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Investigation of the antibiotic resistance and biofilm-forming ability of *Staphylococcus aureus* from subclinical bovine mastitis cases

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ABSTRACT

A total of 112 *Staphylococcus aureus* isolates obtained from subclinical bovine mastitis cases were examined for antibiotic susceptibility and biofilm-forming ability as well as genes responsible for antibiotic resistance, biofilm-forming ability, and adhesin. Antimicrobial susceptibility of the isolates were determined by disk diffusion method. Biofilm forming ability of the isolates were investigated by Congo red agar method, standard tube method, and microplate method. The genes responsible for antibiotic resistance, biofilm-forming ability, and adhesion were examined by PCR. Five isolates (4.5%) were identified as methicillin-resistant *Staph. aureus* by antibiotic susceptibility testing and confirmed by *mec*A detection. The resistance rates to penicillin, ampicillin, tetracycline, erythromycin, trimethoprim-sulfamethoxazole, enrofloxacin, and amoxicillin-clavulanic acid were 45.5, 39.3, 33, 26.8, 5.4, 0.9, and 0.9%, respectively. All isolates were susceptible against vancomycin and gentamicin. The *bla*Z (100%), *tet*K (67.6%), and *erm*A (70%) genes were the most common antibiotic-resistance genes. Using Congo red agar, microplate, and standard tube methods, 70.5, 67, and 62.5% of the isolates were found to be biofilm producers, respectively. The percentage rate of *ica*A, *ica*D, and *bap* genes in *Staph. aureus* isolates were 86.6, 86.6, and 13.4%, respectively. The adhesion molecules *fnb*A, *can*, and *clf*A were detected in 87 (77.7%), 98 (87.5%), and 75 (70%) isolates, respectively. The results indicated that *Staph. aureus* from sublinical bovine mastitis cases were mainly resistant to β-lactams and, to a lesser extent, to tetracycline and erythromycin. Also, biofilm- and adhesion-related genes, which are increasingly accepted as an important virulence factor in the pathogenesis of *Staph. aureus* infections, were detected at a high rate.

Key words: antibiotic resistance, biofilm production, mastitis, *Staphylococcus aureus*

INTRODUCTION

Mastitis is a worldwide problem causing enormous economic losses in dairy industry due to poor milk quality, reduced milk yield, increased usage of drugs and veterinary service, as well as high culling rate of affected cattle and sometimes death due to the disease (Kumar et al., 2010). *Staphylococcus aureus* is one of the leading agents isolated from bovine mastitis cases and is characterized by lower cure rates compared with other mastitis pathogens. This phenomenon is mainly explained by acquisition of antimicrobial resistance and their biofilm-forming ability (Taponen and Pyörälä, 2009).

Misuse and widespread use of antibiotics for the treatment and prevention of bovine mastitis leads to development and emergence of resistance among mastitis pathogens against antibiotics (Oliver and Murinda, 2012). Beta-lactams have been widely used to treat mastitis cases for several decades, but their efficiency is reduced due to β-lactamase synthesis, which is encoded by *bla*Z (Olsen et al., 2006). Another β-lactam resistance mechanism, called methicillin/oxacillin resistance, is mediated by low-affinity penicillin-binding protein (PBP2a) encoded by *mec*A (Sawant et al., 2009).

Another mechanism significantly affecting the effectiveness of treatment of mastitis cases is the production of biofilm. Biofilm formation reduces susceptibility of *Staph. aureus* to various antibiotics by decreasing diffusion of antibiotics inside biofilm matrix and becoming resistant to high concentrations of antimicrobials. Biofilm also helps (1) bacteria adhesion and colonization of mammary gland tissue, (2) evasion from harsh conditions within host and phagocytosis, and (3) persistence of infection. The *ica*A and *ica*D genes, found at the *ica* locus present in *Staph. aureus* and *Stahpylococcus epidermidis*, play a significant role in biofilm formation.

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Whereas *ica*A encodes *N*-acetylglucosaminyltransferase, responsible for the *N*-acetylglucosamine oligomers from UDP-*N*-acetylglucosamine (Arciola et al., 2001), *ica*D plays a critical role in the maximal expression of *N*-acetylglucosaminyltransferase, leading to the phenotypic expression of the capsular polysaccharide (Gerke et al., 1998). In addition to the above mentioned genes, a surface protein called biofilm-associated protein (*bap*) has been reported to be involved in biofilm formation of bovine *Staph. aureus* strains. The *bap* gene implicates biofilm formation by promoting primary attachment and adhesion to inert and live surfaces (Cucarella et al., 2004).

