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Determining the Biofilm Forming Gene Profile of *Staphylococcus aureus* Clinical Isolates via Multiplex Colony PCR Method

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Abstract

Background: Among hospitalized patients, *Staphylococcus aureus* (*S. aureus*) infections pose a serious health threat. The present study investigated the frequency of biofilm forming genes among clinical isolates *S. aureus* and their susceptibility to antibiotics.

Methods: The clinical samples were analyzed via standard biochemical assays for identifying different bacterium, which was then confirmed using the multiplex colony PCR method. Those samples identified as *S. aureus* were examined for the presence of the *cna, fnbA, fnbB* and *pvl* genes. The antibiotic susceptibility of the *S. aureus* isolates was then tested.

Results: Using the standard biochemical assay approach, 54 *S. aureus* strains were identified. However, when using the multiplex PCR method 50 *S. aureus* strains were identified among the clinical samples. The *in vitro* biofilm formation assays determined 3 (6%) strains of *S. aureus* to be strong biofilm forming, 15 (30%) of the isolates were determined to be moderate biofilm forming and, 32 (64%) were determined to be weak biofilm forming. Among the isolated strains, the specific frequencies of the biofilm forming genes were determined to be 31 (62%) for *cna*, 35 (70%) for *fnbA*, 13 (26%) for *fnbB* and 1 (2%) for *pvl*. In 11 (22%) of the isolated strains *fnbA*, *fnbB* and cna genes were all present. All strains were determined to be penicillin, amoxicillin and clavulanic acid resistant.

Conclusions: Due to the increase of the antibiotic resistance in biofilm producing S. *aureus* strains, rapid identification of antibiotic resistance can help to eliminate the infection effectively.

Keywords: Biofilm, Multiplex colony PCR, Pertussis toxin, Spreading factors, *Staphylococcus aureus*.

Introduction

The commensal bacterium, *Staphylococcus aureus* (*S. aureus*), is one of the *Staphylococcus* species that is commonly involved in serious nosocomial and community-acquired infections (1). Infection with *S. aureus* can result in the development of bacteremia, osteomyelitis, skin infections, pneumonia, meningitis and endocarditis (2). The ability of bacteria to form highly organized multicellular complexes called biofilms, is considered to be a significant factor that enhances the virulence of the *Staphylococcus* species (2, 3). Biofilms help bacteria to establish chronic infection and enhance their resistance to the antibiotics by facilitating the transfer of responsible genes such as insertion sequences, especially in Gram positive cocci (4).

Staphylococcus aureus is the most common pathogen involved in infections associated with the implantation of medical devices (5). This bacterium

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is able to colonize implants and establish chronic infections in the patient. The development of these infections commonly results from procedures involving orthopedic implants such as those using prosthetic joints, wires, pins, external fixators, plates, screws, nails and mini-large fragment implants (6). The establishment of *S. aureus* infections results from a combination of host-related factors of the patient and pathogenic factors of the bacterium colonizing the medical device implanted in the patient (2). Biofilms contain channels that allow the bacterium to receive nutrients from the environment. The eradication of biofilms is an incredibly challenging task, as they are often resistant to antibiotic therapy and require surgical intervention. The most effective means of preventing the development of infections by *S. aureus* is through inhibiting the formation of biofilms on the medical devices prior to implantation (7). The formation of biofilms involves two independent processes: (1) the initial attachment of bacteria to a solid surface, and (2) the growth and accumulation of the bacteria (3). To allow for proper attachment and biofilm formation, *S. aureus* contains specific molecules that enable bacterial binding to host cells. Specifically, *S. aureus* binds to cell surface proteins by means of collagen binding proteins (*cna*) and fibronectin binding proteins A and B (*fnbA*, *fnbB*) (8, 9). The *S. aureus* bacterium is capable of producing a vast array of extracellular proteins, including enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ), Panton- Valentine Leukocidin (PVL), toxic shock syndrome toxin 1 (TSST-1), exfoliative toxin (ETA and ETB) and alpha toxin (Hlg) (10). The purpose of this study was to screen for the presence of biofilm-associated genes *(cna, fnbA, fnbB* and *pvl*) by multiplex colony polymerase chain reaction (PCR) and examine the susceptibility of the biofilm forming strains against routine antibiotics.

