Antimicrobial Resistance and Plasmid Typing of Coagulase Positive Staphylococcus Species Isolated from Bovine Mastitis*

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Abstract: In this study, a number of 50 Coagulase positive *Staphylococcus* isolates has been isolated and identified using conventional bacteriological methods from bovine mastitis. Of the total isolates 40 was identified as *Staphylococcus aureus* and 10 was identified as *Staphylococcus intermedius*. This study indicates the increasing significance of *S.intermedius* as the causative agents of bovine subclinical mastitis. All the *Staphylococcus* strains were examined for their susceptibilities to antibiotics and plasmid profiles. Plasmid profiling demonstrated that 38 of 40 *S.aureus* and 9 of 10 *S.intermedius* isolates contained plasmid. Molecular weight of plasmids varied from 1.1 to 19 kb. The highest resistance to chematerapotics used was observed to penicillin. This study suggests that plasmid profile analysis does not appear to be a suitable method for typing *S.aureus* and *S.intermedius*.

Key Words: Plasmid, mastitis, *Staphylococcus aureus*, *Staphylococcus intermedius*

Mastitisli İneklerden İzole Edilen Koagulaz Pozitif *Staphylococcus* Türlerinin Antimikrobiyal Dirençliliği Ve Plazmit Tiplendirmesi

Özet: Bu çalışmada, 50 koagulaz pozitif *Staphylococcus* izolatı mastitisli ineklerden konventiyonal bakteriyolojik metodlar kullanılarak izole edilmiş ve tanımlanmıştır. Toplam izolattan 40 tanesi *Staphylococcus aureus olarak ve 10* tanesi ise *Staphylococcus intermedius* olarak tanımlanmıştır. Bu çalışma, subklinik mastitisli ineklerin etken ajanlar olarak *S. intermedius*'un öneminin arttığını göstermiştir. Bütün *Staphylococcus* suşlarının antibiyotiklere duyarlılıkları ve plazmit profilleri incelenmiştir. Plazmit profili 40 *S. aureus* izolatının 38'nin ve *10 S. intermedius* izolatının 9'nun plazmit içerdiğini göstermiştir. Plazmitlerin moleküler ağırlıkları 1.1 den 19 kb'a kadar değişmektedir. Kullanılan kemoterapotiklere en yüksek dirençlilik penisilinde gözlenmiştir. Bu çalışma, plazmit profili analizinin *S. aureus* ve *S. intermedius*'un tiplendirilmesi için uygun bir metod olmadığını göstermektedir.

Anahtar kelimeler: Plazmit, mastitis, *Staphylococcus aureus*, *Staphylococcus intermedius*

* This study was supported by the grant from the SÜBAP (Grant No:2001/123) and GÜAP (05/2002-21). The study contains some data from the dissertation of E.A.
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Introduction

Staphylococcus aureus is the most prevalent contagious pathogen causing mastitis in dairy cattle [1]. It is also well recognized that this mammary infection is very limited to eliminate, being even culling of the cow most effective way to reduce the incidence of this pathogen in a dairy herd [2]. The extensive use of antibiotics not only in veterinary medicine but also in livestock production for disease prevention or as growth-promoting feed additives has led to a serious increase in, and spread of multiple antibiotic-resistant bacteria including Staphylococci [3, 4].

Knowledge of the epidemiology of staphylococci, particularly *S.aureus*, has been studied by several methods that can discriminate between strains. Methods of typing such as biotyping, phage typing, antibiotic susceptibility patterns, pulsed field gel electrophoresis, random amplified gel electrophoresis, plasmid analysis, and protein electrophoresis used separately or together have been found to be successful in some degree for the *S.aureus* [5, 6, 7, 8, 9, 10]. However, another agent from mastitic udders, a coagulase-positive staphylococcus (CPS), *S.intermedius* still needs to be characterized by the plasmid pattern analysis [11}.

