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ORIGINAL ARTICLE

Biofilm Production and Antibiotic Resistance of *Staphylococcus epidermidis* in Catheter Related Bloodstream Infections

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ABSTRACT

Background: *Most Staphylococcus* epidermidis-*related infections are acquired in hospitals and are associated with the use of medical devices.* ***Objectives:*** *The aim of this study was to detect biofilm producing S. epidermidis from patients with catheter-related bloodstream infections (CRBSI) and determine their antibiotic resistance pattern.* ***Methodology:*** *Blood and catheter tip specimens were collected from 150 patients with CRBSI. Isolated S. epidermidis strains were tested for biofilm production by the modified tissue culture plate (MTCP) method, Congo red agar (CRA) method, and icaA and icaD genes detection by PCR. Antibiotic resistance pattern of both biofilm producing and biofilm non-producing isolates was tested using the disc diffusion method.* ***Results:*** *The prevalence of S. epidermidis in CRBSIs was 13.3%. Biofilm production was detected in 55% and 45% of isolates by the MTCP method and CRA method respectively. IcaA and icaD genes were detected in 20% and 30% of the isolates respectively. The sensitivities of CRA method, icaA and icaD were 81.8%, 36.4%, and 54.5% respectively. All of them showed specificity 100%. Biofilm producing isolates were more resistant to cefotaxime, erythromycin, rifampin, tetracycline, gentamycin, ciprofloxacin and ampicillin than biofilm non-producing isolates. All the isolates were sensitive to linezolid and vancomycin.* ***Conclusion:*** *The MTCP remains the best method for screening of biofilm production. IcaD gene detection is more sensitive for detection of biofilm production in S. epidermidis than icaA gene, however, their presence does not always correlate with phenotypic biofilm production. Biofilm producing S. epidermidis isolates showed more antibiotic resistance than biofilm non-producing isolates.*

INTRODUCTION

Catheter-related bloodstream infections (CRBSIs) are among the most common healthcare-associated infections, and coagulase-negative staphylococci, especially *Staphylococcus epidermidis*, are the most common pathogens involved [1] . *S. epidermidis* is one of the major biofilm-producing bacteria and it works by attaching itself to several surfaces [2]. Biofilm formation facilitates the development of infections and leads to failure of antibiotic therapy, which may result in recurrent infections and the emergence of multi-resistant pathogens [3]. The principal component of the biofilm is a polysaccharide intercellular adhesion (PIA) produced by *ica* operon-encoded enzymes comprising four genes (A, B, C, and D), a transposable element, IS256 and a regulatory gene (*icaR*). PIA plays an essential role in initial bacterial adherence to surfaces and intercellular adhesion of the cells in aggregates [4].

The hospital environment can be colonized by biofilm forming coagulase-negative staphylococci and conveyance of these strains can cause an increased risk of serious nosocomial infections [6]. Existence of a high proportion of *S. epidermidis* strains in cardiovascular infections and in blood samples confirms that this species is one of the most important pathogens causing bloodstream infections because their biofilm formation ability on implanted medical devices [7]. *S. epidermidis* spreads from the external surface of any blood-contacting medical device during the settlement of it by the surgical operation; colonization and biofilm formation on the device's surface may come true and this may be the cause of every reoccurring, persistent infection [8].

Biofilm-associated bacteria are usually less susceptible to antibiotics than planktonic bacteria; this can be explained by many mechanisms, such as the antibiotics binding to biofilm components, reduced penetration of the antibiotic, slower growth of the microorganisms in the biofilm, a high bacterial density and altered gene expression in the bacteria found in the biofilm [9]. Post *et al.* identified biofilm formation and antibiotic resistance as associated with poor clinical outcome in *S. epidermidis* device-related infections [10].

The aim of this study was to detect biofilm producing *S. epidermidis* from patients with catheter-related blood stream infections (CRBSIs) and determine their antibiotic resistance pattern.

METHODOLOGY

The study was conducted on 150 catheterized patients with central venous lines, umbilical catheter or duel lumen short-term hemodialysis catheter who acquired CRBSI after their admission to the intensive care units or hemodialysis unit of Suez Canal University Hospitals, Ismailia, Egypt. All these episodes of CRBSIs have necessitated removal of the catheters. Patients of both sex and all age groups were included in the study after their acceptance to participate.

Specimen collection and processing:

Two specimens were collected from each patient included in this study; a blood specimen and a catheter tip specimen, under complete aseptic conditions. Blood was added to monophasic blood culture bottle (BacT/ALERT FA FAN® Aerobic or BacT/ALERT PF Pediatric FAN; bioMerieux), incubated at 37°C and examined daily for up to 7 days. The subculture of blood culture broth was performed onto blood agar and MacConkey's agar and incubated aerobically at 37°C for 24 hours. Catheter tips were rolled four times across blood agar plates with firm downward pressure and the plates were incubated at 37°C for 24 hours. Cultures yielding 15 or more colonies were considered significant and indicate a catheter-related infection.