Materials and methods

Strain isolation and identification

A total of 200 suspected *staphylococci* spp., were isolated from different clinical samples of patients suspected to have clinical infection from 3 hospitals of Tehran (Sina, Emam Khomeini and Shariati). Samples were isolated from urine, blood, sputum, cerebrospinal fluid (CSF), pleural fluid, and wounds of patients from October 2015 to March 2016. Isolated strains were identified using conventional microbial tests (Gram stain, Oxidase and catalase, Coagulase, mannitol and other special routine tests for identification of *S. aureus* isolates) according to the scheme utilized as previously described (11). All isolates were stored at -20 °C in Brain Heart Infusion Broth with 18% Glycerol.

Confirmation of S. aureus isolates by Polymerase Chain Reaction

Isolated strains were analyzed by multiplex colony PCR. Specifically designed primers (*nucF*: 5-ATGGCTATCAGTAATGTTTCG -3 and *nucR*: 5-TTTAGGATGCTTTGTTTCAGG -3) were used to identify *S. aureus* strains (12). Amplification was done using the Gene Amp PCR system (Applied Biosystem, USA) by colony PCR method in a volume of 25 µl containing: 14 µl master amplicon (Biolab, New England, UK), 1 pmol of each forward and reverse primers, Minor amount of colony as template and 9 µl distilled water. The first cycle of denaturation was at 95 °C for 5 min, followed by 25 cycles at 95 °C for 30 sec, then 55 °C for 1 min, 72 °C for 1 min, and finally a terminal extension for 5 min. The PCR results (318 bp) were visualized with 1.5% agarose gel (KBC, Max Pure agarose, Spain). The molecular approach was optimized by using *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 as the control strains (Fig. 1A).

Antibiotic susceptibility testing

The susceptibility of *S. aureus* isolates to different antibiotics was performed according to the CLSI standard guide lines (15). Specifically, disks (BD BBLTM Sensi DiscTM) contained 30 µg vancomycin, 30 µg nalidixic acid, 25 µg trimethoprim/sulfamethoxazole, 15 µg erythromycin, 5 µg novobiocin, 2 µg penicillin, 30 µg doxycycline, 30 µg ceftriaxone, 10 µg amikacin, 5 µg methicillin, 10/ 20 µg amoxicillin/ clavulanic acid, and 30 µg streptomycin. Evaluation of the results was performed according to the manufacture recommendations for the breakpoints of *Pseudomonas aeruginosa* (Fig. 2).

In vitro biofilm formation assay

The biofilm formation assay was performed as previously described (16). Briefly, the optical density of inoculated colonies on the trypticase soy broth was adjusted to 0.7 in 600 nm. Afterward, 200 µl of 1:200 diluted (OD 0.005 in 600 nm) supernatants in trypticase soy broth supplemented with 1% glucose (TSBg media) was transferred into a polystyrene microtiter plate (Nunc, Roskilde, Denmark). Following a 16 h incubation at 37 °C, supernatants and planktonic cells were decanted by washing three times with phosphate-buffered saline (PBS). All wells received 150 µl of 0.1% crystal violet suspension, additional stain was removed by washing twice with PBS buffer. The absorbance of each well was measured at a wavelength of 595 nm by adding 160 µl alcohol /acetic acid solution (4:1 concentration). Each sample was tested in triplicate and the ability of the bacterium to form biofilms was measured using the following formula. Optical density cut-off value (ODc) = average OD of negative control + 3 X standard deviation (SD) of negative control.

