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Slime Positivity and Antibiotic Resistance in *Staphylococcus aureus* Strains Isolated from Various Animal Clinical Samples

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Summary: In this study, a total of 50 *Staphylococcus aureus* strains isolated from bovine milk, bovine uterus swabs, dog ear swab, dog skin swab and chicken synovial fluid samples were examined in terms of slime factor production and antibiotic resistance. The slime production of strains was determined by using Congo red agar and standard tube methods. Of 50 *S. aureus* strains, 21 (42%) and 18 (36%) were determined as slime positive in the Congo red agar and standard tube methods, respectively. A significant difference was not found between two different methods ($p>0.05$). Standard E-test method was used to detect the antibiotic resistance of isolates. Among the isolates, the highest resistance rate was against penicillin G (20%), followed by cephalothin (16%), oxacillin (16%) and tetracycline (14%). Only one (2%) of the tested strains was resistant to enrofloxacin. However, resistance to erythromycin, trimethoprim/sulfamethoxazole, rifampin and gentamicin were not determined in any strains. When the antibiotic resistance of slime positive and negative strains was compared, resistance to penicillin G, cephalothin and oxacillin was significantly high in slime positive strains ($p<0.05$).

Key Words: Antibiotic resistance, slime factor, *Staphylococcus aureus*

Çeşitli Hayvansal Klinik Örneklerden İzole Edilen *Staphylococcus aureus* Suşlarında Slaym Pozitifliği ve Antibiyotik Direnci

Özet: Bu çalışmada, inek sütü, inek uterus sıvabı, köpek kulak sıvabı, köpek deri sıvabı ve tavuk sinoviyal sıvı örneklerinden izole edilen toplam 50 *Staphylococcus aureus* suşu slaym faktör üretimi ve antibiyotik direnci yönünden incelendi. Suşların slaym üretimi Congo red agar ve standart tüp yöntemleri kullanılarak belirlendi. Elli *S. aureus* suşunun 21'i (%42) Congo red agarda, 18'i (%36) standart tüp yöntemi ile slaym pozitif olarak belirlendi. İki farklı yöntem arasında istatistiksel olarak önemli bir farklılık bulunmadı ($p>0.05$). İzolatların antibiyotik dirençlerinin belirlenmesinde standart E-test yöntemi kullanıldı. İzolatlar arasında en yüksek direnç oranı penisilin G'ye (%20) karşı iken, bunu sefalotin (%16), oksasilin (%16) ve tetrasikline (%14) karşı direnç oranları izledi. Test edilen izolatlardan sadece biri (%2) enrofloksasine karşı dirençliydi. Eritromisin, trimetoprim/sulfametoksazol, rifampin ve gentamisine karşı ise direnç tespit edilemedi. Slaym pozitif ve negatif suşların antibiyotik dirençleri karşılaştırıldığında, slaym pozitif suşlarda penisilin G, sefalotin ve oksasiline karşı direnç önemli oranda yüksekti ($p<0.05$).

Anahtar Kelimeler: Antibiyotik direnci, slaym faktör, *Staphylococcus aureus*

Introduction

Staphylococcus aureus is a common commensal on skin, the mucous membranes of upper respiratory tract, lower urogenital tract and digestive tract in humans and animals. However, this agent causes pneumonia, mastitis, arthritis, synovitis, endocarditis, urinary and genital tract infections, skin lesions, phlebitis, meningitis and food poisoning. Therefore, *S. aureus* is known as the most important pathogen found in *Staphylococcus* genus for human and animals (26, 28).

S. aureus has several virulence factors such as microcapsule, surface proteins, exotoxins, hemolysins and enzymes playing an important role in the pathogenesis of diseases (26, 28). Besides these factors, *S. aureus* can produce a viscous extracellular exopolysaccharide referred as slime or biofilm. Staphylococcal biofilms are encased in an extracellular matrix composed of proteins, polysaccharides, extracellular DNA and presumably host factors (3, 8, 20). This extracellular polysaccharidic layer enhances the adhesion of agent to host tissues and plastic or

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metallic surfaces; contributes to the evasion of bacteria from immunological defense mechanisms; and complicates the pathogen eradication (18). The matrix protects bacteria against high antimicrobial concentration because it impairs the access of antibiotics to bacterial cells and limits the antimicrobial agent diffusion through biofilm (18, 30). Also, extracellular slime production by bacteria provides the protection of cells from the phagocytic activity of macrophages and bactericidal activity of neutrophils (6). Therefore, it has been accepted that slime positive bacterial strains are more often related to significant infections than slime negative strains (3, 4, 7, 24).

