Turk J Vet Anim Sci
30 (2006) 201-206

Research Article

Detection of Slime Factor Production and Antibiotic Resistance in Staphylococcus Strains Isolated from Various Animal Clinical Samples

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Received: 15.04.2005

Abstract: In this study, the production of slime factor in 180 Staphylococcus spp. (90 coagulase-positive Staphylococcus (CoPS) and 90 coagulase-negative Staphylococcus (CoNS)) isolated from various animal clinical specimens was investigated with Congo Red Agar (CRA), Microplate (MP), and Standard Tube (ST) tests and the results were compared to each other. The rate of production of slime factor in all of the Staphylococcus spp. investigated with CRA, MP, and ST tests were 61.1%, 55.5%, and 50.5%, respectively. The existence of Staphylococcus spp. resistance against various antibiotics was also determined by the agar disk diffusion method. The percentage of resistance against penicillin, methicillin, ampicillin, and gentamycin in slime-producing (SP) Staphylococcus spp. was 49.0%, 24.5%, 23.6%, and 13.6%, respectively, whereas for non-slime-producing (NSP) strains it was 42.9%, 15.7%, 14.2%, and 12.9%, respectively. The comparison of SP strains with NSP strains revealed that SP strains had more resistance to those antibiotics. It was determined that all of the Staphylococcus spp. were susceptible to vancomycin. The results observed from this study showed that there was no statistically significant difference between the tests applied (CRA, MP, and ST tests; $X^2 = 0.28$). In conclusion, the CRA test could be used for the detection of slime production in Staphylococcus spp. because it is reliable and practical.

Key Words: Slime production, detection techniques, Staphylococcus spp.

Çeşitli Hayvansal Klinik Örneklerden Izole Edilen Staphylococcus Suşlarında Slime Faktör Üretimi ve Antibiyotik Dirençliliğinin Saptanması

Özet: Çeşitli hayvansal klinik örneklerden izole edilen toplam 180 adet Staphylococcus spp. (90 koagulaz pozitif Staphylococcus (CoPS), 90 koagulaz negatif Staphylococcus (CoNS)'nin slime faktör üretimi Kongo Red Agar (CRA), Mikropleyt (MP) ve Standart Tüp (ST) testleri kullanılarak incelendi ve test sonuçları birbirleriyle karşılaştırıldı. Staphylococcus spp.'lerde CRA, MP ve ST testleri ile incelenen slime faktör üretimi sırasıyla % 61,1, % 55,5 ve % 50,5 oranında pozitif bulundu. Aynı zamanda, Staphylococcus spp.'nin çeşitli antibiyotiklere karşı dirençlilikleri agar disk diffuzyon testi ile araştırıldı. Penisilin, metisillin, ampisillin ve gentamisin gibi antibiyotiklere karşı direnç oranları sırasıyla slime faktör üreten (SP) suşlarda % 49,0, % 24,5, % 23,6, % 13,6 olarak bulunurken slime faktör üretmeyen (NSP) suşlarda % 42,9, % 15,7, % 14,2, % 12,9 olarak tespit edildi. SP ve NSP suşlar antibiyotik dirençliliği yönünden karşılaştırıldığında SP suşların, NSP'lara oranla daha yüksek oranlarda antibiyotik dirençliliği gösterdikleri belirlendi. İncelenen tüm Staphylococcus spp.'leri vankomisine karşı duyarlı olarak bulundu. Test sonuçlarına göre, CRA, ST ve MP testleri arasında istatistiksel açıdan önemli bir farklılık saptanmadı ($X^2 = 0,28$). Bu nedenle, Staphylococcus spp.'de slime üretiminin belirlenebilmesi için CRA testinin pratik ve güvenilir bir teknik olduğu sonucuna varıldı.

Anahtar Sözcükler: Slime üretimi, saptama teknikleri, Staphylococcus spp.

Introduction

In recent years, staphylococci have emerged as important pathogens that can cause substantial economic loss. Staphylococci are the most notable pathogens in foreign body-related infections (1,2).

