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Research Article

Antimicrobial Susceptibility Profiles and Coagulase Gene Polymorphism of *Staphylococcus aureus* Isolated from Bovine Subclinical Mastitis [1] [2]

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Abstract

The purpose of the study was to isolate *Staphylococcus aureus* from bovine subclinical mastitis, determine their antibiotic susceptibilities and investigate the coagulase gene polymorphism by using a PCR-based restriction fragment length polymorphism (RFLP) method. Milk samples from 463 CMT positive udders from 237 cows cultured. The antimicrobial susceptibility of the isolates were determined by disc diffusion method. A total of 82 out of the 83 isolates (98.8%) were found to be resistant at least one out of the 16 antibiotics studied. In this experiment 53 isolates (63.8%) were found to be resistant to penicillin; 52 (62.67%) to trimethoprim/sulphamethoxazole; 51 (61.5%) to ampicillin; 40 (48.2%) to erytromycin; 29 (34.9%) to tetracycline; 18 (21.6%) to ciprofloxacin, 16 (19.3%) to clindamycin, 13 (15.6%) to chloramphenicol; 8 (9.6%) to gentamicin; 5 (6.0%) to cefoxitin; 4 (4.9%) to vancomycin; 3 (3.6%) to cephalotin; 2 (2.4%) nafcillin; one (1.2%) to oxacillin and one to (1.2%) furazolidon. No imipenem resistance was seen in the *S. aureus* isolates. The coagulase gen polymorphism were examined by PCR amplification of coagulase gene followed by *Alu*I digestion of repeating 81 bp DNA sequences. After nested PCR, double bands were produced in 8 of the isolates while there were single band in remaining 75 isolates. Following *Alu*I digestion, isolates that formed single band in length of approximately 300 bp showed 3 different groups.

Keywords: *Bovine subclinical mastitis, Staphylococcus aureus, Antibiotic susceptibility, Coagulase gene polymorphism*

Subklinik Mastitli İneklerden İzole Edilen *Staphylococcus aureus* İzolatlarinin Antibiyotik Duyarlılık Profillerinin Çıkarılması ve Koagulaz Geni Polimorfizmine Göre Tiplendirilmesi

Özet

Bu çalışmanın amacı subklinik mastitisli sığırlardan *Staphylococcus aureus*'u izole etmek, bunların antibiyotiklere duyarlılığını belirlemek ve bir PCR tabanlı restriksiyon fragment lengh polimorfizmi (RFLP) yöntemi kullanarak koagulaz gen polimorfizmi araştırmaktır. 463 sığırdan CMT pozitif olan 237 sığır memesinden süt örnekleri alınarak ekim yapılmıştır. İzolatların antimikrobiyal duyarlılığı disk difüzyon yöntemi ile belirlenmiştir. Toplam 83 izolatın 82'si (%98.8) uygulanan 16 antibiyotikten en az bir antibiyotiğe dirençli bulundu. Bu çalışmada 53 izolat (%63.8) penisiline, 52 izolat (%62.67 trimethoprim/sulphamethoxazole, 51 izolat (%61.5) ampisiline, 40 izolat (%48.2) eritromisine, 29 izolat (%35.0) tetrasikline, 18 izolat (%21.7) siprofloksasine, 16 izolat (%19.3) klindamisine, 13 izolat (%15.6) kloramfenikole, 8 izolat (%9.6) gentamisine, 5 izolat (%6.0) sefoksitine, 4 izolat (%4.9) vankomisine, 3 izolat (%3.6) sefalotine, 2 izolat (%2.4) nafsiline ve 1'er (%1.2) izolat ise oksasilin ve furazolidona dirençli bulundu. *S. aureus* izolatlarında imipenem dirençliliği görülmedi. Koagulaz gen polimorfizmi koagulaz genin tekrarlanan 81 bp DNA dizisinin *Alu*I sindirimini müteakiben koagulaz genin amplifikasyonu ile incelenmiştir. Nested PCR'den sonra izolatların 8'inde çift bant görülmüş kalan 75 izolatta ise tek band vardı. *Alu*I sindirimini müteakiben yaklaşık 300 bp uzunluğunda tek bant oluşturan izolatlar 3 farklı grup göstermiştir.

