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Research Article

Determination of Virulence Factors of Staphylococci Isolated from Bovine Mastitis [1]

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Abstract

Staphylococcal mastitis is one of the major economic problems of cattle. The aim of this study was to identify *Staphylococcus* species that cause mastitis, to examine the virulence factors of these species and to determine the relation between these factors with the pathogenic and nonpathogenic species. In the study, 37 *S. aureus,* 13 *S. hyicus,* 9 *S. simulans,* 8 *S. chromogenes,* 5 *S. lentus,* 5 *S. epidermidis,* 2 *S. haemolyticus,* 2 *S. hominis,* 1 *S. auricularis,* 1 *S. warneri,* 1 *S. sciuri* were isolated and identified. The 41.6% of strains were determined as coagulase positive. In the coagulase positive strains, the rate of protein A, DNase, TNase, capsul, hemolyse, staphylokinase, slime (in agar), biofilm (microdilution) and hemagglutination characteristics were found 71.4%, 48.6%, 11.4%, 40%, 97.1%, 40%, 28.6%, 37.1% and 17.1%, respectively. In the coagulase negative strains, the rate of these characteristics were found 10.2%, 12.2%, 2%, 8.2%, 82%, 32.7%, 12.2%, 12.2% and 10.2%, respectively. The methicillin resistance rates in the coagulase positive and negative strains were determined as 2.9 and 16.3%. In conclusion, it was of the opinion that animals are potential carriers of staphylococcus strains that are pathogen for human.

Keywords: *Bovine, Mastitis, Staphylococci, Virulence*

Sığır İzolatı Stafilokokların Virülens Faktörlerinin Belirlenmesi

Özet

Stafilokokal mastitisler sığırlar için en önemli ekonomik problemlerden birisidir. Bu çalışmada mastitise neden olan stafilokok türlerinin sahip olduğu virülens faktörlerinin belirlenmesi ve patojenik ile apatojenik türler arasında bu faktörlerin ilişkisinin araştırılması amaçlanmıştır. Çalışma kapsamında 37 *S. aureus,* 13 *S. hyicus,* 9 *S. simulans,* 8 *S. chromogenes*, 5 *S. lentus,* 5 *S. epidermidis,* 2 *S. haemolyticus,* 2 *S. hominis,* 1 *S. auricularis,* 1 *S. warneri* ve 1 *S. sciuri* izole ve identifiye edilmiştir. Bu izolatların %41.6'sı koagülaz pozitif olarak belirlenmiştir. Koagülaz pozitif izolatların protein A, DNase, TNase, kapsül, hemoliz, stafilokinaz, slaym (agar'da), biyofilm (mikrodilüsyon) ve hemaglütinasyon özellikleri sırasıyla %71.4, %48.6, %11.4, %40, %97.1, %40, %28.6, % 37.1 ve %, 17.1 olarak tespit edilmiştir. Koagülaz negatif izolatlarda ise bu oranlar sırasıyla %10.2, %12.2, %2, %8.2, %82, %32.7, %12.2, %12.2 ve %10.2 bulunmuştur. Koagülaz pozitif ve negatif izolatlar arasında metisilin direnç oranı %2.9 ve %16.3 olarak saptanmıştır. Sonuç olarak, stafilokok taşıyıcısı hayvanların insanlar için muhtemel infeksiyon kaynağı olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Sığır, Mastitis, Stafilokok, Virulens*

INTRODUCTION

Microbial originated mastitis is considered to be a disease of cattle that causes the vast yield loss in the whole world. Mastitis may occur as acute and chronic with regard to its clinical and subclinical character and yields loss in cattle. Mastitis infections may also end up with economic and production losses and treatment costs can pose a risk in terms of human health. In a review of the etiology of mastitis, these cases have been reported to be isolated from the more than 150 species of bacteria [1]. Clinical signs of mastitis are not specific for the etiologic agents with few exceptions, so it is important to distinguish microbial origin for the treatment of mastitis. Staphylococci are on the first

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line of mastitis agents and incidence of staphylococcal mastitis is seen in the ratio of 60-70% [2,3].