The objective of this study was to develop more detailed information about plasmid pattern characteristics of two member of CPS namely, *S.aureus and S.intermedius* isolated from bovine mammary secretions.

Materials and Methods

Sampling

Staphylococci (n=50) isolated from bovine mammary secretions of cattle with subclinical mastitis at 8 herds from different regions of province Konya were evaluated in the study. Prior to sampling the California Mastitis Test (CMT) were carried out. Milk samples were collected from individual quarters of the cows. Teats were washed with warm water, waited to dry up, disinfected with an individual non-woven moistened with ethanol and the first three squirts of milk were discarded. Milk samples (5 ml) were taken into a sterile tube and kept refrigerated (4 ^{o}C) for bacteriological examination.

Bacterial isolation and identification methods

Milk samples were cultured and bacteriological identification was conducted as described before. An aliqouts of 10 µl from each sample was spread over blood agar plates (Bacto-Agar, Difco Laboratories, MI) containing 5 % defibrinated sheep blood and incubated at 37 ^{o}C for 24 h. Colonies suspected of being staphyloccocci by macroscopical and microscopical examination were sub-cultured to obtain pure cultures on Nutrient Agar (Oxoid). Biochemical tests for catalase positive Gr positive cocci were used for further identification of Staphyloccoci as described [12, 13].

Antibiotic Susceptibility Test

The susceptibility test was performed using the disc diffusion technique on Mueller-Hinton Agar (Difco) as described [14]. Antibiotic discs used in the tests were as follows: Danofloxacine (5 mcg; Pfizer), penicillin G (10 U; Sigma), cloxacillin (5 mcg; Sigma), oxacillin (1 mcg; Sigma), amoxycillin (25 mcg; Sigma), amoxycillin+clavulanic acid (30 mcg; Sigma), methycillin (5 mcg; Sigma), ampicillin+sulbactam (20 mcg; Sigma).

Plasmid DNA analysis

Plasmid DNA of the isolates was prepared according to the method by Maniatis et al [15]. Plasmids were electrophoresed for 4 hours at 100V on a 0.8 % agarose gel in TAE buffer. The gel photographed under UV illumination using poloroid film Sigma 667. The approximate molecular mass of the plasmids was determined (kb) using Lambda-pUC mix Marker 4.

Results

Conventional tests allowed identification of 50 isolates: 40 strains were identified as *S.aureus*, 10 strains were identified as *S.intermedius*. Table 1 summarizes the plasmid profiles of *S.aureus* and *S.intermedius* from cows with subclinically infected udders. Plasmid profiling demonstrated that 38 (95.00 %) of 40 *S.aureus* and 9 (90.00 %) of 10 *S.intermedius* isolates contained plasmid. Figure 1 shows plasmid profiles of some of the isolates from both species.

Seven different molecular weights for each species were identified. Most of the isolotes showed only one plasmid band with size of 19 kb but the rest of the isolates had 2 to 4 bands ranging from >19 to 1.1 kb. The most common plasmid of 19 kb was detected in all strains isolated. Three isolates (2 strains of *S.aureus* and 1 of *S.intermedius*) were plasmid-free. Of thirty-three (86.84 %) *S.aureus* strains contained one plasmid, and 5 (13.16 %) strains contained multiple plasmid. All of the isolates contained multiple plasmid were also found to contain a common plasmid of 19 kb. Multiple plasmids were also detected in 4 (44.44 %) *S.intermedius* strains. Five (55.56 %) of the 9 strains contained a plasmid of 19 kb.

