

Determination of some Virulence Factors in Staphylococcus Spp. Isolated from Various Clinical Samples

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Abstract: The objectives of this study were to identify staphylococci isolated from different animal clinical samples, to examine some of the virulence factors in the isolates, to determine relationships between these virulence factors and coagulase positive Staphylococcus (CoPS)/coagulase negative Staphylococcus (CoNS) strains. A total of 180 strains of Staphylococcus spp. were isolated from bovine mastitis, dogs with otitis externa and chickens with various infections. The isolates were identified as *S. aureus* (29.4%), *S. hyicus* (16.7%), *S. intermedius* (3.9%), *S. chromogenes* (16.1%), *S. lentus* (13.3%), *S. epidermidis* (11.1%), *S. simulans* (7.8%) and *S. haemolyticus* (1.7%). The rate of positiveness for deoxyribonuclease (DNase) test, thermonuclease (TNase) test, presence of the capsule, slime and biofilm formation, hemolysis, and hemagglutination tests for CoPS strains were 42.2%, 43.3%, 53.3%, 77.8%, 74.4%, 58.9%, 46.7%, and for CoNS strains were 54.4%, 5.6%, 13.3%, 44.4%, 36.7%, 28.9% and 41.1%, respectively. The virulence factors which were investigated were determined CoNS and CoPS strains. Thus, it was thought that CoNS strains might be as dangerous as CoPS strains for both animals and humans.

Key Words: Staphylococcus spp., virulence factors, coagulase activity, animals

Çeşitli Klinik Örneklerden İzole Edilen Staphylococcus Spp.'de Bazı Virulens Faktörlerinin İncelenmesi

Özet: Bu çalışmanın amaçları çeşitli hayvansal klinik örneklerden izole edilen stafilokokları tür düzeyinde tanımlamak, bunlarda çeşitli virulens faktörlerinin varlığını incelemek, virulens faktörleri ile koagülaz pozitif Staphylococcus (CoPS)/koagülaz negatif Staphylococcus (CoNS) suşları arasındaki ilişkileri belirlemektir. Bunun için mastitisli sığırlardan, otitis eksternalı köpeklerden ve çeşitli hastalıkları bulunan tavuklardan toplam 180 Staphylococcus spp. izolasyonu yapıldı. İzolatlar *S. aureus* (% 29,4), *S. hyicus* (% 16,7), *S. intermedius* (% 3,9), *S. chromogenes* (% 16,1), *S. lentus* (% 13,3), *S. epidermidis* (% 11,1), *S. simulans* (% 7,8) ve *S. haemolyticus* (% 1,7) olarak tanımlandı. Deoxyribonuclease (DNase) test, thermonuclease (TNase) test, kapsül varlığı, siyme ve biofilm oluşumu, hemoliz, hemagglutinasyon testlerinde CoPS suşlarında sırasıyla % 42,2, % 43,3, % 53,3, % 77,8, % 74,4, % 58,9, % 46,7 ve CoNS suşlarında % 54,4, % 5,6, % 13,3, % 44,4, % 36,7, % 28,9, % 41,1 oranlarında pozitiflik tesbit edildi. İncelenen virulens faktörleri CoNS ve CoPS suşlarında saptandı. Sonuç olarak, hem hayvan hem de insanlar için CoNS türlerinin, CoPS türleri kadar tehlikeli olabileceği düşünülmektedir.

Anahtar Sözcükler: Staphylococcus spp., virulens faktörleri, koagülaz aktivitesi, hayvanlar

Introduction

The staphylococci are most frequently isolated from clinical specimens in the microbiology laboratory with the exception of the Enterobacteriaceae. These bacteria are widespread in nature and can be recovered from environment or as commensally inhabitants of the skin, mucous membranes and other body sites in humans and animals (1,2).

Staphylococcus aureus causes pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, osteomyelitis, endocarditis and superficial skin lesions such as furunculosis. *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* also causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome (1,2).

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Coagulase negative staphylococci (CoNS) are normal and abundant colonizers of humans and become pathogenic only in certain situations. They are commonly isolated in clinical specimens and several species are recognized as important agents of nosocomial infections, especially in neonates (3). CoNS are also involved in animal diseases and several studies have shown that they are the bacteria most frequently recovered from infected bovine and caprine mammary glands (4,5). Two main reasons for the increasing rate of CoNS infections are the spreading antibiotic resistance among CoNS and the increasing use of medical devices in recent years (6). It is, however, difficult to distinguish between pathogenic CoNS and non-pathogenic resident flora, since their virulence factors have not yet been well defined. The pathogenic potential of classical disease-inducing staphylococci, such as *S. aureus*, is associated with a number of biochemical functions which may be considered virulence factors. The virulence factors include surface proteins that promote colonization of host tissues, invasions that promote bacterial spread in tissues (leukocidin, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule), biochemical properties that enhance their survival in phagocytes (catalase production), and membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin). Similar activities have been observed in different species of CoNS (2,5,7).