Microorganisms in staphylococcal infections by binding to the cell surface are dependent on the ability to bear the surface adhesins to colonize tissues. Adhesins of staphylococci, which are the specific receptors for extracellular matrix proteins of the host tissues, are surface proteins. These include fibronectin, fibrinogen, collagen, elastin, a plasminogen, and vitronectin. Staphylococci bare facilitating connection to the traumatized tissue and fibrin blood clots promoted by fibrinogen binding protein (Fbp). In most strains of *Staphylococcus aureus*, there are both fibronectin and fibrinogen binding protein [4]. *S. epidermidis* has solely Fb, a single fibrinogen binding protein. This protein is similar to the fibrinogen receptor of *S. aureus*.

In Turkey, although there are studies dealing with the prevalence of mastitis for the role of *Coagulase Negative Staphylococci*, the studies about virulence factors and their epidemiological aspects are insufficient. In this study, staphylococcal mastitis in cattle in province of Aydin was aimed to be examined in detail. The scopes of this study include precise identification of *Staphylococcus* species, identification of virulence factors, the relationship between nonpathogenic species and epidemiology of infection also.

MATERIAL and METHODS

In this study, 84 Staphylococci strains isolated from milks samples of 120 cows with clinical and subclinical mastitis and 80 healthy cows in different herds in the Aydın region were examined. Milk samples were evaluated by California Mastitis test. The stage of mastitis infection was determined according to the California Mastitis test procedures.

Isolation and Identification of Staphylococci

Milk samples were taken with sterile loop in the volume of 50 µL and inoculated onto Blood Agar (Bacto agar, Difco®, Detroit). The culture media were incubated at 37°C for 24-48 h. The colony morphology of the isolates were examined and staphylococci like (creamy and white or yellow pigmented) colonies were separated for further identification.

The isolated bacterial strains were identified as *Staphylococcus* sp., by the fact that catalase test positive, oxidase test negative and oxidation/fermentation (OF) test positive [5]. Species-specific identification was carried out with a commercial identification kit (Api-Staph, Bio Merieux®, Lyon, France). *Staphylococcus* species were identified with test procedure recommended by the manufacturer. *Staphylococcus* strains were evaluated by the utilization of D-glucose, D-fructose, D-mannose, D-maltose, D-lactose, D-trehalose, D-mannitol, xylitol, D-melibiose, D-raffinose, D-xylose, D-saccharose, methyl a-D-glucopyranoside, N-acetyl-glucosamine acid production, nitrate reduction, and alkaline phosphatase, acetyl-methyl-carbinol, arginine production was examined dihydrolase and urease tests.

Determination of Virulence Factors

The virulence factors of the *Staphylococcus* isolates were determined with regard to coagulase, protein A, DNase, TNase, capsule, slime and biofilm, hemolytic activity, staphylokinase, hemagglutination, methicillin resistance tests.

Coagulase test was performed via tube and slide coagulase tests. In tube coagulase test, 0.5 mLrabbit plasma (bioMerieux®) and 0.1 mL broth of bacterial culture were added together and coagulation was determined as positive reaction. Clumping factor was determined in slide coagulase test, as 1 loop of bacterial colony was homogenized with 50 µL physiological saline, and then 50 µL of rabbit plasma (bioMerieux®) was added. The developing coagulation was determined as positive reaction [5].

Protein A test was applied via commercial latex agglutination test (Staphytect Plus, Oxoid®) and performed by manufacturer's recommendation.

DNase test was performed by inoculating bacterial strains onto DNase agar. After incubation of 3 days at 37°C, one droplet of 1 N HCl was added onto agar plate, a wide pale formation on the agar was determined as positive reaction [5].