Anahtar sözcükler: *Sığır subklinik mastitis, Staphylococcus aureus, Antibiyotik duyarlılığı, Koagulaz gen polimorfizmi*

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INTRODUCTION

Staphylococcus aureus has a wide range host spectrum and can cause serious infections by its methicillin resistant isolates. Long-term antibiotic usage is important in development of resistance against methicillin and other beta-lactam antibiotics. *S. aureus* is one of the most important pathogen for cattle mastitis and it is prevalent all around the world. Despite of strict control measures, control and eradication of *S. aureus* caused intramammary infections are quite difficult and continue as an economical problem. Antimicrobial therapy is one of the measures can be taken in order to control of staphylococcal mastitis. Detection of antibiotic susceptibilities of clinical isolates is necessary not only for treatment but also for preventing spread of resistant isolates. Regional evaluation of antibiotic susceptibilities of *S. aureus* may help veterinary surgeons [1,2]. Although most of the current *S. aureus* isolates have different genotypic and phenotypic characteristics, few are known about geographical distribution of those isolates and types of the pathogens in the herd [3]. Previously, distinct classification methods such as phage typing had been applied to both human and cattle originated *S. aureus* isolates [4,5]. Afterwards, methods such as plasmid analysis, ribotyping, pulsed-field gel electrophoresis, PCR- based fingerprinting, amplification of specific gene regions, and binary typing technics were started to be applied [1,4,6-8]. In recent years, there are some publications about genetic diversity of *S. aureus* isolates in Turkey recovered from subclinical cattle mastitis cases [2,9-13]. The aim of this study was to evaluate the biochemical capacity of the antibiotic resistances of *S. aureus* isolates recovered from subclinical cattle mastitis cases in the Middle Western Anatolia and perform molecular typing on coagulase gene polymorphism.

MATERIAL and METHODS

Bacteriological Studies

Milk samples were collected from 16 different dairy farms located in four different districts of Middle Western Anatolia between January-June 2010. Milk samples were collected in the mid-lactation period. California Mastitis Test (CMT) positive 463 milk samples were collected from 237 cows. The samples were inoculated onto Nutrient agar supplemented with 7% sheep blood, incubated at 37°C for 24-48 h. Eighty three *S. aureus* has been isolated and identified by the conventional tests such as Oxidase, catalase and coagulase positive (slide and tube), susceptibility to furazolidone, hemolysis, pigment formation, O/F, Baird Parker Agar (BP), Egg yolk tellurit, Mannitol Salt Agar (MSA), DNase Agar [14,15].

Gram positive cocci were further identified with conventional biochemical test and API Staph (Bio Merieux, France). The isolates were kept at -70°C in Trypticase Soy Broth (TSB) containing 15% glycerine in order to further use in molecular studies. Antimicrobial suscebtibility tests of the isolates were performed in accordance with National Committee for Clinical Laboratory Standards-NCCLS [16]. The isolates were tested against to the following antibiotics: penicillin (10 IU), gentamicin (10 µg), vancomycin (30 µg), clindamycin (2 µg), trimethoprim/sulfamethoxazole (1.25 µg/23.75 µg), cefalotin (30 µg), imipenem (10 µg), nafcillin (1 µg), furazolidone (100 µg), ampicillin (10 µg), tetracycline (30 µg), oxacillin (1 µg), chloramphenicol (30 µg), cefoxitin (30 µg), erythromycin (15 µg) and ciprofloxacin (5 µg). *S. aureus* ATCC 25923 was used as control strain [15]. Chi square test was used to evaluate the significance between antimicrobial sensitivities or resistances of *S. aureus* isolates.

