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RESEARCH ARTICLE

Identifying the Bacteria Causing Ovine Gangrenous Mastitis and Detection of *Staphylococcus aureus* in Gangrenous Milk by PCR

Osman Yaşar TEL * Faruk BOZKAYA **

* Harran University, Faculty of Veterinary Medicine, Department of Microbiology, TR-63200 Sanliurfa - TURKEY
** Harran University, Faculty of Veterinary Medicine, Department of Genetics, TR-63200 Sanliurfa - TURKEY

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Summary

The objective of this study was to identify bacteria causing gangrenous ovine mastitis as well as to detect and genotype *Staphylococcus aureus* in milk samples by using polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) of coagulase gene. A total of 264 milk samples were collected from Awassi sheep with gangrenous mastitis raised in Sanliurfa province of Turkey. By using bacteriological examination *Staphylococcus aureus* (75%), *Mannheimia haemolytica* (7.2%), coagulase negative staphylococci (6.43%), *Escherichia coli* (4.55%), *Corynebacterium* spp. (0.76%) and *Actinomyces pyogenes* (0.76%) were identified. Bacterial DNA was isolated from 155 randomly selected milk sample. By PCR amplification of coagulase gene *S. aureus* was detected in 91.6% of the milk samples. Three different amplicons of 420 (0.7%), 580 (65.3%) and 780 (34%) bp band sizes, each showing a specific RFLP profile were observed. In conclusion, *S. aureus* was found to be mostly responsible for gangrenous mastitis in sheep raised in this region. *S. aureus* in milk samples could be more rapidly detected by PCR method. Two coa genotype of *S. aureus were* found to be common suggesting that limited number of strains were responsible for most cases of gangrenous mastitis in the region under the study.

Keywords: *Coa gene, Gangrenous mastitis, PCR, Sheep Staphylococcus aureus*

Koyunlarda Gangrenöz Mastitise Neden Olan Bakterilerin İdentifikayonu ve Gangrenli Sütlerdeki *Staphylococcus aureus*'un PCR ile Tespit Edilmesi

Özet

Bu çalışmanın amacı, koyun gangrenöz mastitisine neden olan bakterilerin identifiye edilmesi ve süt örneklerindeki *Staphylococcus aureus*'un polimeraz zincir reaksiyonu (PCR) ile tespit edilerek, koagülaz geni yönünden restriksiyon fragman uzunluğu polimorfizminin (RFLP) belirlenmesidir. Bu amaçla Şanlıurfa ilinde yetiştirilmekte olan gangrenöz mastitisli İvesi koyunlarından 264 adet süt örneği toplanmıştır. Bakteriyolojik incelemede *Staphylococcus aureus* (%75), *Mannheimia haemolytica* (%7.2), koagulaz negatif stafilakok (%6.43), *Escherichia coli* (%4.55), *Corynebacterium* spp. (%0.76) ve *Actinomyces pyogenes* (%0.76) identifiye edildi. Tesadüfi olarak seçilen 155 süt örneğinden bakteriyel DNA izole edildi. Koagulaz geni kullanılarak yapılan PCR amplifikasyonu ile örneklerin %91.6'sında *S. aureus* tespit edildi. Herbiri spesifik RFLP profili gösteren 420 (%0.7), 580 (%65.3) ve 780 (%34) baz çifti uzunluğunda 3 farklı PCR ürünü gözlendi. Sonuç olarak, *S. aureus*'un, bölgede yetiştirilen koyunlarda gangrenöz mastitislerden büyük oranda sorumlu olduğu bulunmuştur. *S. aureus*'un süt örneklerinden izole edilen DNA kullanılarak PCR metoduyla kolaylıkla tespit edilebileceği, iki coa genotipinin yaygın olduğu ve sınırlı sayıda suşun bölgedeki gangrenöz mastitis olgularının çoğundan sorumlu olduğu kanaatine varılmıştır.

Anahtar sözcükler: *Coa gen, Gangrenöz mastitis, Koyun, PCR, Staphylococcus aureus*

INTRODUCTION

Mastitis, which is caused by several etiological agents is considered one of the most important diseases of sheep. *S. aureus* is recognized as the most common aetiological agent of ovine mastitis, followed by minor occurrence of

İletişim (Correspondence)
+90 414 3183918
oyasar@gmail.com

Mannheimia (Pasteurella) haemolytica, *Escherichia coli*, *Clostridium perfringens*, *Streptococcus*, *Pseudomonas* and *Nocardia* genera [1,2]. In sheep production, mastitis has a major economical impact due to reduced milk yield and quality as well as increased use of drugs and veterinary services [3]. Mastitis also negatively effects animal welfare. The annual incidence of clinical mastitis in dairy sheep is generally lower than 5%, but in some herds the incidence may exceed 50%, causing mortality or culling of up to 70% of individuals with gangrenous mastitis [4].