For TNase test, agar containing DNA and 1% toluidine blue was prepared. 100 µL of bacterial culture was added onto the wells on TNase agar. After incubation period, brilliant pink colour formation was determined as positive reaction [5].

Capsule formation was determined via Duguid staining method [6].

Slime formation was determined via development of black colonies on Congo Red agar [7].

Biofilm formation was determined via staining of safranine in microplates [8].

Rabbit and horse erythrocytes were used for hemolytic activity tests.

Staphylokinase activity was determined via screening of pale zone on agar that contains fibrinogen and dog serum [3].

Hemagglutination activity was determined via agglutination of erythrocytes on microplate wells [9].

Methicillin resistance was determined via disc diffusion method on Brain-Heart infusion agar [5].

Statistical Evaluation

The relationship among Coagulase positive, Coagulase

negative strains and virulence factors were statistically evaluated by Chi-square test.

RESULTS

Isolation and Identification of Staphylococci

As a result of isolation procedures in a selective and differential medium, a total of 84 *Staphylococcus* strains were isolated from 120 mastitic milk samples and 80 normal milk samples. The isolated strains were identified as in the number of 37 *S. aureus*, 13 *S. hyicus*, 9 *S. simulans*, 8 *S. chromogenes*, *S. lentus* 5, 5 *S. epidermidis*, 2 *S. haemolyticus*, 2 *S. hominis*, 1 *S. auricularis*, 1 *S. warneri* and 1 *S.* sciuri *(Table 1)*.

The virulence factors of isolates from animals with mastitis and healthy animals were shown in *Table 2* and *Table 3*.

Statistics

Coagulase positive and coagulase negative strains with Chi-square test of significance is based on their virulence factors are given in *Table 4*.

Table 1. *Staphylococcus species isolated from healthy and sick animals*

Strains	Isolation Number	Isolation from Healthy Animals		Isolation From Sick Animals	
		n	%	n	%
S. aureus	37	2	5.4	35	94.5
S. hyicus	13	0	0	13	100
S.simulans	9	0	0	9	100
S. chromogenes	8	5	62.5	3	37.5
S. lentus	5	5	100	0	0
S. epidermidis	5	1	20	4	80
S. haemolyticus	2	0	0	2	100
S. hominis	2	1	50	1	50
S. auricularis	1	1	100	0	0
S. warneri	1	1	100	0	0
S. sciuri	1	0	0	1	100

Protein, DNase and the results of the capsule test were found to be very important. TNase, hemolysis, slime and biofilm formation test results were determined to be significant when the coagulase positive and negative strains are evaluated together. Analyses of staphylokinase and hemagglutination tests were not found significant statistically.

DISCUSSION

Bovine mastitis infections cause heavy economic losses worldwide. Mastitis in Turkey is one of the most important problems of dairy cattle economically. Especially mastitis incidence can be observed up to 40% in dairy industry. Primary factors of mammary infections are often characterized in clinical or subclinical forms of Gram positive bacteria. Staphylococcal mastitis presents the first in line to 60-70%. Staphylococci are resistant bacteria commonly found in nature and natural conditions [2,3]. In this study, a detailed examination of staphylococcal mastitis in cattle is basically intended.

In this study, 84 staphylococcal strains were isolated in the ratio of 44 % *S. aureus*, 15,47%, *S. hyicus*, 10.7% *S. simulans*, 9.5% *S. chromogenes*, 5.95% *S. lentus*, 5.95% *S. epidermidis*, 2.38% *S. haemolyticus*, 2.38% *S. hominis*, 1.19% *S. auricularis*, 1.19% *S. warneri* and 1.19% *S. sciuri*, respectively. A total of 68 strains were identified from mastitis milk samples in the ratio of 51.4% *S. aureus*, 19.1% *S. hyicus*, 13.2% *S. simulans*, 4.4% *S. chromogenes*, 5.8% *S. epidermidis*, 2.9% *S. haemolyticus*, 1.4% *S. hominis*, 1.4% *S. sciuri*, respectively. The species isolated in this study and their isolation rates were found to be consistent with those of other studies in which isolation of *Staphylococcus* spp. from mastitis cases was reported [10-13].