A number of tests are available to detect slime production by staphylococci, including tissue culture plate (11), microplate test (27), standard tube method (11), Congo red agar (17), bioluminescent assay (23) and scanning electron microscopy (15) or fluorescence microscopic examination (33). But, most frequently the Standard tube test and Congo red agar methods are used. By applying these *in vitro* methods to establish the criteria for slime production, strains have been classified as slime producing and non-slime producing (31).

Recently, many studies have mainly focused on the biofilm formation of human clinical isolates (5, 9, 29) and bovine or ovine mastitis isolates (1, 12, 16, 22, 24). But, the investigations about the slime factor production of *S. aureus* isolates obtained from various animal samples are limited (14, 31, 32). Therefore, in the present study, the determination of slime positivity in *S. aureus* strains isolated from different animal clinical sources, including bovine milk, bovine uterus swab, dog ear swab, dog skin swab and chicken synovial fluid, was aimed. Also, the antibiotic resistance of slime positive and negative strains was statistically compared.

Materials and Methods

S. aureus strains

In this study, 50 *S. aureus* isolates were used for detection of their slime factor production and antibiotic resistance. The isolates were obtained from bovine milk with mastitis (n=10 isolates), bovine uterus swabs with fertility problems (n=10 isolates), dog ear swabs with otitis externa (n=10 isolates), dog skin swabs with skin lesions (n=10 isolates) and chicken synovial fluids with synovitis (n=10 isolates). The isolation and identification of *S. aureus* from samples was performed by using standard cultural methods. For this purpose, clinical samples were directly inoculated onto blood agar media (Oxoid CM0271) containing 7% of sheep blood and plates were incubated aerobically at 37 °C for 24-48 hours. After the incubation, each different colony was examined macroscopically (colony morphology, haemolysis, pigment production) and microscopically (Gram staining). Then, each different colony was sub-cultured in blood agar containing 7% of sheep blood and tryptone soya broth (Oxoid CM0129) for further characterization. Oxidase, catalase, Iam and tube coagulase, Voges Prouskauer, fermentation of glucose and mannitol, DNase activity and resistance to bacitracin tests were used for identification of isolates (19, 28).

Detection of slime production

Slime production of 50 *S. aureus* strains was determined by using Congo red agar and standard tube methods. *Staphylococcus epidermidis* ATCC 35984 and *S. epidermidis* ATCC 12228 were used as positive and negative control strains, respectively.

Congo red agar was prepared according to method described by Freeman et al. (17). The composition of medium was Brain Heart Infusion Broth (Oxoid CM0225) 37 g/l, sucrose 50 g/l, agar10 g/l and Congo red 0.8 g/l. The Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121 °C for 15 min) separately and was added when the agar had cooled to 55 °C. The strains were inoculated onto medium and incubated under aerobic conditions for 24 hours at 37 °C. After the incubation, black coloured colonies were regarded as slime positive and pink coloured colonies as slime negative. The experiment was performed in triplicate and repeated three times.

The standard tube method developed by Christensen et al. (11) was used as the second method in the detection of slime production. A loopful of organisms from a single colony in pure culture on blood agar plate containing 7% of sheep blood was inoculated into 10 ml of tryptone soya broth. The tubes were incubated at 37 °C for 18 hours. Then, the contents were decanted and tubes were stained with 1% safranine for 7 min. A positive result was indicated by the presence of an adherent film of stained material on the inner surface of the tube. Presence of stained material at the liquid-air interface alone was not regarded as indicative of slime production. The test was repeated three times for each isolate.

