

## Coagulase Gene Polymorphism of *Staphylococcus aureus* Isolated from Subclinical Bovine Mastitis\*

Özkan ASLANTAŞ<sup>1,\*</sup>, Cemil DEMİR<sup>1</sup>, Hülya TÜRÜTOĞLU<sup>2</sup>, Zafer CANTEKİN<sup>1</sup>, Yaşar ERGÜN<sup>3</sup>, Gökhan DOĞRUER<sup>3</sup>

<sup>1</sup>Department of Microbiology, Faculty of Veterinary Medicine, Mustafa Kemal University, Hatay - TURKEY

<sup>2</sup>Department of Microbiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur - TURKEY

<sup>3</sup>Department of Gynaecology and Obstetrics, Faculty of Veterinary Medicine, Mustafa Kemal University, Hatay - TURKEY

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**Abstract:** This study was conducted to investigate the coagulase gene polymorphism of *Staphylococcus aureus* isolated from subclinical bovine mastitic milk samples from 3 different Turkish cities (Hatay, Gaziantep, and Burdur). Amplification of the coagulase gene from 80 *S. aureus* isolates produced 5 different polymerase chain reaction (PCR) products; 79 isolates showed only 1 amplicon, and 1 isolate showed 2 amplicons. The isolates were grouped into 9 genotypes by analysing the restriction fragment length polymorphism (RFLP) of the gene; the 2 most common genotypes accounted for 73.8% of the isolates. The results indicated that many coagulase gene genotypes are present in the studied regions and only 1 or 2 genotypes predominated.

**Key Words:** *Staphylococcus aureus*, RFLP-PCR, mastitis

### Subklinik İnek Mastitis'lerinden İzole Edilen *Staphylococcus aureus* Suşlarının Koagulaz Gen Polimorfizmi

**Özet:** Bu çalışma, üç farklı şehirden (Hatay, Gaziantep ve Burdur) toplanan mastitisli sığır sütlerinden izole edilen 80 *Staphylococcus aureus* suşunun koagulaz gen polimorfizmini araştırmak amacıyla yapıldı. Seksen *S. aureus* suşu 5 farklı polymerase chain reaction (PCR) ampliconu oluştururken, 79 izolat 1 amplicon ve 1 izolat 2 amplicon oluşturdu. İzolatlar, koagulaz geninin restriction fragment length polymorphism (RFLP) analizi ile 9 genotipe ayrıldı. İki yaygın genotip izolatların % 73,8'ini oluşturdu. Sonuçlar, çalışılan bölgelerde farklı genotiplerin olduğunu ve bunlardan sadece bir veya iki genotipin dominant olduğunu göstermektedir.

**Anahtar Sözcükler:** *Staphylococcus aureus*, RFLP-PCR, mastitis

### Introduction

Although several bacterial pathogens can cause mastitis, *Staphylococcus aureus* is considered the most important causative agent involved in all forms of bovine mastitis (1). From the epidemiological point of view, it is important to determine the origin of micro-organisms involved in the aetiology of the disease. Considerable genetic heterogeneity has been shown in natural populations of *S. aureus* isolates (2,3); therefore, numerous molecular techniques have been developed and used for identification and comparison of *S. aureus* isolates in epidemiological studies. Among these methods, coagulase gene typing is considered a simple and effective

method for typing *S. aureus* isolates from human patients and bovine mastitic milk (4-12). Epidemiological studies based on analysis of the coagulase (*coa*) gene have shown that *S. aureus* isolates could be divided into a number of subtypes, but only a few are responsible for most cases of bovine mastitis in different geographical areas (9,13).

In Turkey, little information is available about the genetic diversity of *S. aureus* isolated from cows with subclinical 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 3 different cities in Turkey.