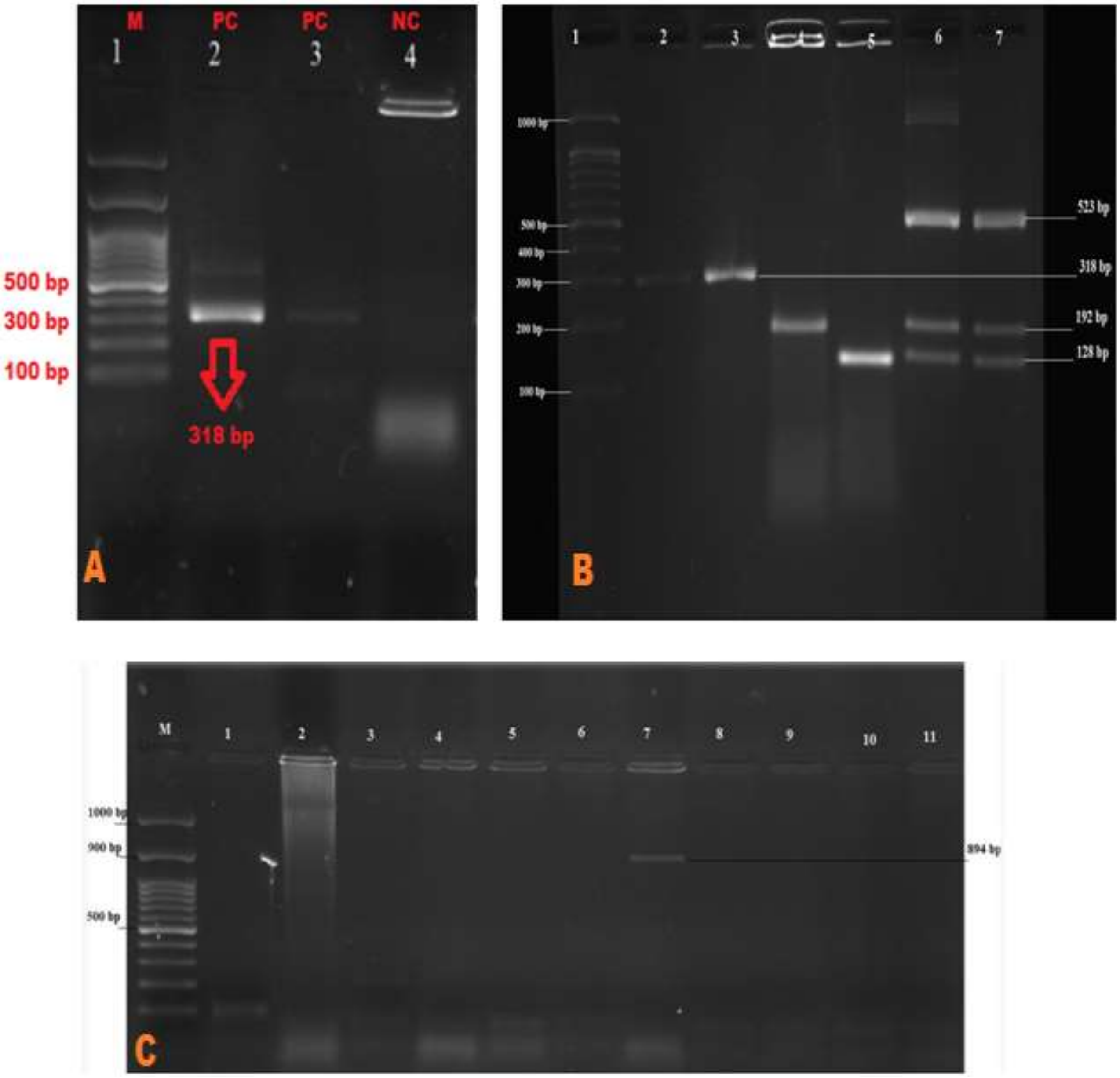

Fig. 1. The amplification of S. *aureus* gene determinants. A) Standardization of PCR by crude DNA and colony as template in 0.8% agarose gel. M; 1 kb DNA ladder, 2; positive control (colony), 3; positive control (crude DNA),4; negative control. B) Standardization of Multiplex colony PCR in 3% agarose gel. M; 1 kb DNA ladder, 2; amplified *nuc* gene from crude DNA, 3; amplified *nuc* gene from a fresh colony, 4, 5 set up of PCR for *cna* and *fnbA* genes, 6,7; optimizations of multiplex PCR by crude DNA and fresh colony. C) Screening of the *pvl* gene in our isolates in 0.8% agarose gel.

