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Full Length Research Paper

Detection of adhesin genes and slime production among Staphylococci in orthopaedic surgical wounds

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This study was aimed at investigating: (i) three adhesin genes (*clf A*, *fnb A* and *cna*) in *Staphylococus aureus* strains, (ii) the presence of slime (*ica A* and *ica D* genes) in both *Staphylococus epidermidis* and *S. aureus* strains isolated from surgical wounds. The slime and adhesin genes were detected by multiplex PCR. The *ica Alica D* positivity rates were determined as 66.2% (104/157) in a total of 157 staphylococcal strains. While the occurance rate of slime genes was 69.6% (48/69) among the *S. epidermidis*, this ratio was 63.6% (56/88) among the *S.aureus* isolates. No statistically significant difference was found between *S. epidermidis* and *S. aureus* isolates in terms of the presence of slime genes (p > 0.05). Among the 88 *S. aureus* strains, almost all of the strains were positive for *fnb A* gene (97.7%). The *cna* and *clf A* positivity rates were detected in 69 (78.4%) and 45 (51.1%) isolates, respectively. The *ica A* and *ica D* genes responsible for slime production have been found to have high prevalence. Also, the frequency of adhesin genes was determined at a high rate in *S. aureus* strains isolated from surgical wounds. Molecular identification of virulent staphylococcal strains may help in management in clinical decision making.

Key words: Adhesins, slime, *Staphylococus aureus*, *Staphylococus epidermidis*, wound infection.

INTRODUCTION

Microbial colonization in wounds is one of the most important problems in the treatment of hospitalized patient. Both *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) are the leading cause of wound and hospital acquired infections worldwide. *S. aureus* and *S. epidermidis* have emerged as the leading cause of postoperative infection in orthopaedic surgery patients. These microorganisms are closely associated with substantial morbidity and mortality. Both *S. epidermidis* and *S. aureus* are concerned polysaccharide intercellular adhesin (PIA, also called biofilm). PIA is one of the most important virulence determinants that facilitate to adherence and colonization of bacteria (Tojo et al., 1998). The mortality and morbidity associated with slime infections are very high among orthopaedic patients. These infections require additional treatment process such as repeated surgeries and prolonged antibiotic therapy (Drancourt et al., 1993; Kumar and Prasad, 2006).

PIA synthesis is closely related with the expression of *ica A* and *ica D* genes. The main function of *ica A* is that it is responsible for the synthesis of the *N*-acetyl -D-glucosamine polymer structure. *ca A* expression in collaboration with *ica D* provides a significant increase in enzymatic activity. The capsular polysaccharide occurs as a result of this coexpression of *ica A* with *ica D* (Gerke et al., 1998).

To enter into host cells and create infection, bacterial adhesion is one of the most significant steps. Bacterial adhesion genes have been shown to play important roles in the initiation of bacterial infection. The microorganisms carrying the adhesion genes are quite important in terms of surgical wound infections.

S. aureus is one of the most important pathogens responsible for both acute and chronic nosocomial infections. Some staphylococcal adhesins such as fibronectin (Herrmann et al., 1988), fibrinogen (McDevitt et al., 1988) and collagen (Patti et al., 1995) are among the most important Staphylococcal virulence factors. It was reported that some these adhesins such as *fnb A* (fibronectin-binding proteins), *clf A* (fibrinogen-binding

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proteins) and *Cna* (collagen-binding protein) can affect the bacterial binding to surface of the host. *Clf A*, *cna*, *fnb A* proteins in the pathogenesis of the various infections have been found to play an important role in the pathogenesis of various infections. *Cna*, *fnb A* proteins have been demonstrated to contribute to tissue colonization in various infections such as medical device-related infections. Patients who have orthopaedic implants inserted are more prone to infections of these kinds of microorganisms, since these adhesins allow the bacteria to be adsorbed on orthopaedic medical devices or the specifically interaction with the plasma or extracellular matrix proteins of the host cell (Jett and Gilmore, 2002; Smeltzer and Gillaspy, 2000; Cheung and Fischetti, 1991).