Some strains excrete a glycocalyx cover that helps them resist humoral and cell-mediated immunological mechanisms, as well as antibiotics. This adherent cover, or "slime", allows the bacteria to adhere to surfaces (3). Slime production could play an important role in the

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adherence of these microorganisms to mucous epithelia (4). Adherence is evidenced by slime production, and this could be quite important for colonization, and hence, its virulence factor (3). Slime can reduce the immune response and opsonophagocytosis, thereby interfering with host defense mechanisms. The ability of an organism to produce slime is significantly associated with its capability to produce diverse illnesses (5).

Coagulase-negative staphylococci (CoNS), which have long been considered relatively inoffensive contaminants, are now recognized to be the cause of several human and animal infections in which their pathogenic role is well established. CoNS are frequently associated with infections in humans following surgical interventions, particularly implants, but are also responsible for disease in intact immunocompetent patients (1,2).

Many strains of cougglase-positive staphylococci (CoPS) and CoNS strains form an adherent bacterial film (slime) whose formation has been implicated as a factor of bacterial virulence. It is postulated that this is a mechanism by which the bacterium adheres to and colonizes certain prosthetic devices. Several reports have shown that CoNS produce enormous amounts of this extracellular substance during growth on inert surfaces, facilitating the adhesion of microorganisms to those surfaces (6,7). Some investigators (8,9) reported that CoNS strains isolated from clinically significant infections produced more slime than saprophytic strains. Similar implications for virulence of biofilm–forming strains are generally thought to exist in *S. aureus* as well (1,4).

Biofilm formation may be determined in several different ways, but most frequently it is demonstrated with the Standard Tube (ST) test, in which bacterial film lining a culture tube is stained with a cationic dye and visually scaled; with the Microplate (MP) test, in which the optical density (OD) of the stained bacterial film is determined spectrophotometrically; or with the Congo Red Agar (CRA) test (4,6). The CRA test was proposed as an alternative to the MP test for screening staphylococcal isolates for slime production. This technique is based on culturing staphylococcal strains on a solid agar medium supplemented with Congo Red dye (4). By applying these in vitro methods to establish the criteria for slime production, strains have been classified as slime producing (SP) and non-slime producing (NSP).

Biofilm forming strains adhere better to surfaces (polystyrene and other materials) and have lower antibiotic susceptibility. Moreover, these traits make them better at eluding host defense systems (10,11). The mechanisms by which the biofilm provides bacteria with higher antibiotic resistance have yet to be completely elucidated (1,12).

The purposes of this study were: 1. To isolate Staphylococcus strains from a variety of clinical samples; 2. To investigate slime production of isolated strains with 3 different methods; 3. To evaluate the occurrence of slime production in both CoNS and CoPS; 4. To determine the sensitivity patterns of CoPS and CoNS strains against different antibiotics, as well as the probable relationship between antibiotic resistance and slime production of isolated strains.

Materials and Methods

Bacterial isolations

In this study, 180 strains of Staphylococcus spp. from bovine mastitis, dogs with otitis externa, and chickens with various infections were examined. For the first isolation, the samples taken from clinical materials (milk, ear swab specimens, lung, trachea, heart, liver, spleen, synovial fluids) were directly streaked on to 7% sheep blood agar (Oxoid) and incubated aerobically at 37 ºC for 48 h. After the incubation period, staphylococci were identified on the basis of colony characteristics (Gram staining, catalase production, oxidase test, and O-F test with glucose), susceptibility to bacitracin disk (0.04 U), and pigment production. Gram-positive, cluster forming, catalase positive, oxidase negative, resistant to bacitracin, and fermentative strains of staphylococci were identified according to standard methods (13). As some pathogenic staphylococci can be negative to the slide coagulase test but positive to the tube test, the tube coagulase test was used with strains that were identified as staphylococci (using rabbit plasma) (13).

Slime production

The isolates were simultaneously examined by 3 methods:

Detection of slime by Congo Red Agar (CRA) method: The method developed by Freeman et al. (14) was used in this study. The composition of medium (CRA) was

brain heart infusion broth (BHIB) 37 g/l, sucrose 50 g/l, agar 10 g/l, and Congo Red 0, 8 g/l. The Congo Red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121 ºC for 15 min and was added when the agar had cooled to 55 ºC. Plates were inoculated and incubated aerobically at 37 ºC for 24 h. Isolates that produced black colonies with dry crystalline consistency were regarded as slime positive, whereas those showing pink colonies were slime negative.