Antibiotic susceptibility test

Resistance to different antibiotics was detected by using standard E-test method. E-test strips were used according to manufacturer instructions (AB Biodisk, Sweden) to determine the minimum inhibitory concentrations (MICs) in µg/ml. Bacterial suspensions were prepared by selecting colonies from overnight cultures on blood agar containing 7% of sheep blood. The pour colonies were suspended in sterile tubes containing 2 ml of saline solution (0.85% NaCl) of equal turbidity to 0.5 McFarland standard. The suspensions were inoculated onto Mueller Hinton agar (Oxoid CM0337) plates using sterile swabs. After the inoculation, penicillin G (PG), cephalothin (CE), tetracycline (TC), erythromycin (EM), trimethoprim/ sulphamethoxazole (TS), rifampin (RI), enrofloxacin (EF), oxacillin (OX) and gentamicin (GM) E-test strips were applied to plates and plates were incubated aerobically at 35 °C for 24 hours. Following the incubation, the MIC values were recorded and results were evaluated according to Clinical and Laboratory Standards Institute (CLSI) (13). *S. aureus* ATCC 29213 was used as control strain.

Statistical analysis

Chi-square test was used between two different methods applied for detection of slime factor production. Antibiotic resistance of slime positive and slime negative strains was compared by Chi-square and Fisher's exact chi-square tests. A probability of $p<0.05$ was considered statistically significant.

Results

Slime production

Of 50 *S. aureus* strains tested, 21 (42%) and 18 (36%) were determined as slime positive in the Congo red agar and standard tube methods, respectively. A significant difference was not found between the two different methods ($p>0.05$).

Antibiotic susceptibility test

In 50 *S. aureus* strains, the highest resistance rate was against penicillin G (20%), followed by cephalothin (16%), oxacillin (16%) and tetracycline (14%). Only one (2%) of the tested strains was resistant to enrofloxacin, while resistance to erythromycin, trimethoprim/sulfamethoxazole, rifampin and gentamicin was not determined in any strains (Table 1). Resistance to penicillin G, cephalothin and oxacillin in slime positive strains was significantly higher than in slime negative strains ($p<0.05$) (Table 2).

Table 1. Antibiotic resistance of 50 *S. aureus* isolates obtained from various animal clinical samples.

Antibiotic	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Range (µg/ml)	Breakpoint (µg/ml)	Resistance	
					n	%
PG	0.064	32	0.002-32	≥0.25	10	20
CE	0.125	256	0.016-256	≥32	8	16
TC	0.5	256	0.016-256	≥16	7	14
EM	0.38	0.5	0.016-0.75	≥8	0	0
TS	0.047	0.25	0.002-0.035	≥4/76	0	0
RI	0.008	0.016	0.002-0.023	≥4	0	0
EF	0.125	0.25	0.002-32	≥4	1	2
OX	0.25	256	0.016-256	≥4	8	16
GM	0.75	1	0.016-2	≥16	0	0

MIC: Minimum inhibitory concentration, PG: Penicillin G, CE: Cephalothin, TC: Tetracycline, EM: Erythromycin, TS: Trimethoprim/sulfametoxazole, RI: Rifampin, EF: Enrofloxacin, OX: Oxacillin, GM: Gentamicin

Table 2. Difference between resistance to antibiotics and slime production in *S. aureus* strains obtained from various animal clinical samples

Antibiotic	Slime Positive (n=21)		Slime Negative (n=29)		Statistically Significant*
	n	%	n	%	
PG	8	38.1	2	6.9	p<0.01
CE	6	28.6	2	6.9	p<0.05
TC	5	23.8	2	6.9	p>0.05
OX	7	33.3	1	3.4	p<0.01

Difference between antibiotic resistance of slime positive and slime negative strains.
PG: Penicillin G, CE: Cephalothin, TC: Tetracycline, OX: Oxacillin

Discussion

This study investigated the slime factor positivity and antibiotic resistance in 50 *S. aureus* strains isolated from different animal clinical samples.