Clumping factor A is a surface-associated adhesins of *S. aureus*. It improves binding of the bacteria to both soluble and immobilized fibrinogen (Hair et al., 2008). *Fnb A* proteins are basic virulence factors of the *S. aureus* cell wall (Schwarz-Linek et al., 2006). The *fnb A* and *cna* adhesins of Staphylococci have been shown to help to tissue colonization in orthopaedic surgical infections (Arciola et al., 2005a).

Wound infections created by biofilm forming *S. aureus* and *S. epidermidis* are particularly challenging infections in terms of treatment. Also, a close relation was reported between slime production and *fnb A* gene. In a study conducted by O'Neil et al., it was determined that the *fnb A* proteins of the *S. aureus* strains promoted the biofilm accumulation (O'Neill et al., 2009).

Molecular methods are the most conveinent tecniques for the detection of the slime and adhesin producing strains (Arciola et al., 2001). The determinations of the slime and adhesin genes are often preferred to PCR-based molecular methods in the various studies. The presences of slime and adhesin genes were detected by multiplex PCR (polymerase chain reaction).

In this study, we aimed to investigate: (i) some adhesin genes (*Clf A, fnb A, cna*) in *S.aureus* strains and (ii) the presence of slime (*ica A* and *ica D* genes) in both *S. epidermidis* and *S. aureus* strains isolated from surgical wounds.

MATERIALS AND METHODS

This study was performed in the department of Microbiology and Clinical Microbiology, Medical Faculty of Mustafa Kemal University, Hatay. Wound samples were collected from the patients in orthopaedics service for the isolation of Staphylococci. A total of 69 *S. epidermidis* and 88 *S. aureus* isolates were included in this study.

Bacteriological specimens

Wound samples were taken from the patients treated in the department of Orthopedics. A total of 157 wound isolates were included in the study. The wound samples were taken from the patients and placed in transport media. They were sent immediately to the microbiology laboratory for the microbial evaluations. And then, the samples were inoculated onto 5% sheep blood agar plates (Difco Laboratories, Detroit, USA) plates. The plates were incubated at 37°C for 48 h. All staphylococcal isolates were identified by conventional microbiological techniques. The identification of Staphylococci was on the basis of colony morphology, gram staining, biochemical tests such as catalase and coagulase tests (Holt et al., 1994). Isolated Staphylococcal strains were stored at -70°C in Mueller-Hinton Broth (Merck, Germany) supplemented with 40% glycerol (v/v).

Bacterial strains

As control strains, *S. epidermidis* ATCC 35984 and ATCC 12228 were included. *S. epidermidis* ATCC 35984 and ATCC 12228 were selected as slime-producing and the non-slime-producing control strains, respectively.

Slime production

The presence of slime production of all staphylococcal isolates was evaluated by Congo red agar method phenotypically as previously described by Freeman et al. (1989). Congo red medium contained the following agents: 37 g/l brain heart infusion broth, 50 g/l sucrose, 10 g/l agar and 0.8 g/l Congo red. Bacterial strains (*S. aureus* and *S. epidermidis* strains) were cultured onto Congo red agar (CRA). The assay plates were incubated at 37°C for 24 h. All plates were examined in terms of color changes after 24 to 48 h of incubation. A black discoloration of the colony was interpreted as a positive test result. When non-slime producing bacteria grew in culture plate, the color of the bacterial colonies did not change. Congo red agar-plate cultures were evaluated by two independent observers for the detection of slime synthesis.

DNA isolation

For nucleic acid isolation from staphylococcal isolates, the frozen samples were thawed rapidly and all bacterial strains were cultivated in brain-heart infusion broth (Merck, Germany) at 37°C with shaking overnight. Total DNA was isolated from 5 ml of a broth culture grown overnight for all bacterial strains used in the study. The DNA extraction procedure has been described previously by Johnson et al. (1991). After incubation, bacterial cells were harvested by centrifugation at 3.000 × g for 10 min. After centrifugation, the cell pellet was re-suspended in in phosphate-buffered saline with 100 g of lysostaphin (Sigma) per ml, and incubated at 37°C for 30 min. The classic phenol/chloroform extraction method was used for nucleic acid extraction from the staphylococcal samples and DNA was precipitated in 1 ml 70% ethanol. The DNA precipitate was dissolved in 50 L of TE buffer (10 mM Tris chloride-1 mM EDTA [pH 8.0], and stored at -20°C until processing.