S. aureus is regarded as pathogenic according to the conventionally coagulase pathotyping feature. However, CNS strains may also be isolated from clinical cases of mastitis [3]. In this study, 41.6% of isolates were CPS. CNS strains were identified in the ratio of 58.3% and found at a higher rate from mastitis milk samples. In this study,

Table 2. *The virulence factors of staphylococci isolated from animals with mastitis*

Strains	n	Clumping Factor n (%)	Coagulase n (%)	Protein A n (%)	DNase n (%)	TNase n (%)	Capsule n (%)	Slime n (%)	Biofilm n (%)	Hemolysis n (%)	Staphylokinase n (%)	HA n (%)
S. aureus	35	28 (80)	28 (80)	30 (85)	21 (60)	3(9)	18 (51)	5 (14)	13 (37)	34 (97)	8 (23)	5 (14)
S.hyicus	13	7 (54)	7 (54)	0	1 (8)	2(15)	0	10 (77)	2 (15)	13 (100)	12(92)	2 (15)
S.epidermidis	4	0	0	0	0	0	0	1 (25)	3 (75)	4 (100)	1(25)	3 (75)
S.chromogenes	3	0	0	0	0	0	0	0	1 (33)	3 (75)	2(67)	1 (33)
S.sciuri	1	0	0	0	1 (100)	0	0	0	0	1 (100)	0	0
S.simulans	9	0	0	0	0	0	0	0	0	9 (100)	0	0
S.hominis	1	0	0	0	0	0	0	0	0	1 (100)	0	0
S.haemolyticus	2	0	0	0	0	0	0	0	1 (50)	2 (100)	1 (50)	0
Total	68	35 (51)	35 (51)	30 (44)	23 (34)	5(7)	18 (26)	16 (24)	20 (29)	67 (99)	24 (35)	11 (16)

Table 3. *The virulence factors of staphylococci isolated from healthy animals*

Strains	n	Clumping Factor n (%)	Coagulase n (%)	Protein A n (%)	DNase n (%)	TNase n (%)	Capsule n (%)	Slime n (%)	Biofilm n (%)	Hemolysis n (%)	Staphylokinase n (%)	HA n (%)
S. aureus	2	0	0	0	0	0	0	0	0	0	0	0
S. epidermidis	1	0	0	0	0	0	0	0	0	1 (100)	1 (100)	0
S. lentus	5	0	0	0	0	0	0	0	0	1 (20)	1 (20)	0
S. chromogenes	5	0	0	0	0	0	0	0	0	4 (80)	3 (60)	0
S.warneri	1	0	0	0	0	0	0	0	0	0	0	0
S.hominis	1	0	0	0	0	0	0	0	0	0	0	0
S. auricularis	1	0	0	0	0	0	0	0	0	1 (100)	1 (100)	0
Total	16	0	0	0	0	0	0	0	0	7 (44)	6 (38)	0

Table 4. *Statistical analyses of virulence factors of staphylococci*

Virulence Factors	Results (n)				P*
	Positive		Negative		
	CoPS	CoNS	CoPS	CoNS	
Protein A	25	5	10	44	0.001
DNase	17	6	18	43	0.001
TNase	4	1	31	48	0.01
Capsule	14	4	21	45	0.001
Slime	10	6	25	43	0.05
Biofilm	13	6	22	43	0.01
Hemolysis	34	40	1	9	0.01
Staphylokinase	14	16	21	33	-
Hemagglutination	6	5	29	44	-

** Statistical analyses: 0.01, not significant; 0.05, significant; 0.001, very significant;* ***CoPS:*** *Coagulase positive strains;* ***CoNS:*** *Coagulase negative strains*

the prevalence of CNS was determined through an epidemiological aspect.