Slime factor production has been considered as an important virulence factor in the pathogenesis of infections because biofilm-associated microorganisms show an innate resistance to antibiotics, disinfectants and clearance by host defense mechanisms (6, 18, 30). Researchers have used various methods to detect slime production by staphylococci (11, 17, 21, 31). We tested 50 *S. aureus* strains by two *in vitro* screening procedures for their slime production ability. Slime production was detected in 42% (21/50) and 36% (18/50) of the *S. aureus* strains by the Congo red agar and standard tube assays, respectively. Similar to other studies (11, 14, 17, 21, 31), statistical analysis did not show a significant difference between the two different methods (p>0.05).

Although limited research exists about biofilms in animals, biofilms are believed to be involved in many diseases such as pneumonia, liver abscesses, enteritis, arthritis, synovitis, wound infections and mastitis (24, 25). The relationship between slime production and virulence has been emphasized in several publications (3, 4, 7, 29), indicating that slime positive bacterial strains are more often related to significant infections than slime negative strains. Türkyılmaz and Eskiizmirliler (31) found the slime factor positivity as 77.7% in 90 coagulase positive staphylococci (CPS) strains isolated from various animal clinical samples by Congo red agar method. Similarly, the slime factor production of 108 *S. aureus* strains was determined as 33.3% by Çiftçi et al. (14). In another study, slime production in 90 CPS strains was reported as 77.8% (32). In our study, 50 test strains were obtained from different clinical materials, including bovine milk, bovine uterus swab, dog ear swab, dog skin swab and chicken synovial fluid, and slime positivity was determined as 42% in these strains. This result was consistent with the findings of similar studies related to slime factor production in *Staphylococcus* strains.

Antibiotics have been commonly used for the treatment of bacterial infections. But, the resistance developed against antimicrobials has complicated the fight to infectious agents in the whole world. Therefore, bacterial identification and susceptibility tests are important for selecting the appropriate antimicrobial agent for treatment. In a research similar to our study, Türkyılmaz and Eskiizmirliler (31) reported that the highest resistance rate was found against penicillin in all CPS strains. Similarly, Çiftçi et al. (14) determined the high resistance rates to penicillin, oxytetracyline and danofloxacin in *S. aureus* strains. In our tested strains, maximum resistance was observed against penicillin G (20%), followed by cephalothin (16%), oxacillin (16%) and tetracycline (14%). Penicillin G is widely used as main antibiotic group in animals for the treatment and prevention of several diseases in Turkey; therefore, a high rate of resistance to this antibiotic was not unexpected. Similarly, the reasons of resistance to cephalothin and tetracycline may be intensive, prolonged and regular use of these antibiotics in large and small animal practices for treatment of various conditions in Turkey.

However, high oxacillin resistance in tested strains was noteworthy. Although penicillinase or β-lactamase resistant penicillins such as oxacillin and methicillin are not used in veterinary medicine in Turkey, the common use of cloxacillin belongs to this group antibiotics, especially in mastitis, may be related to this phenotypic resistance rate.

Several authors have shown in *in vitro* experiments that bacteria growing in a biofilm are 10-1000 times more resistant to antimicrobial agents when compared with planktonic growing bacteria of the same strain (2, 10, 25). In a study, higher resistance rate against tested antibiotics was relatively determined in slime producing CPS strains isolated from various animal clinical materials than non-slime producing strains (31). In the present study, we statistically compared the antibiotic resistance of slime positive and negative *S. aureus* strains. Resistance to penicillin G, cephalothin and oxacillin in slime positive strains was significantly higher than in slime negative strains ($p<0.05$). Similarly, resistance to tetracycline was higher in slime positive strains, but no statistically significant difference was detected among slime positive and negative strains ($p>0.05$). The minority of tetracycline resistant strains may be the cause of this result. These findings were consistent with other studies.

In conclusion, the slime factor producing was evaluated in *S. aureus* strains isolated from various animal clinical samples. According to our results, the resistance to some antibiotics commonly used in Turkey was determined in these slime positive strains. There is a need for awareness that slime positive bacteria can be more pathogen and cause persistent infections. Also, it should be considered that whatever the mechanisms of resistance are, when treating a bacterial infection in animals, it is important to select the correct antibiotics.

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