Molecular Studies

DNA extractions from *S. aureus* isolates were performed by using genomic DNA purification kit (Bio Basic Inc., Totonto, Canada). In addition to the protocol, 11 U lysostaphin was added during lysis phase. Multiplex PCR developed by Maes et al.[17], were performed for the comfirmation of *S. aureus* identification, and for the detection of methicillin resistance. Briefly 2 µL of mecA and nuc primers (10 µmol), 3 µL of 16S rRNA specific primers (10 µmol), 5 µL of dNTP mixture (2.5mM), 5 µL of 10xPCR buffer, 4 µL of $MgCl_2$ (25mM), 0.4 µL of Taq polymerase (TaKaRa, Tokyo, Japan) and 2.5 µL of template DNA were added to PCR mixture and made up to 50 µL by adding distilled water. Amplification conditions consisted of 10 min at 94°C, followed by 23 cycles of 1 min at 94°C, 1 min at 51°C, and 2 min at 72°C, with a final step of 5 min at 72°C. The amplified DNA fragments were evaluated following the gel electrophoresis on a 1.5% agarose (Bio Basic Inc., Totonto, Canada) gel stained with ethidium bromide. Nested PCR followed by *Alu*I restriction enzyme dependent RFLP method was used to determine the polymorphism in coagulase gene regions of *S. aureus* isolates [18]. Primers to replicate the coagulase gene region described previously by Goh et al.[18] were used in nested-PCR assays. PCR mixture was prepared by adding 2 µL of each COA1 and COA4 primers (10 µmol), 5 µL of dNTP mixture (2.5mM), 5 µL of 10xPCR buffer, 4 µL of $MgCl_2$ (25mM), 0.4 µL of Taq polymerase (TaKaRa, Tokyo, Japan) and 26.6 µL ultra distilled water to obtain a 50 µL of final mixture, subsequently 5 µL of DNA extract was added and amplified. Fifty µL of similar mixture was prepared for the second cycle of nested PCR but this time 2 µL of COA2 and COA3 primers (10 µmol) were used as primers and 1.5 µL PCR product obtained from previous amplification as target DNA. DNA amplification was performed by pre-denaturation at 95°C for 5 min followed by 40 amplification cycles of 95°C for 30 sec, 55°C for 2 min, 72°C for 4 min and final extension at 72°C for 5 min. Ten µl of nested PCR product was digested by *Alu*I restriction enzyme (TaKaRa, Tokyo, Japan) according to the manufacturer's recommended protocol. Both PCR

product and restriciton digest fragments were detected by electrophoresis through a 3% agarose gel with Φx 174-Hae III Marker (TaKaRa, Tokyo, Japan) and 100 bp marker (Bio Basic Inc., Totonto, Canada).

Note: Ethics committee approval has been taken from AKU HADYEK on 01.04.2010 with the number 81.

RESULTS

Bacteriological Studies Results

Eighty-three *S. aureus* were isolated from milk samples. According to the results of susceptibility tests, 82 out of 83 isolates (98.8%) were resistant at least one of 16 antimicrobial agents involved in the study. In this experiment 53 isolates (63.8%) were found to be resistant to penicillin; 52 (62.67%) to trimethoprim/sulphamethoxazole; 51 (61.5%) to ampicillin; 40 (48.2%) to erytromycin; 29 (34.9%) to tetracyclin; 18 (21.6%) to ciprofloxacin, 16 (19.3%) to clindamycin, 13 (15.6%) to chloramphenicol; 8 (9.6%) to gentamicin; 5 (6.0%) to cefoxitin; 4 (4.9%) to vancomycin; 3 (3.6%) to cephalotin; 2 (2.4%) nafcillin; one (1.2%) to oxacillin and one to (1.2%) furazolidon. No imipenem resistance was seen in the S. aureus isolates. There were significant differences between antimicrobial sensitivities of *S. aureus* isolates (χ^2=459.03; P<0.01). Fourty one isolates were multidrug resistant (resistant to four and/or more antimicrobial agents). The antimicrobial susceptibilities of those isolates were shown in *Table 1*.

Table 1. *Antimicrobial susceptibilities of S. aureus isolates*

Antibiotic Disc	Disc Content (µg)	Susceptible (%)	Moderate (%)	Resistant (%)
Penicilin	10 Units	36.2	0.0	63.8
SXT*	25	33.7	3.6	62.7
Ampicillin	10	37.3	1.2	61.5
Erythromycin	15	47.0	4.8	48.2
Tetracycline	30	59.0	6.0	34.9
Ciprofloxacin	5	68.6	9.6	21.6
Clindamycin	2	72.3	8.4	19.3
Chloramphenicol	30	79.5	4.9	15.6
Gentamicin	10	84.3	6.1	9.6
Cefoxitin	30	94.0	0.0	6.0
Vancomycin	30	85.5	9.6	4.9
Cefalotin	30	95.2	1.2	3.6
Nafcillin	1	96.3	1.2	2.5
Oxacillin	1	98.8	0.0	1.2
Furazolidon	100	95.2	3.6	1.2
Imipenem	10	100	0.0	0.0
P	-	<0.01		<0.01