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\*\* E-mail: aslantas@mku.edu.tr

## Materials and Methods

### Bacterial isolates

A total of 80 *S. aureus* isolates from Hatay (n = 30), Gaziantep (n = 26), and Burdur (n = 24) were isolated from subclinical bovine mastitic milk samples. 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, haemolysis, 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 (4). Bacterial cell lysate was prepared from 0.5 ml of overnight trypticase soy broth (TSB) cultures. After centrifugation at 12,000 ×g for 10 min, bacterial pellets were washed with 500 µl 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 resuspended in 200 µl of TE buffer (pH 7.5) with 15 U of lysostaphin (2 mg, Sigma, Turkey) per millilitre, and incubated at 37 °C for 1 h. Next, 15 µl of proteinase K (20 mg ml<sup>-1</sup>) (Fermentas, Turkey) 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 ×g for 10 min. The upper phase was carefully transferred into another Eppendorf tube and mixed with 2 volumes of 95% ethanol and stored overnight at -20 °C. The mixture was then centrifuged at 12,000 ×g for 5 min. The DNA pellet was washed with ice-cold 70% ethanol, recentrifuged, and dried by tube inversion. The DNA was suspended in 100 µl of sterile TE, quantified in a spectrophotometer (at 260 nm), and kept frozen at -20 °C.

### PCR amplification

PCR was performed in a 50-µl reaction mixture containing 1-2 µl of template DNA (approximately 500 ng/µl), 5 µl of 10× PCR buffer (750 mM Tris HCl (pH 8.8) 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 0.1% Tween 20), 200 µM of each of the 4 deoxynucleotide triphosphates, 1 U of *Taq* DNA polymerase (MBI Fermentas), 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 Hybaid thermocycler (Hybaid, UK) 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 (12).

### Restriction enzyme digestion

The PCR products were digested with *AluI* (MBI Fermentas) for restriction analysis. For this aim, 12.5 µl of PCR products was mixed with 10 U of enzyme and 10× 1.5 µl restriction buffer, and then incubated at 37 °C overnight.

### Agarose gel electrophoresis

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

### Specificity testing

The DNA of *S. epidermidis* ATCC 12228 and *S. aureus* ATCC 25923 was analysed in order to test the specificity of the primer pair.

### Reproducibility testing

To test PCR reproducibility, 5 randomly selected isolates were tested for 5 consecutive days. RFLP (restriction fragment length polymorphism)-PCR reproducibility was tested by twice submitting 4 different PCR products to *AluI* digestion.

### Data analysis

The size of the PCR and RFLP products were estimated by constructing a standard curve using the molecular weight standard and the distance migrated by each in the gel. Numeric codes were assigned to the PCR genotypes and RFLP patterns (Table).

## Results

Most of isolates (98.7%) produced a single band, with molecular sizes ranging from 730-1050 bp, whereas only 1 isolate yielded 2 amplification products (1.3%) (Figure 1). The products 730 and 970 bp in size

Table. Coagulase genotypes of *S. aureus* strains isolated from cow's milk.

Genotype code	PCR products (bp)	RFLP patterns (bp)	n
I	970	490-320-160	33
II	810	410-240-160	7
III	810	490-240-80	1
IV	810	490-240-160	1
V	890	410-240-160-80	2
VI	810+1050	490-410-320-160	1
VII	890	490-410	4
VIII	730	490-240	26
IX	730	410-320	5

were the most frequent and accounted for 38.8% and 41.3% of the isolates, respectively.

As summarised in the Table, *AluI* restriction enzyme digestion of the PCR products generated 9 different *AluI* restriction patterns (Figure 2). Types I and VIII were the most common, and accounted for 73.8% of the isolates. The number of *AluI* RFLP patterns according to PCR product and genotype frequency is shown in the Table. The amplicons of 730, 810, 890, 970, and 810+1050 bp generated different quantities of fragments, varying from 2 to 4, and their sizes were approximately 80-490 bp.