PCR methods protocols

PCR method for *fnb A*, *cna* and *clf A*

While the oligonucleotide primers for the *fnb A* and *cna* genes were selected from the research of Arciola et al., 2005a, the primer of *clf A* gene was selected from a research article of McDevitt et al. (1995) (Table 1).

The PCR amplification was performed in a 25 L reaction mixture. The PCR was performed under the following parameters: The reaction mixture consisted of 2.5 mL of 10x reaction buffer

Table 1. The primer sequences and predicted sizes used in the multiplex PCRs for *fnb A*, *cna* and *clf A*.

Gene	Primer	Oligonucleotide sequence (5'-3')	Size of amplified product (bp)
clf A	*clf A*-1	CCGGATCCGTAGCTGCAGATGCACC	1000
	clf A-2	GCTCTAGATCACTCATCAGGTTGTTCAGG	
fnb A	*fnb A*-1	GATACAAACCCAGGTGGTGG	191
	fnb A-2	TGTGCTTGACCATGCTCTTC	
cna	*cna*-1	AAAGCGTTGCCTAGTGGAGA	192
	cna-1	AGTGCCTTCCCAAACCTTTT	

Table 2. The primer sequences and predicted sizes used in the multiplex PCRs for *ica A* and *ica D*.

Gene	Primer	Oligonucleotide sequence (5'-3')	Size of amplified product (bp)
ica A	*ica A*-1	CCTAACTAACGAAAG GTAG	1315
	ica A-2	AAGATATAGCGATAA GTGC	
ica D	*ica D*-1	AAACGTAAGAGAGGT GG	381
	ica D-2	GGCAATATGATCAAG ATAC	

without $MgCl_2$ (Promega Corp.); 200 M of each deoxynucleoside triphospate (AB Gene, UK), 2 mM $MgCl_2$; 0.4 µM of primers for *clf A*, *fnb A* and cna and approximately 10 ng of template DNA, and brought up to a 25 L final volume with distilled water. Reactions were hot started for 5 min at 94°C and placed on ice, and 1 U of Taq polymerase (Fermentas, USA) was added. Reaction mixtures were subjected to 25 PCR cycles (1 min at 94°C, 1 min at 55°C and 1 min at 72°C). A final elongation step at 72°C for 10 min was also applied in a thermal cycler (Bioder/Thermal Blocks xp cycler, Tokyo Japan).

PCR method for *ica A* and *ica D*

Primers specific for *ica A* and *ica D* were selected from the studies of Cramton et al. (1999) and Vasudevan et al. (2003), respectively (Table 2). Multiplex PCR assay was carried out for the detection of *ica A* and *ica D* genes in all Staphylococcal strains. The PCR amplification was carried out in a total volume of 25 µl. PCR amplification was achieved as follows: 5 µl of genomic DNA (approximately 50 ng) sample was added to 20 µl of PCR mixture (20 mmol/L Tris -HCl, pH 8.4; 50 mmol/L KCl, 10 mmol/L MgCl2) and 200 µmol/L each of deoxynucleoside triphosphates (dNTPs), 0.6 µmol/L each primers and 1 U *Taq* DNA polymerase. The amplification process was started with an initial denaturation step (94°C, 4 min). Each cycle consists of three steps (denaturation, annealing and extension). Each PCR reaction consisted of 30 cycles of amplification. Amplification consisted of denaturation at 94°C for 45 s, annealing at 52°C for 30 s and DNA chain extension at 72°C for 1 min. And a final extension cycle was performed at 72°C for 7 min.