Staphylococcus aureus have a variety of adhesins playing an important role in the onset of infection by binding host tissues and accepted as an important virulence factors. These adhesins specifically interact adhesive matrix components found on host tissue and designated as the microbial surface component recognizing adhesive matrix molecules (**MSCRAMM**; Patti et al., 1994). Of these MSCRAMM, fibronectin-binding protein A (*fnb*A), clumping factor A (*clf*A), and collagen-binding protein (*cna*) are accepted as important virulence factors in binding to host cell, colonization and invasion (Haveri et al., 2008).

The aims of the current study were (1) to investigate the antibiotic susceptibility and antibiotic resistance genes, (2) determine the ability of biofilm synthesis and the genes responsible for slime synthesis of the isolates, and (3) search for *cna*, *fnb*A, and *clf*A genes coding MSCRAMM in *Staph. aureus* strains from subclinical mastitis.

MATERIALS AND METHODS

Bacterial Isolates

A total of 112 *Staph. aureus* strains isolated from milk samples submitted to the Department of Microbiology laboratory (Mustafa Kemal University) from 2008 to 2010 were studied. The strains were isolated from 330 dairy cattle belonging to 26 family-sized farms (average of 15–20 cattle) located in southern Turkey. Subclinical bovine mastitis cases were detected using California mastitis test. Milk samples were inoculated onto blood agar supplemented with 5% defibrinated sheep blood. The isolates were identified according to classical microbiological methods (Quinn et al., 1998).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates were determined using disk diffusion method according to the guidelines of Clinical Laboratory Standards Institute (2008). The antimicrobials used were penicillin (10 μg), ampicillin (10 μg), amoxicillin-clavulanic acid (20/10 μg), oxacillin (1 μg), vancomycin (30 μg), gentamycin (10 μg), enrofloxacin (5 μg), erythromycin (15 μg), tetracycline (30 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg). *Staphylococcus aureus* (ATCC 29213) was used as a quality-control strain.

Biofilm Formation

Biofilm-forming ability of *Staph. aureus* strains were determined using 3 different methods.

Congo Red Agar Method

Qualitative detection of biofilm production by *Staph. aureus* strains were determined using Congo Red Agar (**CRA**) plates as previously described by Freeman et al. (1989). Strains producing black and rough colonies were considered biofilm producers.

Standard Tube Method

The qualitative assay for biofilm formation was performed according to the method described by Christensen et al. (1985). Presence of adherent film stained with safranine on the inner surface of the standard tubes (**ST**) was accepted as indication of positive result. The biofilms formed were scored as negative (−), weak (+), moderate (++), and strong (+++).

Microplate Method

Quantitative biofilm determination was carried out using the microplate (**MP**) method described by Christensen et al. (1985) in tissue culture plates with 96 flat-bottomed well. All the experiments were repeated at least twice, and the values of optical density were then averaged. A 3-grade scale was used to evaluate the biofilm-forming ability of strains: optical density <0.120 (–); optical density = 0.120–0.240 (+); and optical density >0.240 (++).

DNA Isolation and PCR Amplification of Antibiotic Resistance and Biofilm Genes

Bacterial DNA extraction was performed using commercial DNA extraction kit (InstaGene Matrix, Bio-Rad, Hercules, CA). The PCR amplification of intracellular adhesion genes (*ica*A and *ica*D), *bap* gene, and adhesion molecules (*cna*, *clf*A, *fnb*A) were determined as previously described by Vasudevan et al. (2003), Cu-

Table 1. Antimicrobial resistance phenotype and genotype of *Staphylococcus aureus* isolates