Table 1. Plasmid profiles of *S.aureus* and *S.intermedius* isolated from herds with subclinical mastitis

Staphylococcus sp.	**Number of isolates**	**Plasmid profile** (kb)
S.aureus	33	19
	2	19, 19<
	1	19, 7.7, 2.6, 2.3, 1.8
	1	19, 2.6
	1	19, 2,6, 2,3
	2	0
S.intermedius	5	19
	2	19, 7.7
	1	19<, 19, 2.3, 1.8
	1	19, ~10
	1	0

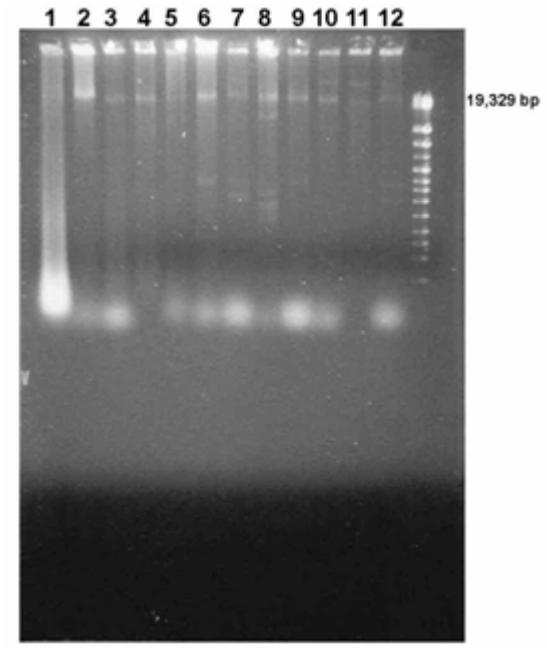

Figure 1. Agarose gel electrophoresis of the plasmids from some of the Staphylococci isolated from bovine mastitis. The strain numbers of 1, 6, 7, 8, 10, 11 corresponds to S.aureus. The numbers 2, 3, 4, 5, 9 and 12 are the strains of S.intermedius. M: Lambda pUC mix marker (19329, 7743, 5526, 4254, 3280, 2690, 2322, 1882, 1489, 1150, 925, 697, 421 bp).

The highest resistance to chemotherapeutics used was observed to penicillin (Table 2). Approximately, 87,50 % of *S.aureus* and 60.00 % of *S.intermedius* isolates were resistant to penicillin, respectively. Thirty four (85.00 %) of 40 *S.aureus* isolates were also resistant to amoxycillin. The corresponding number for the S*.intermedius* was 4 (40.00 %). This antibacterial agent was the second antibiotic to which the highest resistance was detected for the both species of staphylococci. Only one (10.00 %) *S.intermedius* strain was sensitive to danofloxacin, to cloxacillin and to oxacillin. One of each isolates was resistant to methycillin (Table 2).

Table 2. Antibiogram of *S.aureus* and *S.intermedius* isolated from mastitic udders with subclinical mastitis. P: Penicillin, Amox: Amoxycilline, Clox: Cloxacillin, Oxa: Oxacillin, Amox+Cla: Amoxycilline+Clavulanic acid, Amp+Sul: Ampicillin Sulbactam, Dan: Danofloxacin, Met: Methycillin

Staphylococcus sp.	Number of resistant *S.aureus* (%)	Number of resistant *S.intermedius* (%)	Total number of resistant *Staphylococcus sp.* (%)
P	35 (87,5)	6 (60)	41 (82)
Amox	34 (85)	4 (40)	38 (76)
Clox	1 (2.5)	0	1 (2)
Oxa	1 (2,5)	0	1 (2)
Amox+Cla	8 (20)	3 (30)	11 (22)
Amp+Sul	6 (15)	1 (10)	7 (14)
Dan	1 (2,5)	0 (0)	1 (2)
Met	1 (2,,5)	1 (10)	2 (4)

Discussion

Staphylococcal species associated with bovine mastitis have been classified as coagulase-positive or coagulase-negative. Coagulase-positive *S.aureus, S.intermedius* and *S.hyicus* species are considered major pathogens. *S.aureus* continues to be a major cause of bovine mastitis [11]. It was noted that among the biochemical reactions for the identification of *S.aureus* the clumping factor reaction was not absolutely reliable [16, 17]. *S.aureus* isolated from mastitic udders mostly contains plasmids [18, 19]. Plasmid profiles observed in this study suggested no specific plasmid pattern within a species. For the two species evaluated, more than 97.00 % of isolates were positive for plasmids. Plasmids were generally small, which agrees with previous reports [17, 18, 19].