After the amplification of the slime and adhesin genes, 10 µL volumes of PCR samples were mixed with 3 µL of loading buffer (10%, w/v, ficoll 400; 10 mmol/L Tris-HCl, pH 7.5; 50 mmol/L EDTA; 0.25 % bromophenol blue). The PCR products were analyzed in a 2% (w/v) agarose gel in 1xTAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA). Ethidium bromide (0.5 µg/mL TAE)-stained DNA amplicons were visualized using a gel imaging system (Wealtec, Dolphin-View, USA).

Statistical analysis

All data were analyzed by 2 test. The p value <0.05 was considered significant. The statistical analyses in the present study were done by using Statistical Package for Social Sciences (SPSS1 for Windows V. 11.5, Chicago, USA) software.

RESULTS

In the present study, both the presence of slime genes (*ica A* and *ica D*) and phenotpically slime production on congo red agar were investigated in all staphylococcal strains.

The presence of the *ica A* and *ica D* genes was searched in 69 *S. epidermidis*, 88 *S. aureus* isolates and the reference strains. To determine the expected bp lengths (381, for the *ica D*, 1315, for the *ica A* bp), DNA marker with defined molecular weights in the range 100 to 2000 and reference strain were used (Figure 1).

The *ica A*/*ica D* positivity rates were determined as 66.2% (104/157) in a total of 157 staphylococcal strains. While the occurance rate of slime genes was 69.6% (48/69) among the *S. epidermidis*, this ratio was 63.6% (56/88) among the *S. aureus* isolates. No statistically significant difference was found between *S. epidermidis* and *S. aureus* isolates in terms of the presence of slime genes ($p > 0.05$).

S. epidermidis ATCC 35984 was selected as the slime positive control strain (containing both *ica A* and *ica D* genes). On the contrary, *S. epidermidis* ATCC 12228 was selected as a negative control strains (not containing both *ica A* and *ica D* genes).

Slime-forming ability was evaluated on Congo red agar method, phenotypically (Figures 2 and 3).

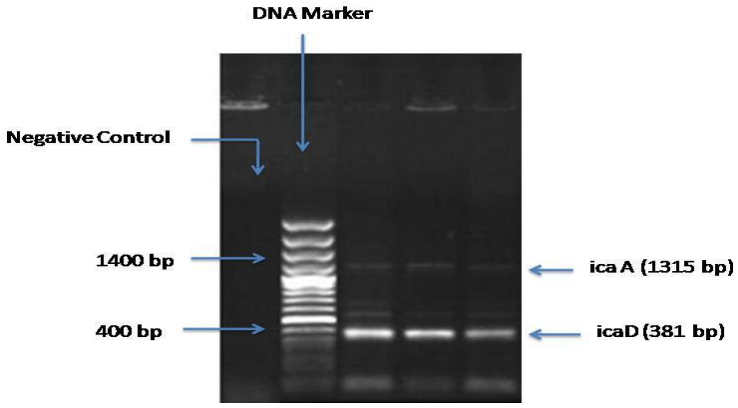

Figure 1. Multiplex PCR amplification products by agarose gel electrophoresis for the *ica A* and *ica D* genes. Lanes 1: Negative control, Lane 2: DNA molecular size marker (100 bp ladder), Lane 3, 4 and 5:; *ica D* (381 bp) and *ica A* (1315 bp).

Figure 2. Congo red agar plate test. Transparent colonies:; the non-slime-producing strains.

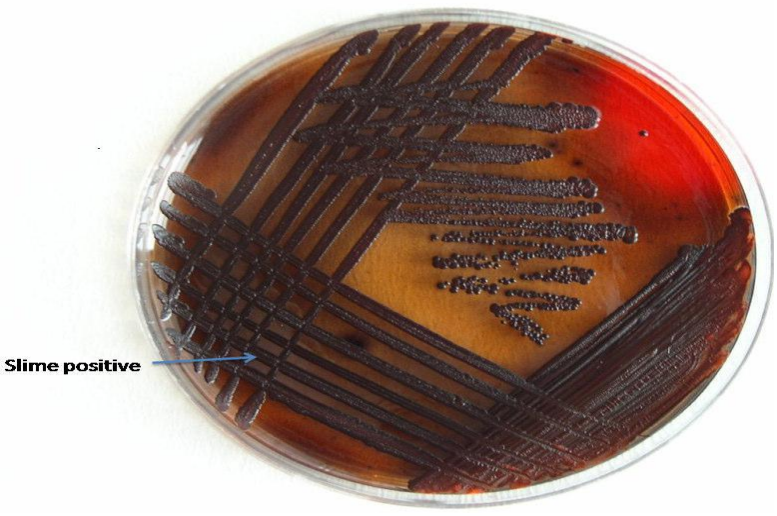

Figure 3. Congo red agar plate test. Black colonies: the slime-producing strains.