There was no amplification product of the DNA from *S. epidermidis*. Reproducibility of the PCR products was demonstrated with 100% of the tested isolates. The types according to city are shown in Figure 3. In Hatay, type I was the most frequent, and accounted for 83.3% of the isolates. In Gaziantep, type VIII was the most frequent, and accounted for 65.4% of the isolates. In Burdur, types I and VIII were the most frequent, and accounted for 29.2% and 33.3%, respectively.

### Discussion

The production of coagulase is an important phenotypic 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 (12). Classification based on RFLP of the *coa* gene of *S. aureus* isolates has been considered a simple and accurate method for molecular typing (4-12). Raimundo et al. (7) reported that this technique could be used in epidemiological investigations of *S. aureus* isolated from bovine mastitis because of its high reproducibility and good discriminatory power, it is the easiest with which to analyse *coa* gene polymorphism among a large number of bacterial isolates, and it generates multiple, distinct polymorphic patterns. In this study, we detected 5 *coa* PCR types and 9 *AluI* RFLP patterns, which indicate considerable heterogeneity in the *coa* gene of *S. aureus* in the studied regions.

Su et al. (9) and Aestrup et al. (13) showed that a few genotypes predominated in each country, and the predominant *coa* genotypes were more resistant to

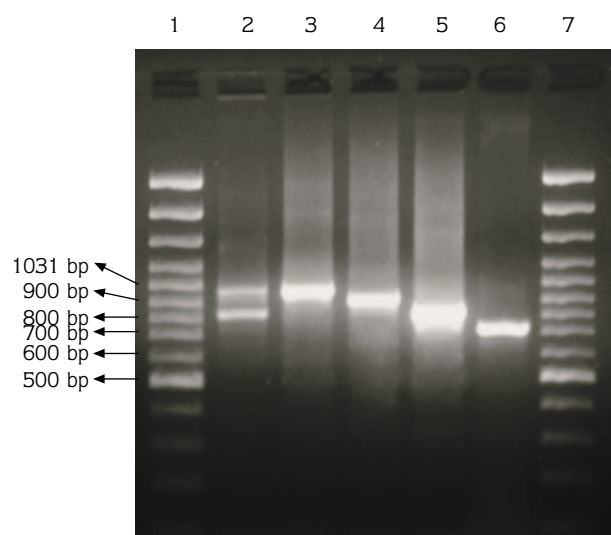


Figure 1. Agarose gel electrophoresis of *S. aureus* PCR products. Lane 1: 100 bp molecular weight standard. Lanes 2-6: Different sizes of *S. aureus* coagulase PCR products. Lane 7: 100 bp molecular weight standard.