Rapid detection of pathogens causing mastitis is important to control the rate of the infection and reduces the time required for return to normal milk production, thereby enhances animal welfare when coupled with appropriate antimicrobial therapy [5]. Currently, the mammary gland pathogens are identified by in vitro culture, which is considered the "gold standard". However, this technique requires two or three days for growing, isolation and identification of the pathogen, therefore is labor intensive and time consuming [6]. Polymerase chain reaction (PCR) based molecular methods have been demonstrated to provide very efficient tools for identification of pathogens in human and different animal species. By means of PCR technique, it is possible to rapidly and easily detect nanograms of pathogens' DNA in animal products or tissues with high specifity and sensitivity [6,7]. Numerous molecular techniques have been used for identification of *S. aureus* isolates in epidemiological studies [8]. Coagulase gene typing has been proven to be a simple and effective tool for identifying and genotyping of coagulase positive *S. aureus* isolates from both human and animal sources [9-11]. Specifity and sensitivity of PCR-based detection methods have been reported to be 100% and up to 10^2 colony forming unit/mL, respectively [12-14]. The amplified DNA fragments of different sizes can be further discriminated by digestion with restriction enzymes such as *Alu*I [15]. PCR -based genotyping method provides a more detailed epidemiological information on *S. aureus* [16,17].

Numerous studies have been carried out on the molecular epidemiology of subclinical and clinical mastitis in sheep [18,19]. However only two studies have been reported on the molecular epidemiology of *S. aureus* isolated from subclinical and ganrenous ovine mastitis in Turkey [20,21]. To our knowledge no information is available on molecular epidemiology of *S. aureus* isolated from sheep with gangrenous mastitis in Turkey. Therefore the objective of this study was to identify bacteria in milk samples from sheep with gangrenous mastitis in Sanliurfa province of Turkey. In addition, the study was carried out to detect *S. aureus* in milk samples of sheep with gangrenous mastitis by using coagulase gene based PCR assay and to compare the results with those of bacteriological examination. Amplified fragments of coagulase gene were digested with *Alu*I for further genotyping in order to determine variations among isolates.

MATERIAL and METHODS

Collection of Milk Samples

A total of 264 milk samples were collected from Awassi sheep with gangrenous mastitis between 2009-2010 in seven different flocks each consisting of 3500-4000 dairy sheep in Sanliurfa, Turkey. All flocks were grazed, with some additional concentrate during spring, summer, and fall. Ewes lambed between December and January, and lambs were kept with their dams for 6-8 weeks. Clinical examinations were performed during full lactation between December and April, when mastitis was most frequently observed. Gangrenous mastitis was diagnosed with clinical examination by observing a cold and blue colored skin of udder and teat as well as presence of blood in the milk sample. A minimum of 5 mL of milk sample was collected from one affected udder lobe in a sterile container, after cleaning the teat with denatured 90% ethanol and discarding the first milk drops. Samples were subjected to microbiological examination in the laboratory within 24 h after collection.

Bacteriological Examination

Milk samples were cultured on 5% defibrinated sheep blood agar and MacConkey agar (Oxoid, Hampshire, England), incubated under aerobic conditions at 37°C for 48 h. Presence of less than five colonies of the same type on the plates was considered contaminant and these colonies were not used for further bacteriological examination while colonies of the same type observed more than five were considered infective agent and used for furthe bacteriological examination [22]. Microorganisms isolated were identified based on macro and microscopical morphology by Gram staining, culture characteristics and biochemical profile [23]. The staphylococcal isolates were identified as *S. aureus* and coagulase negative staphylococci (CNS) by using catalase, tube coagulase, and fermentation test for acid production from glucose, mannitol, and maltose. *Escherichia coli* was identified by catalase, oxidase, growth on MacConkey agar, IMVIC test and metallic sheen on EMB agar. *Corynebacterium* spp. were identified by nitrate reduction, urease activity and the presence of hemolysis on blood agar. *Mannheimia haemolytica* was identified by using the CAMP reaction, the indole, mannitol, sorbitol, trehalose, dulcitol, oxidase and beta-galactosidase tests and presence of haemolysis on blood agar. *Arcanobacterium pyogenes* was identified by Gram staining and the presence of pinpoint colonies surrounded by a narrow zone of clear haemolysis at 48 h.