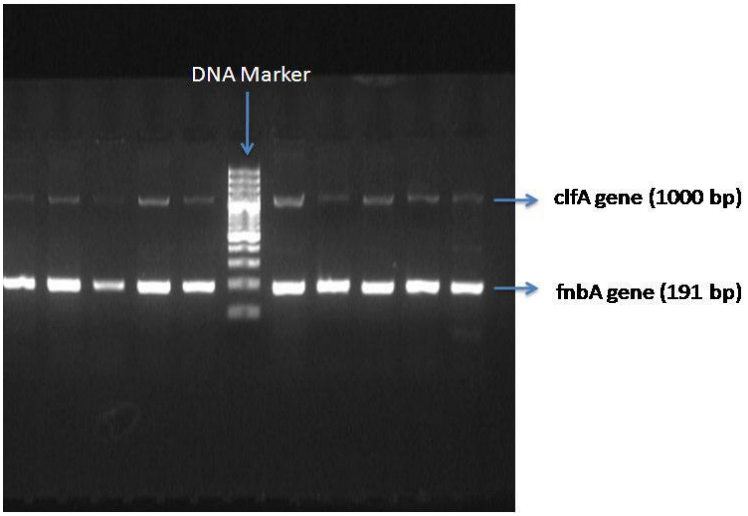

Figure 4. Multiplex PCR amplification products by agarose gel electrophoresis for for the *fnb A* (191 bp) and *clf A* (1000 bp) genes. DNA molecular size marker (100 bp ladder).

61 (58.7%) out of the 104 slime genes-positive strains were able to produce slime as phenotypic. The rates of slime production in *S. epidermidis* and *S. aureus* strains were found to be 58.3% (28/48) ve 59.9% (33/56), respectively.

Detection of adhesins genes *fnb* A, *cna* and *clf* A

In this study, the *clf A* positivity rate was determined as in 45 strains of 88 (51.1%) of *S. aureus* isolates (Figure 4). In addition, 86 strains out of 88 (97.7%) of *S. aureus* analyzed were found to carry the *fnb A* gene. Among the 88 *S. aureus* strains, the *cna A* gene was detected in 69 (78.4%) isolates. Positive strains for *clf A*, *cna* and *fnb A* genes produced a band of 1000, 192 and 191 bp, respectively, in 2% agarose gel (Figures 4 and 5).

Besides, while 2 of 88 *S. aureus* strains had none of these three adhesins genes (*fnb A*, *cna* and *clf A*), 33 strains were positive for *fnb A*, *cna* and *clf A*.

DISCUSSION

S. aureus and *S. epidermidis* are the most common causes of wound infections. The slime synthesis of these bacteria has been extensively investigated in various studies. Recently, attentions focused on the adhesins in staphylococcal infections especially in *S. aureus* infections. It was reported that the protein receptors of the cells assume the important functions for microbial adhesion (Montanaro et al., 1999).

Slime production and adhesins are considered to be a crucial virulence factor among in Staphylococci. In the literature, *fnb*, *clf*, *cna* and PIA have been shown to trigger exacerbation of keratitis (Jett and Gilmore, 2002), osteomyelitis (Smeltzer and Gillaspy, 2000) and medical devices infections. Several studies have been carried out on adhesin and slime genes of *S. aureus*. However, a limited number of studies have been carried out on adhesins and slime genes of both *S. epidermidis* and *S. aureus* isolated from surgical wounds.