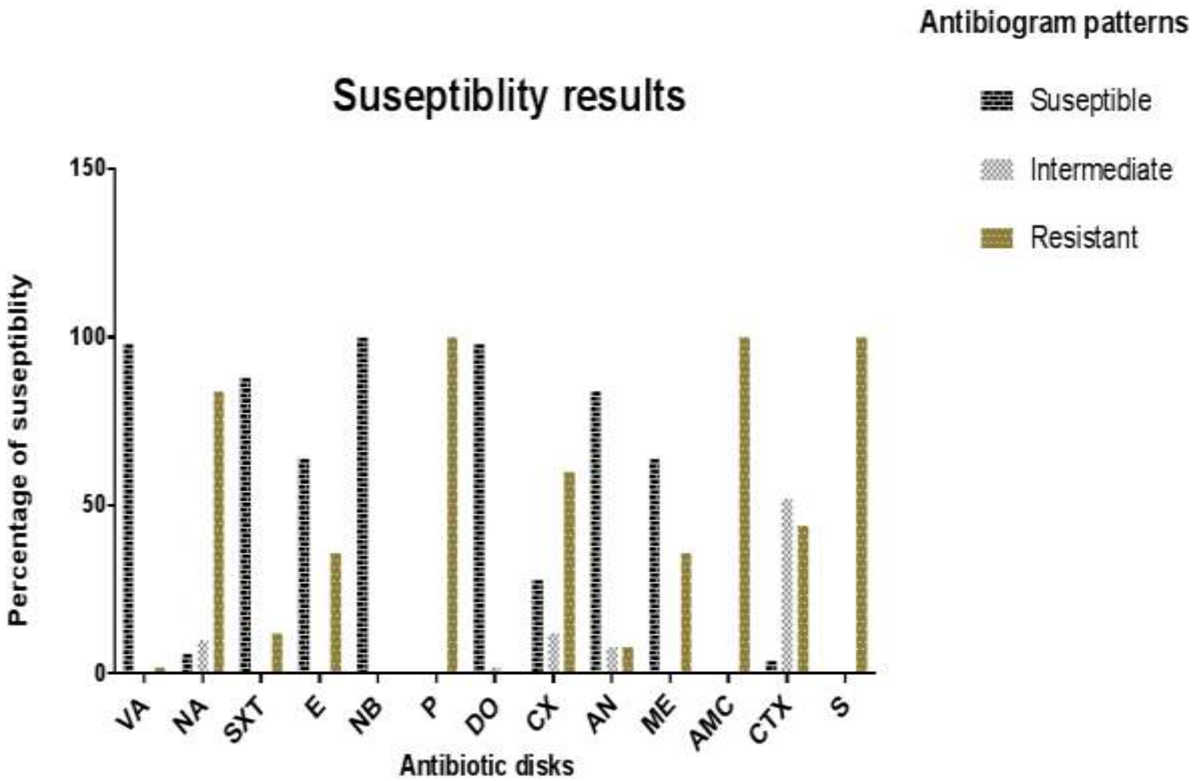

Fig. 2. Susceptibility patterns of *S. aureus* isolates to the antibiotics.
VA; vancomycin, NA; nalidixic acid, SXT; trimethoprim/sulfamethoxazole, E; erythromycin, NB; novobiocin, P; penicillin, DO; doxycycline, CX; Cefotaxime, AN; amikacin, ME; methicillin, AMC; amoxicillin/ clavulanic acid, CTX; ceftriaxone, S; streptomycin.

PCR screening of biofilm-associated genes by Multiplex Colony PCR

All strains were screened for the presence of genes associated with biofilm formation. Specifically, the *cna*, *fnbA*, *fnbB* and *pvl* genes (13). The oligonucleotide primer sequence is specified in Table 1. All targeted genes were generated via amplification reactions using a total volume 50 µl in two series. The mixture contained: 30 µl of master mix amplicon, 1 pmol of each primer, minor amounts of fresh colony as a template using Gene Amp PCR system (Applied Biosystem, USA). Multiplex Colony PCR was performed in two series: first series optimized for sensing of *cna*, *fnbA*, *fnbB* genes and other for *pvl* actual utilizing program an initial cycle of denaturation 95 °C for 2 min, followed by 50 cycles of 95 °C for 30 sec, 55 °C 1 min (60 °C for *pvl*), 72 °C for 1 min with 1 min for final extension. Resulting PCR was visualized with 3% agarose gel (KBC, Max Pure agarose, Spain) for amplified genes. The molecular approach was optimized by *S. aureus* ATCC 25923 as the control strain (Figs. 1B and 1C).

Table 1. Primer sequences used for PCR.