The virulence factors that express toxic, proteolytic and adhesive property were determined in strains of *S. aureus*. Staphylococcal protein A is a surface molecule found more than 95% of *S. aureus* strains [14]. In this study, the presence of protein A was determined only in *S. aureus* strains. These strains were identified as coagulase positive in the ratio of 83%.

Although the presence of DNase activity is a debated issue, Quinn et al.[15] expressed that DNase production could be regarded as a pathogenicity factor for Staphylococci from bovine clinical samples. In a previous study, it was determined that only *S. chromogenes* showed positive activity with regard to DNase production of strains isolated from bovine milk [16]. In our study, *S. aureus* strains expressed DNase and TNase activity. However, in this study, DNase production rate of *S. aureus* strains was found in the ratio of 60%. This positive result is observed to be a lesser extent in comparison with another study [17].

Capsule formation plays quite a great role in the formation of staphylococcal mastitis. *S. aureus* isolates have capsular polysaccharides in the ratio of 90% and 11 serotypes have been identified before [18]. Capsule has been proposed as an adherence factor for *S. epidermidis* [19]. In a survey research conducted in the United States, the prevalence of capsular serotypes was determined from *S. aureus* strains isolated from bovine mastitis. Compared with the predominant serotype 5 in France, the dominant group in the United States was determined as serotype 8 [20]. It has been shown that CNS *(S. haemolyticus*, *S. hyicus*, *S. simulans*, *S. warneri*, *S. lentus)* (15.5%) have capsule in less proportion [21]. In this study, capsule formation was determined in the ratio of 48.6%. This result is in line with the literature data obtained before, since the presence of capsular polysaccharide indicated mostly in *S. aureus* strains. Biofilm formation was indicated as the most important virulence factor in *S. epidermidis* and other CNS isolated from infections caused by medical instruments. In a study previously done, biofilm formation of *S. aureus* strains isolated from orthopedic implants has been detected in the ratio of 88.9%. The biofilm formation of *S. epidermidis* was also detected in the ratio of 88.9% [22]. In other studies, biofilm formation of *S. aureus* was reported in the ratios of 18-80%. Baselga et al.[23] reported slime production of staphylococci in the ratio of 12%. In this study slime production of isolates were detected in the ratio of 19%. The results of these two methods were compared and no significant differences were observed. In this study, biofilm formation was detected in higher proportion for coagulase positive strains.

Cytotoxic molecules important for *S. aureus* are classified in four types as alfa toxin, beta toxin, delta toxin and gamma toxin. In previous studies, delta toxin production of CNS was reported in the ratio of 97% [24]. In another study, beta and delta hemolysin production of *S. aureus* strains were reported in the ratio of 24%. In this study, beta and delta hemolysin production ratios of *S. aureus* strains were detected as 51.35% and 32.43% respectively. Alfa hemolysin was detected in *S. hyicus* strains at highest proportion. In previous studies, delta hemolysin was reported as the most common hemolysin in *S. epidermidis*

strains [25]. In this study, delta hemolysis was observed in the ratio of 40% in *S. epidermidis* strains. CPS produced beta hemolysin in the ratio of 51.42% while CNS strains produced in the ratio of 36.73%.