Both *S. epidermidis* and *S. aureus* are able to form biofilms. One of the major virulence factors of *S. epidermidis* and *S. aureus* is the slime production (Freeman et al., 1989; Montanaro et al., 1999). These bacteria have been known as a main cause of nosocomial infections. It is known that slime formation will significantly enhance the pathogenesis of staphylococcal infections. Furthermore, the slime formation allows staphylococci to escape host defenses and resist the antimicrobial action of antibiotics. Detection of slime production in staphylococcal strains isolated from clinical wound samples is important. It helps to know virulence factors for bacterial pathogenicity. In the present study, the occurance of slime genes and slime production in staphylococci were tested by multiplex PCR and Congo red agar method, respectively.

Staphylococci are the most important cause of wound infections. It is well known that slime layer delays the healing of wound infection. Slime layer protect the microorganism from the effect of antimicrobial agent neutrophil attacks. Biofilm presence is frequently associated with chronic wounds. In a study carried out by Yazdani et al. (2006), the biofilm forming capacity and the presence of slime genes were investigated among *S. aureus* isolated from wound infections. In their study, 50 *S. aureus* were identified, 26 of them (52%) were found as positive in terms of biofilm forming and 24 (48%) isolates were negative (Yazdani et al., 2006). These

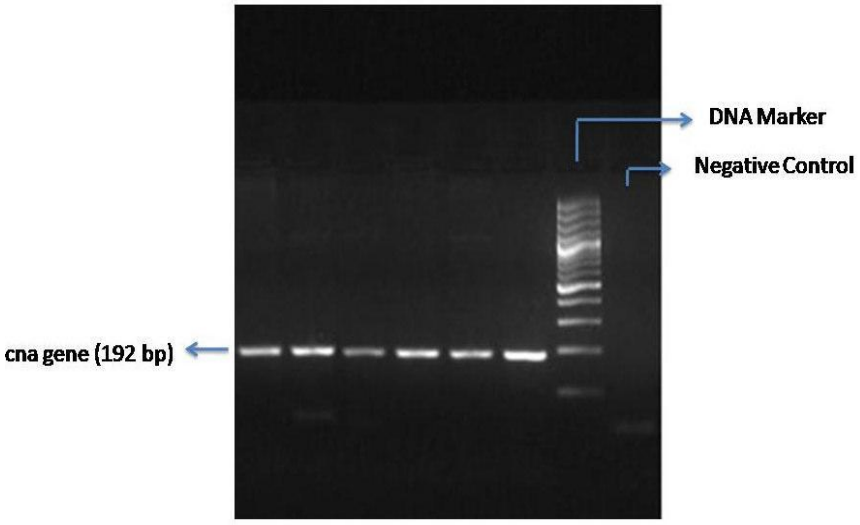

Figure 5. Multiplex PCR amplification products by agarose gel electrophoresis for the *cna* (192 bp). DNA molecular size marker (100 bp ladder).

results are in agreement with our study among the *S. aureus* and *S. epidemidis.* There was no statistically significant difference in terms of the presence of slime genes between *S. aureus* (51%) and *S. epidermidis* (50%) strains (p>0.04).

In another study, slime production was investigated among the 113 *S. epidermidis* strains isolated from biomaterial-associated infections. The *ica A ica D*-positivity rates were determined as 57.5% of all the strains (Arciola et al., 2002). Similarly, among the patients with orthopaedic prosthesis infections in ortopedic unit, the rate of slime production was detected as 56.1% (69/123) of the *S. epidermidis* isolates by cultures on CRA (Arciola et al., 2003).

In a previous study conducted by Arciola et al. (2005b), they searched all five individual genes of ica locus in *S. epidermidis* isolated from 400 clinical isolates from prosthesis-associated infections. Among these isolates, the *ica* positivity rate was detected as 57%. Similar results were found in our study among the *S. epidemidis* strains.

S. aureus is a known significant pathogen. This bacterium has developed the various bacterial adhesion mechanisms. Among these mechanisms the host protein-binding receptors commonly termed adhesins are quite important (Patti et al., 1992). Adhesins are not absolutely necessary for the occurrence of infections; these factors enhance the infection severity. In *S .aureus* strains, the significant adhesin genes such as *clf*, *fnb* and *cna* have been found among the strains isolated from clinically various infections. However, little is known about surface adhesins of *S. aureus* strains isolated from surgical wound infections.