** Trimethoprim/sulfamethoxazole*

Molecular Studies Results

As the result of multiplex PCR, presence of nuc gene was determined and *S. aureus* identifications were comfirmed in 83 isolates which were identified by conventional methods previously. One isolate which was determined to have phenotypic resistance to meticillin was also genotypically positive in presence of mecA. All 83 *S. aureus* isolates showed differences in coagulase gene region polymorphism. After nested PCR, double bands were produced in 8 of the isolates while there were single band in remaining 75 isolates. Following *Alu*I digestion, isolates that formed single band in length of approximately 300 bp were also showed 3 different groups. Fragments of 81 bp or multiplies were evaluated for RFLP type classification *(Table 2)*. As the result of RFLP typing out of 55 isolates from district A (4 farms), 44 isolates were type II and 11 isolates were type I. Out off 15 isolates from district B (5 farms) eight of them were type I, four of them were type II and three of them were type III in RFLP profile. Isolates from district C (3 farms) were distributed into type II and type IV profiles. And finally the isolates from district D (4 farms) were belonging to type I and type IV. The antimicrobial resistnace profiles within the groups were shown in *Table 3*.

As a consequence, according to antibiogram results, staphylococci have gained resistance to some commonly used antibiotics. That's why it is recommended that it should not be used antibiotic without making an antibiogram.

DISCUSSION

β-Lactam antibiotics are commonly used in cattle mastitis treatment. Penicillin resistance may be related to national policies about usage of antimicrobial drugs and differencies about animal raising systems [5]. The highest penicillin resistance was reported to be in Ireland (71.4%) and England (67.3%) within the European countries followed by 50% in USA. It was rather low in Denmark (18.7%) and in Norway (2%) which were the Scandinavian countries [5,19]. It was also shown by Sori et al.[20] that resistance against penicillin was quite high (87.2%) in South-West Ethiopia. There are studies about cattle mastitis showing that penicilin resistance is high in staphylococcus bacteria from isolates in different regions of Turkey. Guler et al.[2] reported the highest resistance was against penicilin and ampicillin as high as 63.3%. In Aydin region resistance against penicillin, SXT and erythromycin were 81%, 17% and 7% respectively [21]. In another study from different region, resistance of *S. aureus* isolates against penicillin G, tetracycline, erythromycin and oxacillin were 85.4%, 39.6%, 5.2% and 3.1%, respectively in mastitic cow milk [10]. The results of this study showed that penicillin resistance (63.86%) in Middle Western Anatolia of Turkey was higher than other antibacterials. This situation is relatively similar to that in other countries such as Ireland, England and USA. It is quite higher than those in Denmark

Table 2. *Typing of isolates based on PCR and AluI digestion*

Number of Isolates (%)	RFLP Type	Sizes of PCR Products (approx.bp)	*Alu*I Profiles*		
			81 (bp)	162(bp)	243(bp)
21 (25.3%)	Type-I	300	+	+	+
51 (61.4%)	Type-II	300	+	-	+
3 (3.6%)	Type-III	300	-	-	+
8 (9.6%)	Type-IV	290, 870	+	-	+

** Results for only fragments of 81 bp or multiples are shown*

Table 3. *Distribution of S. aureus isolates within dairy farms, antimicrobial sensitivities and RFLP types*

Origin of the Samples (number of farms)																	
	Antimicrobial Agent	A(4)				B(5)				C(3)				D(4)				Total
		RFPL Type				RFPL Type				RFPL Type				RFPL Type				
		I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV	
Number of antimicrobial resistant isolates	P	6	33	-	-	5	4	1	-	-	-	-	1	1	-	-	2	53
	GM	3	2	-	-	1	-	-	-	-	1	-	-	-	-	-	-	7
	VA	2	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	4
	DA	1	7	-	-	4	-	-	-	-	-	-	1	2	-	-	1	16
	SXT	7	30	-	-	5	-	2	-	-	2	-	4	-	-	-	2	52
	CF	1	1	-	-	-	-	-	-	-	-	-	1	-	-	-	-	3
	IPM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	NAF	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1
	FX	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1
	AMP	7	29	-	-	5	2	2	-	-	1	-	3	1	-	-	1	51
	TE	3	15	-	-	6	2	1	-	-	-	-	1	-	-	-	2	30
	OX	-	2	-	-	1	-	-	-	-	-	-	2	-	-	-	-	5
	C	1	8	-	-	1	-	1	-	-	1	-	-	1	-	-	1	14
	CTX	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6
	E	5	24	-	-	7	2	-	-	-	2	-	1	1	-	-	1	43
	CIP	4	8	-	-	1	-	-	-	-	2	-	1	-	-	-	1	17
RFPL Type	I	11	-	-	-	8	-	-	-	-	-	-	-	2	-	-	-	21
	II	-	44	-	-	-	4	-	-	-	3	-	-	-	-	-	-	51
	III	-	-	-	-	-	-	3	-	-	-	-		-	-	-		3
	IV	-	-	-	-	-	-	-	-	-	-	-	6	-	-	-	2	8