Staphylokinase, which is known as one of staphylococcal extracellular proteins, converts plasminogen to plasmin. However, there is no conclusive evidence that staphylokinase is a virulence factor. In this study, staphylokinase production was determined in the ratio of 40.47% in Staphylococcal strains. Staphylokinase production was detected in *S. hyicus* strains at highest proportion. Coagulase positive strains produced staphylokinase in the ratio of 40% while coagulase negative strains produced in the ratio of 32.65%. However, this topic is excluded from discussion since there is no proper literature for staphylokinase production. The studies for detection of haemagglutination feature in Gram positive microorganisms are insufficient and they have focused on *S. saprophyticus*. In latter studies, it was determined an association between biofilm formation and hemagglutination feature of *S. epidermidis* [9]. In this study, the haemagglutination feature was determined in the ratio of 60%. These results correlate with the data obtain in previous studies. In another study, agglutination ratio was determined as 33% for coagulase negative staphylococci except *S. epidermidis* [9]. The haemagglutination feature of *S. aureus* was found in the ratio of 23% in a study performed previously [26]. In this study, haemagglutination ratio was determined in the ratio of 14.28% for coagulase positive Staphylococci and 16.32% for coagulase negative Staphylococci. In general, this ratio was determined as low when compared with both coagulase positive and negative strains. Haemagglutination feature was found in the ratio of 13.5% only in *S. aureus* strains.

The formation of a large increase of methicillin resistance has been observed in recent years particularly in nosocomial infections [27]. Methicillin resistant *S. aureus* strains have been isolated in a study conducted in the Netherlands, albeit at a lower prevalence of staphylococci isolated from animals. Although methicillin-resistance in coagulase-negative strains was determined in the ratio of 4%, resistance in coagulase positive strains was not determined [28]. Methicillin resistance of coagulase negative strains was found in the ratio of 8% in a study conducted in United States [29]. The first MRSA strain in animals has been reported in mastitis cattle in 1972, and this number has increased in the following years [30,31]. Among the studies on methicillin resistance in mastitis milk specimens in Turkey, there is a proportional difference for MRSA. Ak et al.[10] and Güler et al.[11] reported that oxacillin resistance was absent in Staphylococcal strains causing mastitis in Konya. Kırkan et al.[12] reported that 60% of the oxacillin resistance was detected in *S. aureus* strains studied in Aydın region. Türkyılmaz et al.[13] reported that cefoxitin resistance was 17.2% in *S. aureus* strains in the same region. In this study, methicillin resistance was determined in the ratio of 2.9% for coagulase positive strains and 16.3% for coagulase negative strains. The results correlate with previous studies. *S. haemolyticus* strains exhibit a tendency to develop versatile resistance to drugs [32]. *S. haemolyticus* was determined as one of the methicillin resistant CNS [28]. In this study, two of the methicillin resistant strains were detected as *S. haemolyticus*. This result correlates with the data reported in previous studies. The study has also demonstrated that methicillin resistance should be investigated for antibiotic susceptibility testing of staphylococcal mastitis for methicillin resistant strains that are resistant to all β-lactam antibiotics. As the development of methicillin resistance in veterinary medicine may increase in the following years, methicillin resistance should be considered as an important factor in the treatment of staphylococcal mastitis. The wider and more comprehensive epidemiological studies to be carried out in this regard will clarify the situation in Turkey of the infections caused by methicillin-resistant staphylococcal strains.

In this study, the identification of *Staphylococcus* species isolated from milk samples of healthy and mastitic animals, and the virulence factors of isolates were examined. The relationship between these factors and the strains regarded as pathogen and nonpathogenic was determined also. The results indicate that isolated strains showed wide range of virulence factors. Thus, CNS were determined to have an important role in the pathogenesis of mastitis. Staphylococcal mastitis etiology, epidemiology and virulence factors were examined on the basis of the region and the prevalence of CNS were determined epidemiologically. It is considered that presence of methicillin-resistant staphylococci might create a significant problem for public health in this situation. As a result, it was concluded that coagulase-negative staphylococci also can play a role in the pathogenesis of mastitis, animals might transmit the pathogen staphylococci to human and in order to launch an effective treatment against mastitis, the exact etiology, pathogenesis and epidemiology of the infection should be determined. The large amount of staphylococcal agents found in bovine milk represents a health hazard to the animals and emphasises the need for improved hygiene practise at levels in the dairy.

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