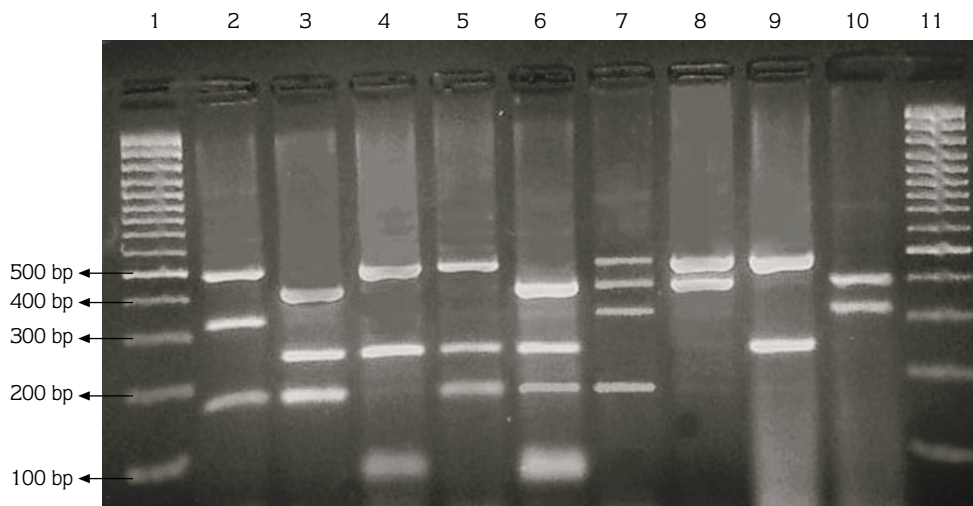


Figure 2. Agarose gel electrophoresis of *AluI* restriction fragments of PCR products. Lane 1: 100 bp molecular weight standard. Lanes 2-10: RFLP patterns obtained after treatment of coagulase PCR products with *AluI* endonuclease. Lane 2: Type I (fragments: 490, 320, and 160 bp). Lane 3: Type II (fragments: 410, 240, and 160 bp). Line 4: Type III (490, 240, and 80 bp). Line 5: Type IV (fragments: 490, 240, and 160 bp). Line 6: Type V (fragments: 410, 240, 160, and 80 bp). Line 7: Type VI (fragments: 490, 410, 320, and 160 bp). Line 8: Type VII (fragments: 490 and 410 bp). Line 9: Type VIII (fragments: 490-240 bp). Line 10: Type XIX (fragments: 410-320 bp). Lane 11: 100 bp molecular weight standard.

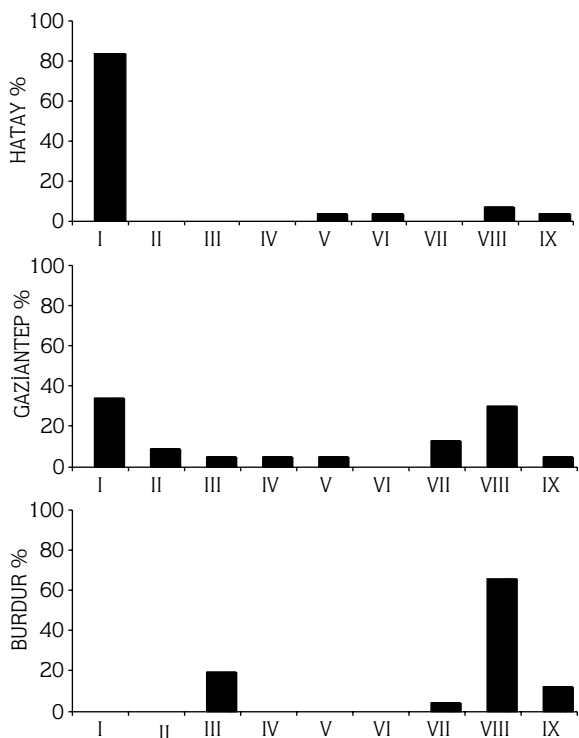


Figure 3. Frequency of coagulase genotypes in strains of *S. aureus* isolated from Hatay, Gaziantep, and Burdur.

neutrophil phagocytosis and death than those with the rare genotypes. In this study, the most common genotype in Hatay was type I, which accounted for 83.3% of the total genotypes. In Gaziantep, type VIII was the most common genotype, accounting for 65.4% of the total, whereas, types I and VIII were the most common genotypes in Hatay, accounting for 29.2% and 33.3%, respectively. This distribution may be explained by the co-evolution of the pathogens and their host, as well as by differences in reservoirs, herd management, and the environment of each geographical area. These characteristics also indicate the necessity to know the ecological and epidemiological profile of a specific region and herd before applying mastitis control measures (9).

Until recently, single banded *coa* PCR products were reported in *S. aureus* strains isolated from bovine milk samples; however, double-banded amplification products have also been reported recently from *S. aureus* strains derived from bovine mastitis in Brazil (4). In the current study, double-banded amplification product was detected only in 1 *coa* positive isolate. Goh et al. (12) explained the presence of double-banded amplification products with different allelic forms of the *coa* gene.

In conclusion, this study has shown that mastitis in the studied regions was caused by *S. aureus* strains harbouring more than one *coa* genotype, and that only 1 or 2 genotypes predominated. However, further studies using a large collection of strains should be conducted to

determine the common characteristics of the predominant strains. The information gathered could be used to develop more efficient staphylococcal mastitis control programmes.

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