***P:** penicillin, **GM:** gentamicin, **VA:** vancomycin, **DA:** clindamycin, **SXT:** trimethoprim/sulfamethoxazole, **CF:** cefalotin, **IPM:** imipenem, **NAF:** nafcillin, **FX:** furazolidon, **AMP:** ampicillin, **TE:** tetracycline, **OX:** oxacillin, **C:** chloramphenicol, **CTX:** cefoxitin, **E:** erythromycin, **CIP:** ciprofloxacin, **-;** no growth*

and Norway. Besides the penicillin, resistance against trimethoprim/sulphamethoxazole and ampicillin were considerably high. These antimicrobial agents are usually preferred for the treatment of mastitis cases in Turkey. Coagulase production is an important phenotypic specification to identify *S. aureus*. Gene coagulase is the most important virulance factor for *S. aureus*. It is reported that both counts and localisations of *Alu*I restriction regions in 3'ends of gene coagulase contain a sequence of 81 base pairs which is different between *S. aureus* isolates [18]. The classification of *S. aureus* isolates depending on gene coa is a simple method for molecular typing and can be assumed as a validation test [18,22]. Aslantas et al.[9] reported that RFLP models of gene coagulase of *S. aureus* isolates from cattle mastitis showed a great diversity. They determined that coagulase gene polymorphism of *S. aureus* isolates from mastitic cow milk by RFLP method using *Alu*I enzyme produced 9 different genotype strains. Rodrigues da Silva and Silva [22], reported that there were 49 different types of RFLP samples after digestion with *Alu*I enzyme. Raimundo et al.[23] examined coa gene type of 151 samples of cattle *S. aureus* isolates from 76 farms

by using Coag2 and Coag3 primers and found 6 types of PCR. Su et al.[3] investigated coa gene diversity in *S. aureus* isolates from 4 countries. They reported 5 genotypes were dominant for each country. However, dominant types changed according to the geographical regions. Karahan and Cetinkaya [24] reported that 83.9% were produced single band and 16.1% produced 2 bands after coa gene amplification in PCR results of 161 coa positive *S. aureus* isolates. They found that 23 different types of restriction profiles in RFLP results by using *Alu*I enzyme. Guler et al.[2] investigated 125 *S. aureus* isolates which had antibiotic resistance and found that there were 4 types of coagulase gene. In the present study, 90.4% of the isolates produced single band and 9.6% of them produced double bands after coa gene amplification. In this study, 51 out of 83 *S. aureus* isolates were obtained from 7 dairy farms and each of them produced bands approximately 300 bp in length. After RFLP those isolates produced 2 bands (81, 243 pb). Remaining 32 isolates were obtained from 9 different dairy farms and produced 2 types of PCR products of 300 and 290, 870 bp. It was also found that 24 samples to have 2 different types of 300 bp product after RFLP (81, 162, 243 bp and 243 bp) while PCR products of 290, 870 bp in length were produced 2 bands of 81, 243 bp in length after RFLP. This study showed that there were 4 different types of *S. aureus* as Type I, II, III and IV in Middle Anatolia region upon classification by PCR-RFLP. Besides Type II was the most common one to be present in 61.4% of the isolates. Eventually, 33 out of 51 Type II isolates were found to be resistant to penicilin. 6 out of 8 Type IV isolates were resistant to trimethoprim/sulfamethoxazole and, 13 out of total 21 Type I isolates were resistant to ampicillin amongst antibiotics involved in the trial. All of 83 isolates were sensitive to imipenem. However, only 1 isolate were resistant to nafcillin and furazolidon *(Table 3)*.

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