Identification of *S. epidermidis:*

Isolated bacterial colonies were Gram stained and tested by catalase and coagulase tests. Gram-positive cocci that were catalase positive and coagulase negative were further identified as *S. epidermidis* by their susceptibility to novobiocin, positive urease reaction, mannitol non-fermentation and acid production from D-mannose fermentation. Isolates that were confirmed as *S. epidermidis* were tested for biofilm production by phenotypic and genotypic methods.

Phenotypic detection of biofilm production:

Biofilm production by *S. epidermidis* was phenotypically detected by two methods; modified tissue culture plate method and congo red agar method. *S. epidermidis* ATCC 35984 (slime producer) and *S. epidermidis* ATCC 12228 (slime non-producer) were used as positive and negative controls respectively.

a. *Modified tissue culture plate (TCP) method*:

S. epidermidis isolates from fresh agar plates were inoculated onto trypticase soy broth with 1% glucose and incubated for 24 hours at 37°C and then diluted (1 in 100) with fresh medium. Individual wells of sterile, flat-bottom tissue culture plates were filled with 0.2 ml aliquots of the diluted cultures. One well was filled with broth to serve as a control to check sterility and non-specific binding of media. Positive and negative controls were also included in the wells; after incubation for 24 hours at 37°C, the content of each well was gently removed by tapping the plates and the wells were washed four times with 0.2 ml of phosphate buffer saline (pH 7.2) to remove free-floating planktonic bacteria. Twenty-five microliters of crystal violet 1% was added to each well and the plates were incubated at room temperature for 15 minutes, after which they were rinsed thoroughly and repeatedly with water. Crystal violet-stained biofilm was then solubilized in 200 µl of 95 % ethanol of which 125 µl were transferred to a new polystyrene microtiter dish to be read with a micro-ELISA auto reader (STAT FAX-2100, Fisher Bio block Scientific) and the values of absorbance were considered as an index of biofilm formation capacity of each tested strain. To compensate for background absorbance, Optic Density (OD) readings of wells with ethanol were used as blank and subtracted from all tests' values [11].

The isolates were classified into three categories, non-adherent (OD $\leq$ 0.111), moderately adherent (OD >0.111 to $\leq$ 0.222), and strongly adherent (OD > 0.222). Non-adherent isolates were considered as negative for biofilm formation, while the moderately or strongly adherent were considered positive [3].

b. Congo Red Agar (CRA) method:

Congo red agar was prepared by adding 36g of 5% sucrose and 0.8g of congo red stain (Sigma) to one liter of brain heart infusion (BHI). *S. epidermidis* isolates were cultured on CRA and incubated aerobically at 37°C for 24 - 48 hours. Biofilm-producing *staphylococci* grew as black colonies while biofilm non-producing strains grew as red colonies [12].

Genotypic detection of biofilm formation:

Genomic DNA was extracted by the boiling method as described by Pérez-Roth *et al.,* 2001[13]. Amplification of *icaA* and *icaD* genes by PCR method was carried out in a thermal cycler (Techneprogene) using two pairs of primers. The sequences of *IcaA* and *IcaD* primers are shown in the table 1.

Table 1: Primer sequence of *IcaA* and *IcaD* genes*:*

Primer	*Sequence*	*Nucleotide position*	*Product size*
IcaA forward primer	5'-TCTTGCAGGAGCAATCAA-3'	1337 - 1356	188 bp
IcaA reverse primer	5'-TCAGGCACTAACATCCAGCA-3'	1505 - 1524	
IcaD forward primer	5'-ATGGTCAAGCCCAGACAGAG-3'	1963 - 1982	198 bp
IcaD reverse primer	5'-CGTGTTTTCAACATTTAATGCAA-3'	2138 - 2160	

The 25µl reaction volume contained 2.5 µl of each of the forward and reverse primers (1 µM each), 150 ng (5 µl) of the extracted DNA, 100 µM each of dATP, dCTP, dGTP, and dTTP, 1 U of *Taq* DNA polymerase, PCR buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100) and 2.5 mM $MgCl_2$. The amplification protocol was incubation at 94°C for 5 minutes, followed by 50 cycles of 94°C for 30 seconds (denaturation), 55.5°C for 30 seconds (annealing), 72°C for 30 seconds (extension), and 72°C for 1 min after the conclusion of the 50 cycles. After the first 30 cycles, a further 1 U of *Taq* DNA polymerase was added. After amplification, 10 µl of the PCR mixture was analyzed by agarose gel electrophoresis (2% agarose in Tris-borate-EDTA and stained with ethidium bromide). A 100 bp DNA ladder was used as a molecular weight marker.

