3.3)	170	5
1 (1.1)	110	3

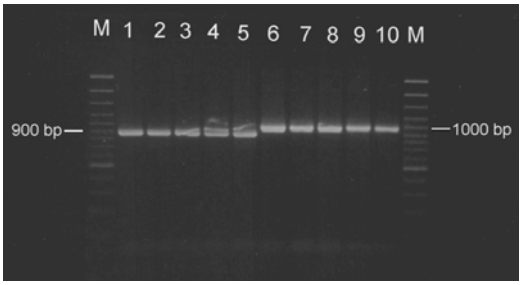

Fig. 1: Ethidium bromide-stained agarose gel electrophoresis of *clf*A PCR results in *coa*-positive *S. aureus* isolates. M: 100 bp DNA Ladder (Fermentas, SM 0321), 1-5: field isolates forming bands at the size of 900 bp; 6-10: field isolates forming bands at the size of 1000 bp.

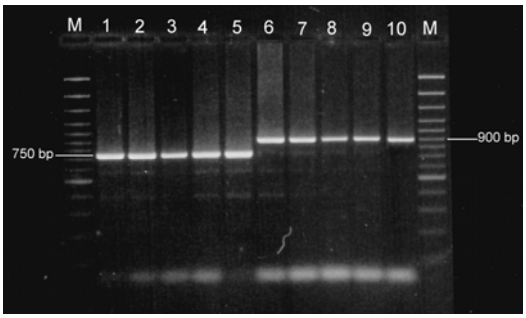

Fig. 2: Ethidium bromide-stained agarose gel electrophoresis of *spa*- IgG PCR results in *coa*-positive *S. aureus* isolates. M: 100 bp DNA Ladder (Fermentas, SM 0321), 1-5: field isolates forming bands at the size of 750 bp; 6-10: field isolates forming bands at the size of 920 bp.

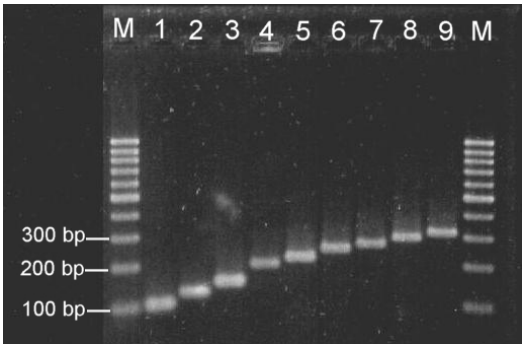

Fig. 3: Ethidium bromide-stained agarose gel electrophoresis of *spa*-X PCR results in *coa*-positive *S. aureus* isolates. M: 100 bp DNA Ladder (Fermentas, SM 0241), 1: 110 bp; 2:140 bp, 3: 170 bp 4: 190 bp 5: 220 bp, 6: 240 bp, 7: 270 bp, 8: 290 bp, 9: 320 bp.

DISCUSSION

Although most of the *coa* positive *S. aureus* isolates were determined to carry *clfA* gene, eight isolates were negative for this gene in the present study. A great majority of the previous studies reported the presence of 100% positive correlation between *clfA* and *coa* genes in the bovine *S. aureus* isolates (Akineden *et al.,* 2001; Stephan *et al.,* 2001; Salasia *et al.,* 2004; Reinoso *et al.,* 2008). However, epidemic *clfA* negative isolates of human origin has been noted (Schwarzkopf *et al.,* 1993). A study conducted by Yavuz and Esendal (2002) suggested that determining clumping factor in phenotypic terms was an indispensable test for the discrimination of *Staphylococcus* species. However, it should not be ignored that many isolates were found positive in terms of coagulase, which were, on the other hand, found negative in terms of phenotypic clumping factor (Yavuz and Esendal, 2002). Hence, determining *clfA* gene was considered to be a useful method in order to avoid possible false identification results in phenotypic tests. Amplicons at two different molecular sizes were obtained in the PCR analysis of the isolates for *clfA* gene in this study. Stephan *et al*. (2001) and Salasia *et al*. (2004) obtained only one amplicon at 1000 bp molecular length from all of the isolates they examined and they did not report any polymorphism for this gene domain. However, Akineden *et al*. (2001) and Reinoso *et al*. (2008) reported amplicons at 900-950 bp molecular length in fewer isolates as well as amplicons at 1000 bp in most of the isolates they examined. Even though sequence variances were reported in previous studies, there is still insufficient knowledge related to the polymorphism in this gene.

In the PCR amplification of *spa*-IgG, two different amplification products were determined with the one at the size of 920 bp being the most common. Likewise, in previous studies, it was reported that the isolates with the PCR product at the size of 920 bp were predominant (Stephan *et al.,* 2001; Salasia *et al.,* 2004). On the other hand, Reinoso *et al*. (2008) reported that while the isolates originating from cattle mastitis produced amplicons at 920 bp, human isolates formed PCR products at the size of 750 bp. When the results of this and previous studies are evaluated together, it can be stated that there is a relationship between *S. aureus* isolates of bovine and human origin. This situation may stem from a contamination of milking workers during the milking process. Seki *et al*. (1998) reported that the isolates forming amplicons at the size of 920 bp had five repeat units, whereas amplification products at the size of 750 bp had four repeat units. Because IgG binding region of *spa* is comprised of 170 bp domains, one IgG domain was considered to be missing in the isolates forming amplicons at the molecular length of 750 bp in this study.

S*pa*-X has been regarded as an appropriate target gene domain for determining the differences between *S. aureus* isolates in a short time (Frenay *et al*., 1996; Annemuller *et al*., 1999; Lange *et al*., 1999; Reinoso *et al*., 2008). PCR products at nine different molecular size with various repeat units were noted in the PCR amplification of *spa*-X with 10 repeat units at the size of 290 bp being the most common in the present study. The number of repeat units may reveal the associations between the isolates of different origins or different geographical locations. Frenay *et al*. (1994) reported an association between the potential distribution of *S. aureus* and the number of repeat units. They defined the isolates with more than seven repeat units as epidemic isolates and with seven or fewer repeat units as non-epidemic isolates. The present study revealed that field isolates with more than seven repeat units were more prevalent in eastern Turkey with 56 (60.9%) of the 92 cattle mastitis isolates. Similar results were reported by other researchers from Turkey as well (Kuzma *et al*., 2005). The finding that

eight isolates which were negative for *clfA* but positive for S*pa* may also bring into mind the possibility of deletion in the *clfA* gene in some isolates those differed from most of the isolates.

General comparison of the virulence genes examined in this study showed that the isolates forming approximately 900 bp amplicons for *clfA* gene was observed to produce amplicons at the size of 750 bp for IgG domain and at the size of 140 bp with four repeat units for X domain of *spa* gene, while they produced an amplicon at the size of 950 bp with the exception of only one isolate in the *coa*-PCR (Karahan and Çetinkaya, 2007). This finding may be significant to enlighten the reasons of polymorphism in *clfA* gene, which yet to be explained, in future studies.

In conclusion, C*lf*A, *spa*-IgG and *spa*-X, which are important virulence genes, were observed to be present in *coa*-positive *S. aureus* isolates obtained from dairy cows with subclinical mastitis in eastern Turkey. The PCR amplification results of X domain of *spa* gene indicated that it can be proposed as an alternative method to *coa*-PCR in order to gain detailed information about the distribution of Stapylococcal isolates.

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