DNA Extraction of Bacterial DNA from Milk

A total of 155 randomly selected milk samples were used for DNA extraction. One mL of each sample was transferred into a microtube and centrifuged at 14.000 rpm for 4 min. The supernatant was discarded and the pellet

was resuspended and washed 2-3 times with TES buffer (Tris-HCL 10 mM; EDTA 1 mM, SDS 1% (w/v), pH 8.0) until a clear solution was obtained. Pellets were resuspended in 500 µL of TES buffer and 0.3 mg of proteinase K (Fermentas Life Sciences, Burlington, Ontario, Canada) was added. The samples were incubated on a water bath at 56°C for 2-3 h. DNA was extracted using phenol/chloroform method, followed by an isopropanol precipitation described by Sambrook *et al.*[24]. DNA pellets were finally solved in 100 µL of TE buffer (10 mM Tris-Hcl, 1 mM EDTA, pH 8,0).

Polymerase Chain Reaction Assay (PCR) and Restriction Fragment Length Polymorphism Analysis (RFLP)

Coagulase gene typing was carried out by using the procedure described by Hookey *et al.*[16]. The 3'-end region of the *coa* gene was amplified with use of the following primer pairs: 5'-ATAGAGATGCTGGTACAGG-3'(bases 1513-1531); and 5'-GCTTCCGATTGTTCGATGC-3'(bases 2188-2168). The PCR reaction was carried out in 25 µL of reaction volume containing 2.5 µL of 10X reaction buffer, 0.6 µM of each primer, 60 µM of each dNTP, 3 mM $MgCl_2$, 1,25 U of Taq- DNA polymerase (Fermentas, Vilnius, Lithuania) and 2 µL of extracted DNA. As positive control *S. aureus* ATCC 29213 was used. As template for negative control sterile water was added. Amplification was performed with a thermocycler (Takara, Otsu, Japan) by using the following program: 45 s at 94°C, followed by 30 cycles of 20 s at 94°C, 15 s at 57°C, and 15 s at 70°C with a final step at 72°C for 2 min. Amplified products were separated by 2% agarose gel electrophoresis stained by ethidium bromide and visualized by using a UV transilluminator.

For RFLP analysis 10 µL of PCR products were digested with *Alu*I (Fermentas, Vilnius, Lituania) according to instructions of the manufacturer. Fifteen microliters of the digestion products were electrophoresed on 2% agarose gel, and visualized under UV illumination.

The sensitivity of PCR was tested by using 10-fold serial dilutions (CFU/mL) of a *S. aureus* strain isolated in UHT milk. Specificity of the PCR was checked by using DNA isolated from the strains of CNS, *M. haemolytica* and *E. coli* isolated in this study.

RESULTS

Bacterial Identifications

A total of 264 milk samples was subjected to bacteriological examination, and 250 (94.7%) samples showed bacterial growth while 14 (5.3%) samples showed no bacterial growth on blood agar. The bacteria species isolated and identified from these milk samples are shown in *Table 1*.

Results of PCR

The detection limit of the PCR assay was $6x10^2$ cfu/mL of *S. aureus*. All strains used for the specificity of the PCR test gave negative results. Amplification of the *coa* gene yielded a single product in 141 of 155 (91%) milk samples, while no amplification product was obtained in 14 samples (9%) *(Fig. 1)*. All samples from which only *S. aureus* was isolated with bacteriological examination (109 of 155 samples) yielded a PCR amplicon for coagulase gene. On the other hand a PCR product was obtained in 32 of 46 samples from which *S. aureus* was not isolated. There were no culture positive samples that were negative by using PCR method. By using PCR amplification of *coa* gene *S. aureus* was detected in 29.3% further samples in comparison to bacteriological examination. Furthermore by using PCR *S. aureus* was detected in 27 of 37 samples (73%) from which other bacteria were isolated *(Table 2)*.