Antibiotic Susceptibility Testing:

Antibiotic susceptibility testing was performed according to the recommendations of Clinical and Laboratory Standard Institute (CLSI) [14]. The disc diffusion method was used for testing Cefoxitin 30µg, Erythromycin 15 µg, clindamycin 2 µg, Linezolid 30 µg, Sulphamethoxazole-Trimethoprim 25 µg, ampicillin 10 µg, Cefotaxime 30 µg, tetracycline 30 µg, Gentamicin 10 µg, Ciprofloxacin 5 µg and Rifampicin 5 µg. All antibiotic discs were purchased from Oxoid, Thermo Fisher Scientific Inc. Company. Vancomycin susceptibility was tested using the broth dilution method to determine its minimal inhibitory concentration (MIC). Vancomycin powder was purchased from Sigmatech, Inc.

RESULTS

The study included 150 patients with CRBSI. Strains of *S. epidermidis* were isolated from only 20 patients. The prevalence of *S. epidermidis* in CRBSIs in this study was 13.3%.

Phenotypic detection of biofilm production:

Biofilm production ability of the twenty *S. epidermidis* isolates was tested by both modified TCP method and CRA method. Using the modified TCP method, 11 strains (55%) were biofilm producers; 6 (30%) were strongly adherent and 5 (25%) were moderately adherent. Nine strains (45%) were biofilm non-producers. Using the CRA method, 9 strains (45%) were positive for biofilm production and 11 strains (55%) were negative.

Genotypic detection of biofilm formation:

The conventional PCR revealed that four *S. epidermidis* strains (20%) were positive for *icaA* gene (figure 1) while *icaD* gene was detected in six strains (30%) (figure 2). All the four strains which were positive for *icaA* were also positive for *icaD.* All *icaD* positive strains were also positive for phenotypic detection of biofilm production by both the modified TCP and CRA methods. Table 2 shows the rate of detection of biofilm production by all methods used in this study.

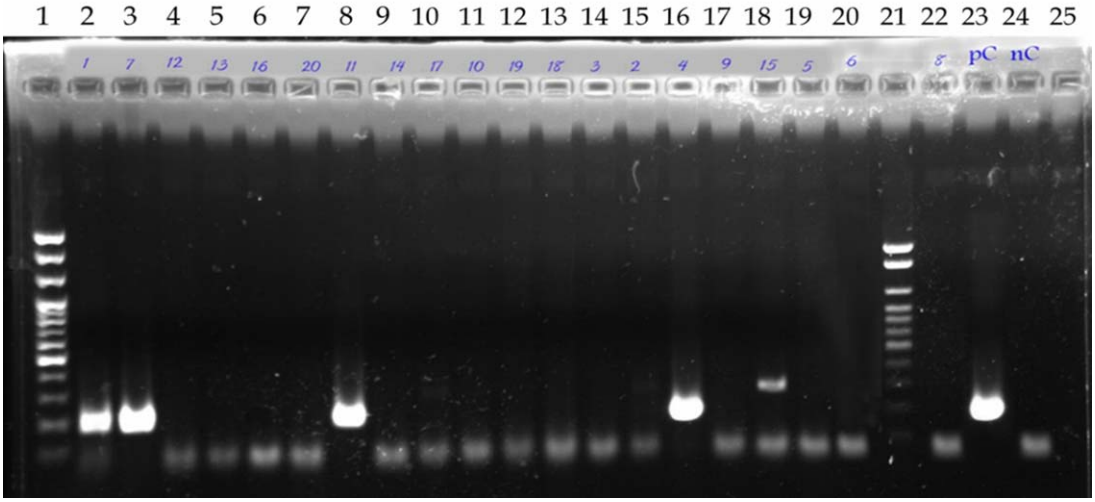

Fig. 1: Agarose gel electrophoresis of PCR products for *icaA* gene. Lanes 1 and 21: MW ladder, L23: positive control, L24: negative control, four strains (strains number 1, 7, 11 and 4) showed specific bands of the expected size of *icaA* amplicons (188 bp).