Phenotype[1]	Genotype	Isolates, no.
P, AMP	*bla*Z	17
P, AMP, TE	*bla*Z, *tet*K	10
TE, E	*tet*K, *erm*C	7
P, AMP, TE, E	*bla*Z, *tet*K, *erm*C	6
P, TE, E	*bla*Z, *tet*M, *erm*A	2
P, E, AMP	*bla*Z, *erm*C	2
OXA, TE	*mec*A, *tet*K	2
P, TE, E, STX	*bla*Z, *erm*C, *tet*M	2
E, P	*bla*Z, *erm*C	2
TE, E	*tet*K, *tet*M, *erm*C	2
P, TE, E	*bla*Z, *tet*M, *erm*A	1
E	*erm*A, *erm*C	1
P, AMP, TE	*bla*Z, *tet*M	1
P, AMP, OXA	*bla*Z, *mec*A	1
P, AMP, STX	*bla*Z	1
P, AMP, TE, E	*bla*Z, *tet*M, *erm*A	1
P, AMP, OXA, TE, E	*bla*Z, *tet*M, *mec*A, *erm*A	1
P, AMP, OXA, TE, E, ENR, STX	*bla*Z, *mec*A, *tet*M, *erm*A, *erm*C	1
P, AMP, E, AMC	*bla*Z , *erm*A	1
P, AMP, E, STX	*bla*Z, *erm*A, *erm*C	1
P, AMP, TE, STX	*bla*Z, *tet*K, *tet*M	1
Total		63

[1]P = penicillin, AMP = ampicillin, TE = tetracycline, E = erythromycin, OXA = oxacillin, AMC = amoxicillin-clavulanic acid, SXT = trimethoprim-sulfamethoxazole, ENR = enrofloxacin.

carella et al. (2004), and Arciola et al. (2005). Antimicrobial resistance genes related to methicillin (*mec*A), penicillin (*bla*Z), tetracycline (*tet*M, *tet*K, *tet*L, *tet*O), aminoglycoside [*aac(6′)-Ie-aph(2″)-Ia*, *ant(4′)-la* and *aph(3′)-IIIa*], and macrolide (*erm*A, *erm*B, and *erm*C) were investigated as previously reported by Jensen et al. (1999), Vesterholm-Nielsen et al. (1999), Strommenger et al. (2003), and Choi et al. (2003) using PCR assay.

RESULTS

Antimicrobial Susceptibility Testing and Resistance Genes

The data on the antimicrobial susceptibility of 112 *Staph. aureus* strains and resistance genes are given in Table 1. Five isolates (4.5%) were found as methicillin-resistant *Staph. aureus* (**MRSA**) by antibiotic susceptibility (resistant to oxacillin) and confirmed by *mec*A detection. Various rates of resistance to penicillin (45.5%), ampicillin (39.3%), tetracycline (33%), erythromycin (26.8%), trimethoprim-sulfamethoxazole (5.4%), oxacillin (4.5%), enrofloxacin (0.9%), and amoxicillin-clavulanic acid (0.9%) were detected. All isolates were susceptible to vancomycin and gentamicin. All penicillin-resistant isolates contained *bla*Z. Of 37 tetracycline-resistant isolates, 25 possessed *tet*K, 9 had *tet*M, whereas 3 carried both *tet*K and *tet*M. Among the erythromycin-resistant isolates (n = 30), *erm*A was detected in 21 isolates, *erm*C was detected in 6 isolates, *erm*A and *erm*C were detected in 3 isolates. None of the isolates carried aminoglycoside-resistance genes.

Biofilm Formation and Biofilm-Related Genes

Out of 112 *Staph. aureus* isolates, 79 (70.5%) isolates by CRA method, 75 (67%) isolates by MP method, and 70 (62.5%) isolates by the ST method were found as biofilm producers (Table 2). Both *ica*A and *ica*D were detected in 97 (86.6%) isolates, and *bap* were detected in 15 (13.4%) isolates. Comparison of CRA, MP, and ST methods with PCR results is given in Table 3.

Adhesion Genes

Genes *cna*, *fnb*A, *clf*A were detected in 98 (87.5%), 87 (77.7%), and 75 (70%) isolates, respectively.

Table 2. Screening of 112 *Staphylococcus aureus* isolates for biofilm production by Congo red agar (CRA), standard tube (ST), and microplate (MP) methods

	Screening method		
Biofilm formation	CRA, no. (%)	MP, no. (%)	ST, no. (%)
Strong	43 (38.4)	27 (24.1)	35 (31.3)
Moderate	36 (32.1)	48 (42.9)	22 (19.6)
Weak	0 (0)	0 (0)	13 (11.6)
None	33 (29.5)	37 (33)	42 (37.5)

Table 3. Evaluation of Congo red agar (CRA), standard tube (ST), and microplate (MP) methods considering PCR as the reference method