The objectives of this study were to identify the species level of different coagulase positive staphylococci (CoPS) and CoNS strains isolated from various animal clinical samples, to examine the expression of some potential virulence factors in Staphylococcus isolates and to determine the relationships between these virulence factors and CoPS/CoNS strains.

Materials and Methods

1. Isolation and identification of isolates: A total of 180 strains of Staphylococcus spp. from bovine mastitis, dogs with otitis externa and chickens with various infections were examined. 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 and incubated aerobically at 37 °C for 48 h. After incubation, suspect colonies were examined by Gram staining. The colonies

with morphologies compatible with Staphylococcus spp. were transferred to Tryptic Soy Broth (TSB) (Oxoid) and Tryptic Soy Agar (TSA) (Oxoid). After growth, staphylococci were identified on the basis of colony characteristics, Gram staining, pigment production, hemolysis and the following biochemical reactions: catalase activity, coagulase test (rabbit plasma), oxidase test, O/F test with glucose, resistance to bacitracin (0.04 U), mannitol fermentation on Chapman Agar, urease, nitrate reduction, novobiocin resistance, phosphatase, deoxyribonuclease (DNase) test, carbohydrate (xylose, sucrose, trehalose, maltose, fructose, lactose, mannose) fermentation tests (8-10).

2. Determination of some virulence factors:

a. Coagulase Test: Coagulase activity was determined by the method described by Quinn et al. (2). This test was performed as a Tube Coagulase (TC) test. Several colonies of each organism were mixed with 0.5 ml of citrated rabbit plasma in a sterile test tube. The tube was incubated at 37 °C and examined after 4 and 24 h. Clot formation at either reading was recorded as positive.

b. DNase test: This test was carried out by using commercially available DNase agar (Difco). Spot inoculation were done on the DNase agar and incubated at 37 °C for 4 days. After incubation, 1 N HCl was poured on the agar. Clearing around the bacterial growth was evaluated as positive (11).

c. Thermonuclease (TNase) test: TNase activity was determined by the method described by Hawkey and Lewis (12). For the detection of TNase activity, toluidine blue-deoxynucleic acid agar was prepared and poured onto plastic petri dishes. The formation of a pink halo (1 to 3 mm wide) surrounding each test well, indicating the presence of thermostable nuclease.

d. Capsule Test: Capsule test was done by the reported by Gerhardt et al. (13). A loopful of Indian ink and a small amount of bacteria were mixed on the microscope slide. A cover slide was put on top of the slide. The capsule was seen on the brown-black ground as a clear zone surrounding the microorganism.

e. Slime Formation: The Congo Red Agar (CRA) method developed by Freeman (14) was used in this study. The composition of medium was Brain Heart Infusion Broth (BHIB) (Oxoid) 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 (121 °C for 15 min) separately and was added when the agar had cooled to 55 °C. Plates were inoculated and incubated aerobically at 37 °C for 24 h. Isolates which produced black colonies with dry crystalline consistency were regarded as slime positive, whereas those showing pink colonies were slime negative.

f. Biofilm Formation: Quantitative determination was carried out by the Microplate method (MP) proposed by Pfaller et al. (15) using tissue culture plates of 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 °C, the contents were aspirated and plates were washed twice with phosphate-buffered saline (PBS, pH: 7.2). The wells were stained with 0.25% safranin 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, and the values of optical density (OD) were then averaged. A 3-grade scale was used to evaluate the strains slime producing ability: no biofilm producer or (-): $OD < 0.500$; (+): $0.500 < OD < 1.500$; (+ +): $OD > 1.500$.

g. Hemolysis: Alpha-hemolysin was evaluated on TSA supplemented with 5% washed rabbit erythrocytes. The plates were incubated for 24 h at 37 °C, when positive samples showed a wide zone of complete hemolysis with

blurred edges. Beta-hemolysin was evaluated by plating strains on 5% sheep blood TSA. The plates were incubated at 37 °C for 24 h and then overnight at 4 °C, when positive strains showed a wide zone of incomplete hemolysis with sharp edges. Non-hemolysis on 5% sheep blood TSA was evaluated as gamma hemolysis (1,16).

h. Hemagglutination: Hemagglutination test was performed in a manner similar to the method described by Rupp and Archer (17). The test was performed in U-shaped 96-well microtiter plates. The bacterial suspensions in PBS were adjusted to McFarland standard 1. Two serial dilutions of the bacterial suspension were made in the microtiter plates to give a total volume of 50 μ l per well. Then, 50 μ l of the 1% human erythrocyte suspension in PBS was added to each well. After shaking, the plates were incubated at room temperature for 2 h and the hemagglutination was recorded as positive or negative.