The three types of PCR products observed were as follows: A (420±20 bp, n=1) B (580±20 bp, n=92), C (780±20 bp, n=48) *(Fig. 2)*. Three distinct RFLP patterns were observed among the 141 isolates examined after *Alu*I digestion of the PCR products. Only PCR amplicons of 780 bp band size were cutable giving three distinct bands, while the amplicons of 420 and 680 bp band size were not cutable with *Alu*I giving only one band *(Table 3)*.

Table 1. *Bacteria isolated by using bacteriological examination and samples positive for S. aureus by PCR of coa gene*
Tablo 1*. Bakteriyolojik incelemede izole edilen bakteriler ve PCR yöntemiyle tespit edilen S. aureus pozitif örneklerin sayısı*

Species	Number of Isolates (Frequency %)	Samples Used for PCR	Samples Positive for *S. aureus* by PCR
S. aureus	198 (75)	109	109
M. haemolytica	19 (7.2)	17	12
CNS	17 (6.43)	5	5
E. coli	12 (4.55)	10	9
Corynebacterium spp.	2 (0.76)	2	1
A. pyogenes	2 (0.76)	2	0
No growth	14 (5.3)	10	5
Total	264	155	141

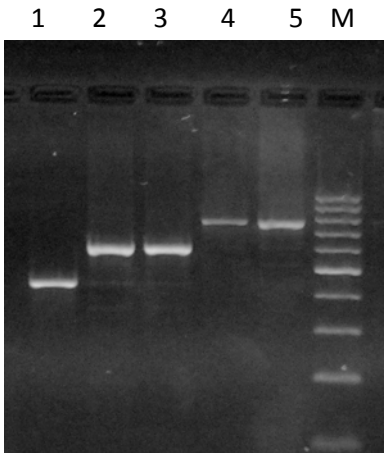

Fig 1. Coagulase gene PCR products of *S. aureus* DNA isolated from milk samples. Lane 1, type A; lanes 2-3, type B; lanes 4-5, type C, lane M, Molecular marker (100 bp ladder)

Şekil 1. Süt örneklerinden izole edilen *S. aureus* DNA'sına ait koagulaz geni PCR ürünleri. 1, tip A; 2-3, tip B; 4-5, tip C, M, Moleküler markör (100 bp ladder)

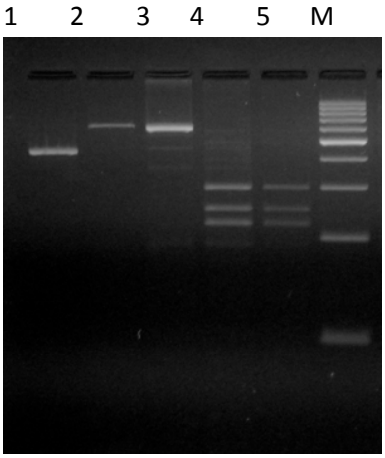

Fig 2. Restriction fragments of PCR products generated by cutting with *Alu*I. Lane 1, profile I; lanes 2-3, profile II; lane 4-5, profile III; lane M, molecular marker (100 bp ladder)

Şekil 2. PCR ürünlerinin *Alu*I ile kesimi sonucu ortaya çıkan fragmentler. 1, profil I; 2-3, profil II; 4-5, profil III; M, moleküler markör (100 bp ladder)

Table 2. *Comparison of bacteriological and PCR examination*
Tablo 2*. Bakteriyolojik inceleme ve PCR sonuçlarının karşılaştırılması*

PCR Examination	Bacteriological Examination		
	***S. aureus* Positive**	***S. aureus* Negative**	**Total (Frequency)**
PCR positive	109	32	141 (91%)
PCR negative	0	14	14 (9%)
Total	109 (70%)	46 (30%)	155 (100%)

Table 3. *PCR and RFLP patterns of coagulase gene of S. aureus*
Tablo 3*. S. aureus koagulaz geninin PCR ve RFLP profilleri*

Coagulase Genotype				No. of Samples	Frequency (%)
PCR Type Code	(Approx. bp)	RFLP Profile	(Approx. bp)		
A	420±20	I	420±20	1	0.7
B	580±20	II	580±20	92	65.3
C	780±20	III	220+250+300	48	34

DISCUSSION

Ovine mastitis is an endemic problem which is hardly treated and controlled. To determine the appropriate treatment and control measures it is important to rapidly detect the aetiological agents of the disease.