The specific surface proteins (*fnb A*, c*lf A*, *cna*) are expressed mainly by *S. aureus* strains (Foster and McDevitt, 1994). These specific surface proteins provide the specific interaction between bacteria and extracellular matrix proteins of the host cell. As a result, they contribute to bacterial colonization (Zmantar et al., 2008).

One of the most important staphylococcal adhesins is *cna*. In the literature, in large-scale studies, *cna* is accepted to be one of most important virulence factors in the pathogenesis of orthopaedic infections. It has been reported that *cna* gene was found to express in more than half of the staphylococci isolated from orthopaedic infections (Holderbaum et al., 1987). In a study conducted by Ryding et al. (1997), it was reported that the *cna* gene encoding a collagen adhesin was found in appraximately 60 of *S. aureus* isolates. In our study, the presence of *cna* (78.4%, in 69 of 88 *S. aureus*) was consistent with the literature.

Fnb A has been reported in a high proportion among the Staphylococcal strains isolated from various clinical infections (higher than 95%) (Peacock et al., 2000; Rice et al., 2001). As previously reported, *fnb A* adhesin gene was found to be very frequent in *Staphylococcal* strains. In a study conducted by Arciola et al. (2005a) the significant virulence factors (fibronectin (*fnb A*, *fnb B*) and *cna* adhesins) of *S. aureus* strains isolated from orthopaedic surgical infections were examined. In their study, the *cna* positivity rate was found to be 46%. Prevalence of the *fnb A* genes were almost detected in all surgical samples (98%) (Arciola et al., 2005a). Similar results have been found in studies of adults. *Fnb A* ratio (97.7%) in our study was found very similar to the findings from Arciola et al. (2005a's) study.

The bacterial adhesion thought to be an important step in the beginning of the infections (Projan and Novick, 1997). *Clf A* is a fibrinogen-binding surface protein of *S. aureus*. It account for interceeding the adhesion of

S. aureus to matrix proteins (McDevitt et al., 1995). And, it prevents phagocytosis during bacterial infection (Higgins et al., 2006). It showed that *clf A* is a significant virulence factor in various experimental infections such as endocarditis and sepsis (Vernachio et al., 2003; Josefsson et al., 2001).

In a study conduct by Higgins et al. (2006), *clf A* was shown to be important an as protein A as in *S. aureus* strains. In their study, *clf A* was also found to be antiphagocytic in a Gram-positive host. It was found that *clf A* inhibited phagocytosis in the absence of fibrinogen and showed enhanced inhibition in the presence of fibrinogen. In our study, the existence of *clf A* gene (51.1%) have been identified in more than half of *S. aureus* strains isolated from the wounds samples.

In our study, the percentage of three adhesins in *S. aureus* strains was found in quite high rates. The rate of *fnb A* gene among these adhesins were highest. It was followed by *clf A* and *cna* , respectively (*fnb A* positive: 97.7% of the strains, *clf A* positive: 51.1% of the strains, and *cna* positive: 78.4% of the strains). In our study, these findings were consistent with the earlier reported studies in the literature (Arciola et al., 2005a; Holderbaum et al., 1987; Rice et al., 2001).

In conclusion, in surgical wounds, some bacterial virulence factors such as slime production and adhesins may complicate wound healing and cause serious medical problems. Also, they may lead to increasing antimicrobial resistance.

The *ica A* and *ica D* genes responsible for slime production among the Staphylococci isolated from surgical wound samples have been found in a significantly high frequency. Slime production in *S. aureus* and *S. epidermidis* strains was found to be significantly similar to each other. The frequency rate of adhesin genes was very high in *S. aureus* strains isolated from surgical wounds. While *clf A* and *cna* genes were found in more than half of these strains, almost all of the strains were positive for *fnb A* gene. Molecular identification of virulent staphylococcal strains may help in clinical decision making. The necessary precautions should be taken to prevent infections caused by virulent Staphylococci.

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