Primer sequence (5-3)	Size of product (bp)	References
nuc F: ATGGCTATCAGTAATGTTTCG *nuc* R: TTTAGGATGCTTTGTTTCAGG	318	(12)
fnbA F: CATAAATTGGGAGCAGCATCA *fnbA* R: ATCAGCAGCTGAATTCCCATT	128	(13)
fnbB F: GTAACAGCTAATGGTCGAATTGATACT *fnbB* R: CAAGTTCGATAGGAGTACTATGTTC	523	(13)
cna F: AAAGCGTTGCCTAGTGGAGA cna R: AGTGCCTTCCCAAACCTTTT	192	(13)
Pvl (*luk*pv)F: ATCATTAGGTAAAATGTCTGGACATGATCCA *pvl* (*luk* pv) R: GCATCAACTGTATTGGATAGCAAAAGC	892	(14)

Results

A total of 183 clinical samples were analyzed. Among the samples, 27% were identified to be *S. epidermidis*, 27% were *S. aureus* and 46% were other staphylococcus species. Fifty isolates were confirmed as *S. aureus* strains utilizing the multiplex colony PCR method. The distribution of *S. aureus* in the clinical samples was: Blood - 4 (8%); Tracheal aspirate - 18 (36%); Urine 11 (22%).

When screening for the genes associated with attachment to the host cells, *fnbA* and *cna* (98% and 84%) were determined to be the predominant genes found within the clinical isolates. These genes were found to occur at a much higher frequency than the *fnbB* and *pvl* genes (26% and 2 %, respectively). Among the strong biofilm forming isolates, the frequency of *fnbA* and *cna* genes were higher than in those of the moderate biofilm forming strains.

Table 2. Frequency of biofilm related genes in clinical isolates.

Screened genes	*fnbA, fnbB*	*fnbA, cna*	*fnbA, pvl*	*fnbB cna*	*fnbB, pvl*	*cna, pvl*	One gene +	*fnbA, fnbB, cna*	All genes +	None genes +
Percentage	18	52	2	4	2	0	76	6	0	22

Comparative analysis for the biofilm assay for confirmed *S. aureus* strains demonstrated that 6% of the samples produced strong biofilm, 30% of the samples were moderate biofilm forming strains, and 64% of the isolates were weak biofilm forming. All of the strong biofilm forming strains were isolated from the trachea. These strong biofilm forming strains were resistant to methicillin. The targeted genes (*fnbA*, *fnbB*, and *cna*) were found to be present in the strong biofilm forming strains. Details are listed in Table 2.

Susceptibility testing indicated, that: vancomycin (98%), doxycycline (98%), novobiocin (100%), Amikacin (84%) and methicillin (64%) were effective forms of antibiotics against most of the clinical isolates. The *S. aureus* strains were highly resistant to penicillin (100%), AMC (100%), streptomycin (100%), nalidixic acid (84%) and methicillin (36%) (Fig. 2).

Our findings indicate of the 50 *S. aureus* strains retrieved from the clinical isolates 49 (98%) isolates of them were resistant against more than one antibiotic. The percentage of multi drug resistant (three to nine antibiotics simultaneously) strains was as follows: 11 (22%), 13 (26%), 11 (22%), 4 (8%), 5 (10%), 3 (6%) and 1 (2%). Only 1 (2%) of the isolated strains was resistant to all of the antibiotics.

Discussion

Staphylococcus aureus is a common bacterium colonizing the human skin and mucous membranes. However, *S. aureus* is also a major causative agent of hospital and community-associated infection that can result in life-threatening disease (3, 10). Attempts to control the extent of antibiotic-resistant *S. aureus* strains have relied on three factors: ensuring proper hand hygiene among healthcare workers, restricting the use of antibiotics, and promptly identifying and isolates infected patients (17, 18).

Although biochemical assays have been described as an appropriate means of identifying *S. aureus* strains this approach is subpar. With the use of molecular approaches such as PCR, identification of the exact bacterial strains can be more precise (19). Our current study utilized both the standard biochemical assay for determining the strains of the clinical isolates, as well as the molecular PCR approach. Using the biochemical assay, 54 of the clinical isolates were determined to be *S. aureus*. (54 strains were identified by biochemical methods with Gram stain, oxidase and catalase cconsumption pattern to mannitol, but only 5 of them were suspected in the evaluation of coagulation activity. Using the molecular method, these 5 isolates were deleted for further evaluation purposes). When using PCR, only 50 (27%) isolates were determined to be *S. aureus*. Previous work has shown similar results in the accuracy of detecting the specific strains. The use of PCR has shown to have higher sensitivity than the biochemical assay. Therefore, PCR may be a better method to use for fast and precise identification of bacteria from clinical samples (12).