3. *Statistical Analysis*: Significant differences between CoPS, CoNS and virulence factors were determined by chi-square test (18).

Results

1. *Isolation and identification of isolates*: Origin of isolates and species distribution of the *Staphylococcus* spp. are given in Table 1.

Table 1. Origin of isolates and species distribution of the *Staphylococcus* spp.

Isolates	Origin of isolations			n	%
	Chicken	Cattle	Dog		
<i>S. aureus</i>	14	20	19	53	29.4
<i>S. hyicus</i>	9	13	8	30	16.7
<i>S. intermedius</i>	1	1	5	7	3.9
<i>S. chromogenes</i>	9	11	9	29	16.1
<i>S. lentus</i>	8	9	7	24	13.3
<i>S. epidermidis</i>	9	4	7	20	11.1
<i>S. simulans</i>	8	2	4	14	7.8
<i>S. haemolyticus</i>	2	-	1	3	1.7
TOTAL	60	60	60	180	100

n: The number of isolates.

From a total of 180 staphylococci isolates, 90 isolates were identified as CoPS, whereas 90 isolates were CoNS by the tube coagulase test. Distribution of the species of these strains was *S. aureus* 29.4%, *S. hyicus* 16.7%, *S. intermedius* 3.9%, *S. chromogenes* 16.1%, *S. lentus* 13.3%, *S. epidermidis* 11.1%, *S. simulans* 7.8%, and *S. haemolyticus* 1.7%.

2. Virulence Factors:

The distributions of the virulence factors of CoPS and CoNS are given in Table 2.

The rate of positiveness for DNase test, TNase test, presence of the capsule, slime and biofilm formation, hemolysis, and hemagglutination tests for CoPS strains were 42.2%, 43.3%, 53.3%, 77.8%, 74.4%, 58.9%, 46.7%, and for CoNS strains were 54.4%, 5.6%, 13.3%, 44.4%, 36.7%, 28.9% 41.1%, respectively.

3. Statistical Analysis: The distribution of the virulence factors of CoPS, CoNS strains and significance are given in Table 2.

The relationship between CoPS and CoNS strains was statistically significant for TNase, capsule, hemolysis, slime and biofilm formation whereas DNase and hemagglutination tests were non-significant ($P < 0.001$).

Discussion

In this study, the examination of some virulence factors for the Staphylococcus isolates, and the determination of the relationships between these virulence factors and CoPS/CoNS strains were aimed.

Coagulase activity, hemolysis, capsule and slime formation of the Staphylococcus spp. were regarded as pathogenicity criterions in laboratory (1,6,12). A number of biochemical activities are considered to contribute to the virulence of pathogenic staphylococci. DNase is not entirely reliable as an indicator of pathogenicity (2). It was reported that CoNS also have DNase activity (2). In this study, the rate of DNase positiveness was 42.2% and 54.4% for CoPS and CoNS, respectively. These results were parallel with the other studies (19,20). Like the coagulase test, TNase test has been considered to be specific to *S. aureus* (5). The TNase test detects production of thermostable deoxyribonuclease and is highly reliable, comparable in results to the TC test (21). Previous studies (21,22) have suggested the simultaneous use of TC and TNase tests. In the present study, TNase activity was positive at a rate of 43.3% and 5.6% for CoPS and CoNS, respectively. Test findings revealed that the TNase test mostly resulted in positiveness, especially for *S. aureus* strains; these results

Table 2. The distribution of the virulence factors of CoPS, CoNS strains.