Method	Biofilm-producing strains	Strains positive for *ica*AD	Strains negative for *ica*AD	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %
CRA	79	73	6	75.25	60.0	92.4	27.27
MP	75	71	4	73.19	73.33	94.66	29.72
ST	67	62	5	63.91	66.66	92.53	22.22

DISCUSSION

Many previous studies showed that *Staph. aureus* was the most important microorganism isolated from subclinical mastitis cases in the world (Taponen and Pyörälä, 2009). Similarly, previous studies carried out in Turkey also revealed that *Staph. aureus* was the most common agent with various isolation rates varying between 24.63 and 39.04% (Gulcu and Ertas, 2004; Macun et al., 2011; Yesilmen et al., 2012).

In our study, *Staph. aureus* isolates showed higher rate of resistance to penicillin (45.5%). This result is not surprising, because β-lactams are widely prescribed agents to cure bovine mastitis cases in Turkey. Previous studies conducted in Turkey revealed that prevalence of penicillin resistance were 75% in Marmara region, 62% in Central Anatolia, and 63.3% in Burdur, respectively (İkiz et al., 2013; Güler et al., 2005; Turutoğlu et al., 2006). Resistance rate to tetracycline (33%) was higher than those from findings of İkiz et al. (2013; 16.6%) and Güler et al. (2005; 27.9%), but lower than findings (61.2%) of Turutoğlu et al. (2006), suggesting that resistance rates for *Staph. aureus*, such as those for other bacteria, vary regionally and are influenced by antibiotic usage. The erythromycin-resistance rate (26.8%) was inconsistent with previous studies conducted by Ünal and İstanbulluoğlu (2009) in Kırıkkale and Tel et al. (2012) in Şanlıurfa, who reported resistance rates of 4.3 and 9.3%, respectively. But, Ikiz et al. (2013) reported erythromycin resistance rate as 33.33%. In previous studies, generally low or no resistance against trimethoprim-sulfamethoxazole was reported in Turkey (Güler et al., 2005; Ünal and İstanbulluoğlu, 2009; Tel et al., 2012). Similarly, low resistance (5.4%) was found against this agent in current study. In contrast to these studies, high levels resistance were reported by Turutoğlu et al. (2006) and İkiz et al. (2013) (45.6 vs. 58.83%, respectively). One of the striking results of the study was very low resistance prevalence against enrofloxacin (0.9%) and amoxicillin-clavulanic acid (0.9%), as these drugs are critically important for the treatment of staphylococcal infections in veterinary medicine (Beco et al., 2013).

The biofilm-forming ability of staphylococci has increasingly been accepted as an important virulence trait in addition to exotoxins and surface proteins produced by staphylococci (Vancraeynest et al., 2004). Among the methods used for determining biofilm-forming ability of *Staph. aureus* from bovine mastitis cases, it was observed that the highest positivity was obtained by the CRA method (Vasudevan et al., 2003; Turkyilmaz and Eskiizmirliler, 2006; Dhanawade et al., 2010). In a similar manner, in the current study, higher positivity rate was found by CRA (70.5%) than by MP (67%) or ST (62.5%). A discrepancy has also been reported between the results of phenotypic and genotypic methods used for the determination of biofilm forming ability of *Staph. aureus* isolates. Indeed, 18 *ica*A- and *ica*D-positive isolates were negative by all 3 phenotypic methods used in our study. Cramton et al. (1999) suggested that this discrepancy may arise from point mutations in the *ica* locus or other yet unknown factor that negatively affects biofilm synthesis. Baselga et al. (1993) indicated that phenotypic expression of the biofilm synthesis was quite sensitive to in vitro conditions. Ciftci et al. (2009) mentioned possible role of genes in the *ica* locus involved in controlling slime expression. Nourbakhsh and Namvar (2016) investigated possible role of 12 genes involved in biofilm formation in MRSA and found that all strains had biofilm-producing ability with different degrees due to the different prevalence rates of these genes. Pereyra et al. (2016) emphasized that overexpression of *ica*D and *fnb*B genes was necessary to reach the highest invasion rates, irrespective of genes related to adherence and biofilm formation. Another gene involved in biofilm formation and persistence of *Staph. aureus* on the mammary gland epithelium is *bap* (Cucarella et al., 2001), which was detected only in 15 (13.4%) of the isolates in the current study. Our results are comparable to the study carried out by Zuniga et al. (2015), who reported that 15.8% of *Staph. aureus* strains obtained from bovine subclinical mastitis cases in Brazil harbored this gene. However, Salimena et al. (2016) in Brazil detected *bap* in 95.6% of the isolates, which is the highest prevalence rate reported to date, whereas some authors did not detect this gene among *Staph. aureus* from subclinical bovine mastitis cases (Sung et al., 2008; Vautor et al., 2008; Szweda et al., 2012; Xu et al., 2015). Khoramrooz et al. (2016) in Iran and Darwish and Asfour (2013) in Egypt detected this