Detection of slime with the Standard Tube (ST) method: The qualitative assay for biofilm formation was performed according to the method described by Christensen et al. (8). For this purpose, a loopful of organisms from a single colony in pure culture on blood agar plate was inoculated onto 5 ml of trypticase soy broth (TSB) (Oxoid). The inoculated tubes were incubated at 37 ºC. After 24 h, the contents were decanted. The tubes were then 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.

Detection of slime by Microplate (MP) method: Quantitative determination was carried out with the MP method proposed by Pfaller et al. (7) using tissue culture plates with 96 flat-bottomed wells. Each well was filled with 0.2 ml of 10^5 CFU/ml of a bacterial suspension in TSB. After 48 h incubation in aerobiosis at 37 ºC, the contents were aspirated and the plates were washed twice with phosphate-buffered saline (PBS; pH: 7.2). The wells were stained with 0.25% safranine for 30 s. The plates were read in an enzyme-linked immunosorbent assay (ELISA) reader (BioTek, ELx808) to 490 nm. Sterile TSB was used as a negative control. All the experiments were repeated at least twice, the values of optical density were then averaged. A 3-grade scale was used to evaluate the strain's slime producing ability; (-): ODs < 0.500; (+): ODs 0.500-1.500; (++): ODs > 1.500.

Susceptibility Tests

For the susceptibility test, isolates were suspended in TSB and the suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard. The antibiotic susceptibility test was performed with the agar disk diffusion method (15). Isolates were categorized as susceptible, moderately susceptible, and resistant, based upon interpretive criteria developed by the Clinical and Laboratory Standards Institute (CLSI) (16). Penicillin (10 IU), methicillin (5 µg), gentamycin (10 µg), ampicillin (10 µg), and vancomycin (30 µg) were used for antimicrobial susceptibility tests.

Statistical Evaluation

Significant differences between CoPS, CoNS, and CRA, ST, and MP tests were determined by chi-square test (17).

Results

Bacterial isolations

According to the CRA test, the origin of isolates and distribution of the Staphylococcus spp. are given in Table 1.

Table 1. According to the CRA test, origin of isolates and distribution of the Staphylococcus spp.

Staphylococcus spp.	Origin of isolations							
	Chicken		Cattle		Dog		TOTAL	
	N	n	N	n	N	n	N	n
CoPS	24	16	34	30	32	24	90	70
CoNS	36	18	26	10	28	12	90	40
TOTAL	60	34	60	40	60	36	180	110

N: the number of isolated strains
n: number of slime producing strains

Of 180 staphylococci isolates, 90 isolates were identified as CoPS and the remaining 90 isolates were CoNS.

Slime Production

The summarized results of CRA, MP, and ST tests, and the distribution of slime production in staphylococci strains isolated from different clinical materials are shown in Table 2.

Percentage of slime production of CoPS and CoNS strains, and all of the Staphylococcus spp. measured as positive with CRA, MP, and ST tests were as follows: 77.7%, 74.4%, and 66.6%; 44.4%, 36.6%, and 34.4%; 61.1%, 55.5%, and 50.5%, respectively.

Antibiotic Resistance

The antibiotic susceptibility test results of the SP and NSP staphylococci strains collected from various clinical samples were shown in Table 3.

Percentage of resistance against selected antimicrobial agents (penicillin, methicillin, ampicillin, gentamycin) of SP (n = 110) and NSP (n = 70) strains was 49%, 24.5%, 23.6%, 13.6%, and 42.9%, 15.7%, 14.2%, and 12.9%, respectively. Maximum resistance was noted against penicillin, whereas all the strains were susceptible to vancomycin.

Statistical Evaluation

There was no statistically significant difference between the tests applied (CRA, MP, and ST tests; X^2 = 0.28).

Table 2. The results of slime factor production.

Test	Slime Production					
	CoPS (=90)		CoNS (=90)		Total (=180)	
	n	%	n	%	n	%
CRA	70	77.7	40	44.4	110	61.1
MP	67	74.4	33	36.6	100	55.5
ST	60	66.6	31	34.4	91	50.5

n: number of slime producing strains

Table 3. In vitro antibiotic susceptibility of SP and NSP staphylococci isolates.