Virulence factors	The results of the tests				(X ²) test
	Positive		Negative		
	CoPS (%)	CoNS(%)	CoPS (%)	CoNS(%)	
DNase	38 (42.2)	49 (54.4)	52 (57.8)	41 (45.6)	2.7 -
TNase	39 (43.3)	5 (5.6)	51 (56.7)	85 (94.4)	34.8*
Capsule Formation	48 (53.3)	12 (13.3)	42 (46.7)	78 (86.7)	32.4*
Slime Formation	70 (77.8)	40 (44.4)	20 (22.2)	50 (55.6)	21.0*
Biofilm Formation	67 (74.4)	33 (36.7)	23 (25.6)	57 (63.3)	26.0*
Hemolysis	53 (58.9)	26 (28.9)	37 (41.1)	64 (71.1)	16.4*
Hemagglutination	42 (46.7)	37 (41.1)	48 (53.3)	53 (58.9)	0.6 -

- : non-significant, *: significant ($P < 0.001$)

were parallel with the other studies (21,22). Because of the protection of the microorganisms from phagocytosis, capsule formation is an important virulence factor for many microorganisms. In this study, the rate of capsule formation positiveness was 53.3% and 13.3% for CoPS and CoNS, respectively. Revealing of the capsule formation, especially in many *S. aureus* strains, agreed with another study (23). It has been thought that testing for biofilm formation could be a useful marker for the pathogenicity of staphylococci (14,15). Slime production may reflect the microorganism's capacity to adhere to specific host tissues and thereby to produce invasive microcolonies (8,9). In our study, the rate of CRA and MP methods positiveness was for CoPS 77.8% and 74.4% and for CoNS 44.4% and 36.7%, respectively. These results showed that slime and biofilm formation in staphylococci were important virulence factors, which were reported in previous studies (5,15,24). Damage to host cells is in part mediated by staphylococcal hemolysins, which contribute importantly to virulence in *S. aureus*. Alpha hemotoxin assembles into transmembrane pores in many nucleated cells, and readily lyses sheep or rabbit erythrocytes, while horse or human red cells are less susceptible. Beta hemolysin has sphingomyelin-specific phospholipase activity, resulting in partial cell lysis (25). In the present study, it was determined that 58.9% of the CoPS and 28.9% of CoNS strains have hemolysis (alpha or beta) characteristics. In generally, beta hemolysis was seen in CoPS strains while alpha hemolysis was detected in the CoNS strains (25,26). This showed that beta hemolysis incidence was higher than that in another study previously performed (26). This situation might have originated from CoPS strains of which nearly 58.9% consisted of *S. aureus* with beta hemolysis characteristics (25,26). Hemagglutination rates were 46.7% and 41.1% for CoPS and CoNS, respectively. In another study, the hemagglutination rate was reported as 33% for CoNS except *S. epidermidis* (17).

S. aureus is the most common CoPS isolated from bovine mastitis. Erganiş et al. (20) reported that 53.8% of CoPS (14 strains) isolated from bovine and sheep mastitis were *S. aureus*. Çitak et al. (27) reported that 704 of 851 *Staphylococci* isolates from milk samples were *S. aureus*. Altay et al. (28) reported that 44 CoPS (30 *S. aureus*, 9 *S. delphini*, 3 *S. intermedius*, 1 *S. schleiferi*, 1 *S. hyicus*) and 74 CoNS (11 *S. hyicus*, 16 *S. simulans*, 8 *S. saprophyticus*, 6 *S. epidermidis*, 4 *S. schleiferi*, 4 *S. arlettae*, 4 *S. lentus*, 4 *S. gallinarum*, 3 *S. chromogenes*, 3 *S. warneri*, 3 *S. haemolyticus*, 2 *S. caprae*, 2 *S. auricularis*, 2 *S. xylosus*, 2 *S. cohnii*) from poultry. In this study similar strains were identified. *S. hyicus* was an opportunistic pathogen found in pigs and cattle. In this study, isolation of *S. hyicus* from chickens was parallel with another study (28). Previous studies (29,30) reported that the CoPS strains, such as *S. intermedius* and *S. aureus*, were frequently isolated from dogs with otitis externa. In the present study, CoPS species were the most prevalent and they made up 53.3% of the total isolates. CoNS species constitute a major component of the normal microflora of dogs and they were considered important opportunistic pathogens (30). Nevertheless, the presence of these species in animals has received little attention to date (31). In conclusion, further studies are necessary in order to evaluate the real role of *Staphylococcus* spp. in the etiology of major infections in domestic animals.

In this study, isolation of staphylococci was performed from various animal materials and some of the virulence factors of these strains were also determined. The results of the present study revealed that CoNS isolated from animals have virulence factors and might have an important role in the pathogenesis of infections. In conclusion, it was thought that CoNS species could be as dangerous as CoPS for both animals and humans.

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