Use of plasmids patterns in conjunction with antibiogram susceptibilty profiles in *S.aurues* strains were found to be of little value by this study. This is in agreement with previous studies [17, 20]. In addition, *S.intermedius* strains have also same characteristics as the evidenced by the present study.

Three strains of isolates were found to be free of plasmids in this study. *In vitro* and *in vivo* studies have showed that variability and instability of plasmid content in staphylococcal isolates could occur [21, 22]. These type of changes have been suggested to be related the observation that staphylococcal self-transmissble plasmids are capable of mobilizing smaller plasmids into recipient isolates [23]. The primary mechanisms that contribute to exchange of plasmids among staphylococcal isolates have been reported to be appeared by conjugation and transduction [20]. This could explain that isolates from the same udder obtained over the time may be the same strain, regardless of the plasmid profile.

Although some of the *S.aureus* strains showed resistance to only one chemotherapeutic, they were found to contain 3 plasmid (Isolate No: 17, 24; data not shown) suggests that plasmid observed in the strains could be correlated with the chemotherapeutics which have not been examined by this study.

Two points regarding 19 kb plasmid needs tobe emphasize. Firstly, although *S.intermedius* caused mastitis cases have been seen rarely, the ratio of the presence of the plasmid (19 kb) by

two species (92.00 %) was high. This suggests that acquiring the plasmid by the *S.intermedius* strains may be common. However, future studies examining plasmid patterns of this species isolated by different georaphic regions will help this to make clear. The second point is that 2 methycillin resistant strains (one of each species) contained one or two plasmids. But one plasmid in each strain was 19 kb plasmid. This should be a matter of concern on spread of methycillin resistance since MRSA is a major problem.

Since the virulence plasmids in staphylococci have been reported to be not common and plasmids in staphylococci might be correlated with resistance to antibiotics [17] by the results of this study, it may be speculated that this should be the case for not only *S.aureus* but also for *S.intermedius*.

We conclude that plasmid profile analysis does not appear to be an adequate method to differentiate isolates of two staphylococci recovered from bovine mastitis.

References

[1] Gudding R, McDonald JS, Cheville NF (1984) **Pathogenesis of *Staphylococcus aureus*, bacteriological, histologic, and ultrastructural pathologic findings**. *Am J Vet Res* 45, 2525-2531.

[2] Francis GP, Carrol PJ (1986) **Antibiotic resistance patterns of *Staphylococcus aureus* strains isolated from clinical bovine mastitis**. *Vet. Rec* 188: 361-366.

[3] Linton AH (1984) **Antibiotic resistance in *Staphylococcus aureus* and Streptococcus.** *Brit Med Bull* 40: 7781.

[4] Moellering RC (1998) **Antibiotic resistance: lessons for the future.** *Clin Inf Dis* 27, 135-140.

[5] Parisi JT, Hecht DW (1980) **Plasmid profiles in epidemiologic studies of infections by *S. epidermidis*.** *J Inf Dis* 141, 637-643.

[6] Wachsmuth I, Kiehlbauch J, Bopp C, Cameron D, Strockbine N Wells JABP (1991) **The use of plasmid profiles and nucleic acid probes in epidemiologic investigations of foodborne, diarrheal diseases**, *Inter J Food Microbiol* 12, 77-90.

[7] Dorn, C, Silapanuntakul R, Angrick E, Shipman L (1992). **Plasmid analysis and epidemiology of *Salmonella enteritidis* infection in three commercial layer flocks**, *Avian Dis* 38, 844-851.