The ability to produce biofilms on the surface of medical devices has been considered to be one of the most frequent causes of nosocomial sepsis and nosocomial infections in hospitalized patients. Infection among these patients results in an extremely high rate of morbidity and mortality (19). Biofilms are highly resistant to both innate and adaptive host defense mechanisms. Increasing recognition has been given to the important role that biofilms play in the establishment of chronic bacterial infections (1). Our study examined the biofilm formation abilities of different *S. aureus* isolates. Samples were categorized into either weak, moderate or strong biofilm formers. Among the clinical isolates, 30% of the *S. aureus* strains were moderate-biofilm formers 6% as strong biofilm formers and 64% of them were determined as weak biofilm formers. According to previous findings, biofilm forming ability of the vancomycin resistant *S. aureus* (VRSA) isolates were 54.5% and 27.3% for the strong and moderate biofilm forming strains, respectively (20). In this study, out of the 50 *S. aureus* isolates, 2 (4%) were determined to be vancomycin-resistant. The *in vitro* biofilm forming assay results for these isolates revealed that this isolated VRSA strain was strong biofilm forming (20).

Adherence of *S. aureus* to the human body occurs through the use of microbial surface components such as collagen binding protein (*cna*) and fibronectin binding proteins A and B (*fnbA*, *fnbB*) (9). In our study, the frequency of *fnbA* are very similar to a study from 2013 examining *S. aureus* strains among hospitalized children. However, the frequencies of the genes *fnbB* and *cna* show very different results (21). This may be due to differences in sample collection sites. In the 2013 study, the clinical samples of urine, sputum, wound biopsies, and respiratory chips were collected from hospitalized patients from the intensive care unit (ICU), and the infectious disease and burn wards. A previous study examining *S. aureus* strains isolated from hemodialysis catheters of Mexican patients showed the prevalence of *fnbB* to be 31% and *cna* to be 43%, which is also different from our study. However, all samples were collected via swabs from the insertion site of the hemodialysis catheters which may account for the differences among the results (14). In a separate study, the frequency of *pvl* was observed to be 51%, which is far different from the 2% observed in our study (22). The differences in the frequencies of the *pvl* gene in the *S. aureus* isolates may be due to the differences in the number of MRSAs strains present among the collected samples (22).

Our study has shown for the first time that the frequency of biofilm-related genes can be accurately determined using the Multiplex Colony PCR method. Within our specific isolates of *S. aureus,* the PCR results show the *fnbA* and *cna* genes to be to the more dominant genes. The frequency of identifying the *fnbA* gene is relatively similar to previous findings (23). In another study, the frequency of the *cna*, *fnbA* and *fnbB* genes were 43%, 19% and 31%, respectively. These findings are different from our results (24). The differences may be attributed to the type of samples examined.

Antibiotic susceptibility testing is the main protocol for determining the proper antibiotics to be used to clear bacterial infections (15). Complete or relative resistance to β-lactams is a characteristic feature of the genus staphylococci (15). Bactericidal antibiotics (e.g., ampicillin or penicillin G) for strains susceptible to penicillin and, glycopeptide antibiotics (e.g., vancomycin) are the best drugs for treating *Staphylococcus* infections as they do not exhibit high level resistance to these antibiotics (25). Our findings show almost all isolates to be susceptible to a combination of vancomycin and amikacin. Penicillin, streptomycin, amoxicillin/clavulanic acid and nalidixic acid were determined to be insufficient for eradicating the *S. aureus* infections. The susceptibility testing showed the MDR strains to be a major problem with *S. aureus* infections. The presence of genes for attachment and the formation of biofilms was examined. Strains with more biofilm forming genes have an increased ability to colonize the human body, exhibit enhanced pathogenesis and antibiotic resistance. Due to the existence of multi-drug resistance, rapid identification and precise characterization of the biofilm related genes, and thus, potential pathogenicity of the *S. aureus* strains, is essential to prevent the spread of infections throughout hospital wards.

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