gene in 5 and 2.5% of their isolates, respectively. To our knowledge, this is the first time *bap* has been shown among *Staph. aureus* isolates of bovine mastitis origin in Turkey.

In the current study, all penicillin-resistant isolates (45.5%) were also positive for *bla*Z. Similarly, da Costa Krewer et al. (2015) also reported higher rate (97.6%) of *bla*Z among penicillin-resistant *Staph. aureus* from bovine mastitis in the northeast of Brazil. Another mechanism is methicillin resistance; MRSA has gained increasing importance in veterinary medicine in the last 2 decades, as MRSA show resistance not only β-lactams but also other classes of antimicrobials (Lee., 2003; Baptiste et al., 2005). In the current study, all oxacillin-resistant isolates were also positive for *mec*A (3.6%). In a previous study, Ciftci et al. (2009) detected presence of *mec*A in only 4 of 59 (6.7%) *Staph. aureus* isolates from bovine mastitis in Turkey.

Staphylococcal adhesins have been shown to be crucial for binding host surface. Thus, adhesins contribute to tissue adhesion and colonization in various infections, which is considered a critical stage in the initiation of infection (Klein et al., 2012; McCormack et al., 2014; Zuniga et al., 2015). However, little is known about surface adhesins of *Staph. aureus* strains isolated from bovine mastitis cases in Turkey. Our study marks the first time presence of some important adhesin genes (*cna*, *fnb*A, and *clf*A) were investigated in *Staph. aureus* from bovine mastitis in Turkey.

The *cna* gene was detected in 87.5% of the isolates in the present study, which is very high percentage compared with previous studies. Zuniga et al. (2015) and Ikawaty et al. (2010) reported this gene in 47.4 and 49% of their isolates, respectively. A lower percentage of the *cna* gene was also identified in 10.7, 22.4, 22.5, and 31.9% of *Staph. aureus* isolates subclinical bovine mastitis by Xu et al. (2015), Klein et al. (2012), Khoramrooz et al. (2016), and Ote et al. (2011), respectively.

The gene encoding the fibronectin-binding protein A (*fnb*A) was detected in 77.7% of *Staph. aureus* isolates, which is nearly similar to findings of Khoramrooz et al. (2016), Xu et al. (2015), Ote et al. (2011), and Zuniga et al. (2015), who detected this gene in 72.5, 70, 83.8, and 84.2% of the isolates, respectively. However, Ikawaty et al. (2010) and Kumar et al. (2011) reported higher prevalence rates of 96 and 100% of the *Staph. aureus* isolates, respectively. Also, a lower prevalence rate (50.6%) was also reported by Klein et al. (2012).

Another important adhesin belong to MSCRAMM is *clf*A. This adhesin promotes virulence in invasive infections using different mechanisms, such as coating the bacterium with plasma fibrinogen and splitting of the complement opsonin C3b (McCormack et al., 2014). In our study, *clf*A was detected in 70% of *Staph. aureus* isolates, which is high compared with previous studies carried out by Klein et al. (2012; 50.6%) and Ikawaty et al. (2010; 21%). In contrast, Xu et al. (2015), Ote et al. (2011), and Pereyra et al. (2016) reported higher prevalence rate in their studies (89.3, 96.9, and 100%, respectively).

In conclusion, our study showed that *Staph. aureus* isolates of bovine subclinical mastitis carried widely both biofilm and adhesin genes involved in the pathogenesis of *Staph. aureus* infections, which indicate potential virulence of the isolates. In addition, high resistance was observed against β-lactams as well as moderate resistance against tetracycline and erythromycin, which are widely used in veterinary practice. Therefore, to achieve effective treatment of bovine mastitis cases and to prevent emergence of antibiotic-resistant bacteria, particular attention should be given to isolation of causative agent and determination of antimicrobial susceptibility.

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