Antibiotic	Number of SP resistant isolates				Number of NSP resistant isolates				Number of total resistant isolates			
	CoPS (=70)		CoNS (=40)		CoPS (=20)		CoNS (=50)		SP (=110)		NSP (=70)	
	N	%	n	%	n	%	n	%	n	%	n	%
Penicillin	42	60.0	12	30.0	12	60.0	18	36.0	54	49.0	30	42.9
Gentamycin	7	10.0	8	20.0	4	20.0	5	10.0	15	13.6	9	12.9
Ampicillin	18	25.7	8	20.0	4	20.0	6	12.0	26	23.6	10	14.2
Methicillin	21	30.0	6	15.0	5	25.0	6	12.0	27	24.5	11	15.7
Vancomicin	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0

n: number of resistant isolates

Discussion

In the present study, slime factor production of 90 CoPS and 90 CoNS was investigated with 3 different traditional methods. Then the resistance of SP and NSP staphylococci against various antibiotics was measured.

It has been thought that testing for biofilm formation could be a useful marker for the pathogenicity of staphylococci (8,9). However, some authors found little or no correlation between biofilm formation, in vitro, and clinical outcome of the infection (18). Baselga et al. (4) reported that positivity in slime production in various clinical specimens was 47.7%. Ammendolia et al. (19) reported that positivity in slime production of CoPS and CoNS strains was 88.9% and 47.8%, respectively. Mohan et al. (20) found the slime production of *S. epidermidis* strains to be positive (48.7%). In this study, CRA, MP, and ST methods were used for slime detection. Slime production that was defined as positive with CRA, MP, and ST tests in CoPS and CoNS strains was 77.7%, 74.4%, and 66.6%, and 44.4%, 36.6%, and 34.4%, respectively.

Woznicova et al. (21) compared the sensitivity and specificity of the CRA and ST tests and found the CRA method to be more specific. In this study, the slime production of all staphylococci as determined with CRA, MP, and ST tests was 61.1%, 55.5%, and 50.5%, respectively. The main limitation of the tube test is its qualitative nature; the ST test displayed a good correlation with the MP and CRA method for strongly positive biofilm isolates. However, it is very difficult to evaluate weak biofilm-producing strains with the ST method. The tube adherence assay is simple and easy to perform, but reading the results may be difficult, especially if the observer is not familiar with this technique (8,22). In the present study, however, there was no statistically significant difference between ST, CRA, and MP tests ($X^2 = 0.28$). For that reason, the CRA test was preferred for the detection of slime producing staphylococci.

Bacterial identification and susceptibility tests are important for selecting the appropriate antimicrobial agent for treatment. In the present study, results of antibiotic susceptibility tests showed multi-drug resistance. Variability in sensitivity and resistance patterns were similar to those reported by Goel et al. (23), Pathak et al. (24), and Mohan et al. (20). Maximum resistance was observed against penicillin in all staphylococci isolates (SP = 49%; NSP = 42.6%), followed by methicillin (SP = 24.5%; NSP = 15.7%), ampicillin (SP = 23.6%; NSP = 14.2%), and gentamycin (SP = 13.6%; NSP = 12.9%). All isolates investigated in this study were susceptible to vancomycin. The susceptibility to vancomycin in the investigated strains emphasized its importance. Davenport et al. (9) had mentioned a link between the production of slime and the resistance of infection. Diaz-Mitoma et al. (25) also found an association between antibiotic failure and slime production. In the present study, in slime producing strains, high resistance was determined against all antibiotics tested.

Keskin et al. (26) reported that CoNS strains isolated from cow milk samples with mastitis and of chicken lesions were more slime productive and adherent compared to the strains isolated from healthy animals. In this study, it was determined that CoNS strains isolated from various cases had slime production capability by using different methods. Çıtak (27) et al. investigated slime production of CoPS and CoNS isolated from raw milk samples using the CRA method and they reported that slime production was an important virulence factor for identifying pathogenic staphylococci; the results of the present study are in agreement.

In conclusion, slime production was found to be a factor of virulence in the identification of staphylococcal infections. The CRA method was a practical, reliable, and inexpensive technique for the investigation of slime producing staphylococci strains. Further studies based on molecular methods for the investigation of slime factor production, in both coagulase positive and coagulase negative staphylococci, should be conducted.

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