S. aureus has been reported to be the most common aetiological agent causing clinical mastitis (29-65% of isolated bacteria) while CNS (3-19%), *E. coli* (3-19%) and *M. haemolytica* (1.5-4.3%) have also been less frequently observed [2,25-27]. In the present study also *S. aureus* was found to be the most frequently isolated species while other bacteria species such as *M. haemolytica*, CNS and *E. coli* was isolated less frequently in agreement with those reported by other researchers [2,25,28]. In the present study no growth of bacteria could be detected in 5.3% of the samples by using bacterial examination. Culture of bacteria in milk samples from infected animals may yield no growth due to low numbers of bacteria, the presence of leukocytes and residual therapeutic antibiotics inhibiting the growth of bacteria [6,7].

Production of coagulase is an important phenotypic feature used worldwide to identify *S. aureus* [29,30]. Amplification of coagulase gene by PCR has been used for identification and genotyping of *S. aureus* isolated from different animal species with mastitis [12,31]. By means of PCR amplification of *coa* gene *S. aureus* was detected in all samples assigned as *S. aureus* positive (n=109) and in 32 milk samples assigned as *S. aureus* negative with bacteriological examination. Therefore by using PCR method *S. aureus* was detected in 29.3% more samples compared to bacteriological examination. By using PCR *S. aureus* was

detected in 73% of the samples from which other bacteria were isolated, suggesting a mixed infection caused by *S. aureus* and other bacteria. A high specificity of the PCR method has also been reported by several authors [12,16]. The sensitivity of the PCR method used in this study was comparable to those reported by Kumar [32] and Riffon *et al.*[6].

Three different PCR amplicons having band sizes of 420, 580 and 780 bp were observed. The amplicon of 420 bp band size was observed in one sample. Amplicons of similar band sizes have also been reported by other authors [18,20,28,33]. However, several researchers have observed four [18], to nine [20] different PCR amplicons of *S. aureus* strains isolated from sheep with mastitis. Moroni *et al*.[19] have found only a single amplicon of 760 bp of *coa* gene in *S.aureus* from milk of alpine goats. Da Silva *et al.*[34] reported 10 amplicons of different sizes ranging from 480 to 1080 bp for *S. aureus* isolates from goats with mastitis in Brazilian dairy herds. In the present study, 580 and 780 bp amplicons were observed for 99% of the isolates included into PCR amplification. In different studies amplicons of similar fragment lengths (580±20 and 820±20 bp) were accounted for 67% [18] and 88.9% [31] of *S. aureus* strains isolated from bovine mastitic milk. However prevalence of amplicons of similar fragment lengths varied from 10.2% [33] to 20% [18] of *S. aureus* strains isolated from sheep milk or milk products. On the other hand amplicon of 660 bp fragment length has been reported to be the most frequent one in sheep [18,33]. Different RFLP patterns of *S. aureus* strains have been reported by several researchers [18,31,33]. The RFLP pattern of type B, which was not cutable by *Alu*I in the present study was similar to type B1 found by Katsuda *et al.*[31] in cows. The frequency of the type B (65.3%) in this study was comparable to that found by Katsuda *et al.*[31] for the type B1 (59.6%) which had the same fragment length as the type B in this study.

The results indicated a predominance of two *coa* genotypes, suggesting that limited number of closely related *S. aureus* strains were responsible for most cases of gangrenous mastitis in the region under the study. These findings are consistent with those reported about bovine mastitis by other researchers who have reported the presence of a limited number of predominated types within each herd and country [10,11,35]. The predominance of the two coagulase genotypes of *S. aureus* observed in this study might be due to their resistance to environmental conditions or immun response of the host. Su *et al.*[11] have reported that predominant genotypes of *S. aureus* are more resistant to neutrophil killing than the rare types.

In conclusion, *S. aureus* was found to be mostly responsible for gangrenous mastitis in sheep raised in the region under the study. The presence of *S. aureus* in the milk samples from sheep with gangrenous mastitis could be detected more rapidly by PCR than bacteriological examination studied. By using PCR and RFLP method two *coa* genotype of *S. aureus* found to be common in the region studied suggesting that limited number of closely related strains were responsible for most cases of gangrenous mastitis in the region under the study. Further studies are required to determine the epidemiological distribution of different *S. aureus* strains in milk samples collected from sheep with gangrenous mastitis in other regions of Turkey. Investigation on the distribution of *S. aureus* strains with different *coa* genotypes isolated from subclinical and clinical mastitis cases might give valuable information on the epidemiology of gangrenous mastitis in the region.

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