[8] Neuwirth C, Siebor E, Lopez J, Pechinot A, Kazmierczak A (1996) **Outbreak of TEM-24-producing *Enterobacter aerogenes* in an intensive care unit and dissemination of the extended-spectrum beta-lactamase to other members of the family *Enterobacteriaceae***, *J Clin Microbiol* 34, 76-79.

[9] Shi ZH, Liu PYF, Lau YJ, Hu BS, Lin YH (1996) **Comparison of polymerase chain reaction and pulsed-field gel electrophoresis for the epidemiological typing of *Campylobacter jejuni***, *Diag Microbiol Inf Dis* 26, 103-108.

[10] McClure PJ (2000). ***Microbiological hazard identification in the meat industry. HACCP in the meat industry.*** (M Brown, Ed), CRC Press Woodhead Publishing Limited, Cambridge, 157-176.

[11] Honkanen-Buzalski T (1990) **The role of coagulase-negative Staphylococcal species in bovine mastitis. International Symposium on Bovine Mastitis**, Indianapolis, USA. September 13-16, 98-102.

[12] Collins CH, Carter GR, Chengappa MM (1991) ***Essentials of Veterinary Bacteriology and Micology.*** 4th Ed. Lea & Febiger, London.

[13] Erganiş O, Kuyucuoglu Y, Ok U (1995) **İnek ve koyun mastitislerine sebep olan koagulaz negatif ve pozitif *staphylococ*'ların biyotiplendirilmesi.** *Veterinarium*. 6, 23-27.

[14] Bauer AV, Kirby WMM, Sherris JC, Turc M (1966) **Antibiotic susceptibility testing by a standardized single disc method.** *J Clin Pathol* 45, 493-494.

[15] Maniatis R., Fritsh EF, Sambrook J (1982). **A laboratory Manual. In: *Molecular Cloning*,** Cold Spring Harbors, New York.

[16] Garcia ML, Moreno B, Bergdoll MS (1980) **Characterization of staphilococci isolated from mastitis cows in Spain.** *Appl Env Microbiol* 39, 548-553.

[17] Baumgartner A, Nicolet J, Eggimann M (1984) **Plasmid profiles of *Staphylococcus aureus* cousine bivine mastitis.** J Appl Bacteriol *56, 159-163.*

[18] Tenover FC, Arbeit R, Archer G, Biddle J, Byrne S, Goering R, Hancock G, Hebert GA, Hill B, Hollis R, Jarvis WR, Kreiswirth B, Eisner W, Maslow J, McDougal LK, Miller JM, Mulligan M, Pealler MA (1994) **Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus.*** *J Clin Microbiol* 32, 407-415.

[19] Nascimento JS, Santos KRN, Gentilini E, Sordelli D, Bastos MCF (2002) **Phenotypic and genetic characterisation of bacteriocin-producing strains of *Staphylococcus aureus* involved in bovine mastitis.** *Vet Microbiol* 85, 133-144.

[20] Matthews KR, Jayarao BM, Oliver SP (1992) **Plasmid pattern analysis of Staphylococcus species isolated from bovine mammary secretions.** *J Dairy Sci* 75, 3318-3323.

[21] Kloos WE, Orban BS, Walker DO (1981) **Plasmid composition of Staphylococcus species.** *Can J Microbiol* 27: 271-275.

[22] Mickelsen PA, Plorde JJ, Gordon KP, Hargiss C, McClure J, Schoenknecht FD, Condie F, Tenover FC, Tompkins LS (1985) **Instability of antibiotic resistance in a strain of *Staphylococcus epidermidis* isolated from an outbreak of prosthetic valve endocarditis.** *J Infect Dis* 150: 50-57.

[23] Bialkowska-Hobrazanska H, Jaskot D, Hammerberg O (1990) **Evaluation of restriction endonuclease fingerprinting of chromosomal DNA and plasmid profile analysis for characterization of multiresistant coagulase negative staphylococci in bacteremic neonates.** *J Clin Microbiol* 28: 269-276.