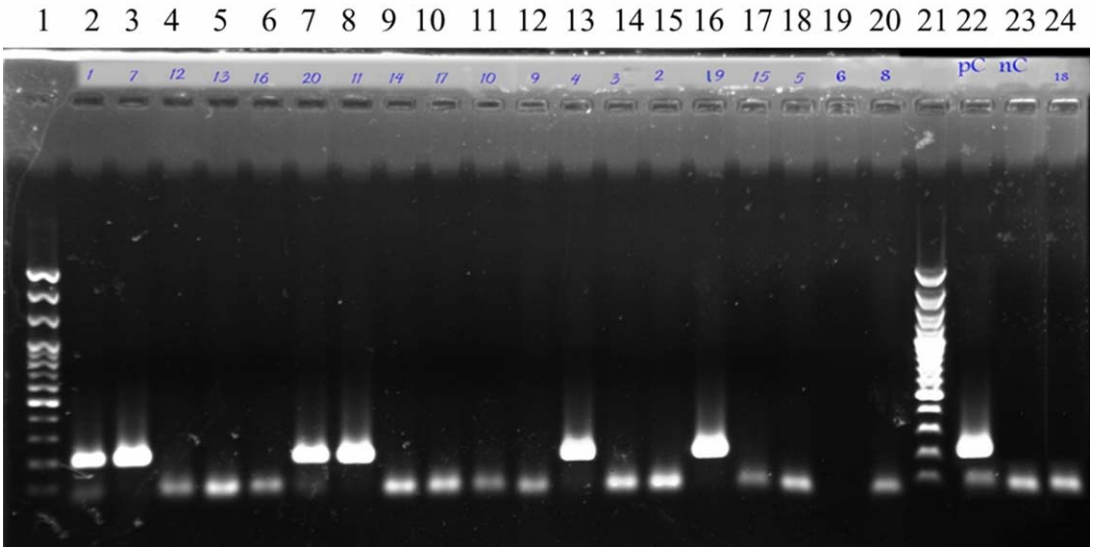

Fig. 2: Agarose gel electrophoresis of PCR products for *icaD* gene. Lane 1 and 21: MW ladder, L22: positive control, L23: negative control, six strains (strains number1, 7, 20, 11, 4 and 19) showed specific bands of the expected size of *icaD* amplicons (198 bp).

Table 2: Rate of detection of biofilm production in *S. epidermidis* by the phenotypic and genotypic methods (n=20):

Method	***MTCP***	***CRA***	**IcaA *PCR***	**IcaD *PCR***
Positive	11 (55%)	9 (45%)	4 (20%)	6 (30%)
Negative	9 (45%)	11 (55%)	16 (80%)	14 (70%)

Considering the modified TCP method as the gold standard method for detection of biofilm production, the CRA and PCR methods were evaluated. Their sensitivities, specificities, positive predictive values (PPV), negative predictive values (NPV), and accuracies were calculated as shown in table 3. The CRA method was more sensitive (81.8%) than the *icaA* PCR and *icaD* PCR methods with the *icaD* PCR being more sensitive (54.5%) than the *icaA* PCR method (36.4%). In addition to its higher sensitivity, the CRA method was more accurate and with higher NPV than the PCR methods. All the evaluated methods showed specificity 100%

Table 3: Statistical evaluation of the CRA, *icaA* PCR, and *icaD* PCR methods for detection of biofilm production in *S. epidermidis* in comparison to the MTCP method:

Method	***Sensitivity***	***Specificity***	***PPV***	***NPV***	***Accuracy***
CRA	81.8%	100%	100%	81.8%	90%
IcaA PCR	36.4%	100%	100%	56.3%	65%
IcaD PCR	54.5%	100%	100%	64.3%	80%

Antibiotic Susceptibility Testing:

The antibiotic susceptibility of both biofilm producing and biofilm non-producing *S. epidermidis* strains showed that biofilm producing isolates were more resistant than biofilm non-producing isolates to erythromycin, gentamycin, rifampin, tetracycline, cefotaxime, ciprofloxacin, and ampicillin. All biofilm producers and biofilm non-producers were sensitive to linezolid and vancomycin. Table 4 shows the antibiotic susceptibility pattern of biofilm producing and biofilm non-producing isolates.

Table 4: Results of antibiotic susceptibility testing for biofilm producing and non-biofilm producing *S. epidermidis*.

Antibiotic	*Biofilm producers (N = 11)*				*Biofilm non-producers (N = 9)*			
	Sensitive		**Resistant**		**Sensitive**		**Resistant**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Cefoxitin 30 µg	4	36.3	7	63.7	2	22.2	7	77.8
Erythromycin 15 µg	2	18.1	9	81.9	2	22.2	7	77.8
Clindamycin 2 µg	8	72.8	3	27.2	6	66.7	3	33.3
Linezolid 30 µg	11	100	0	0	9	100	0	0.0
Sulphamethoxazole-trimethoprim 25 µg	8	72.8	3	27.2	5	55.6	4	44.4
Rifampicin 5 µg	3	27.3	8	72.7	8	88.9	1	11.1
Gentamicin 10 µg	0	0	11	100	5	55.6	4	44.4
Tetracycline 30 µg	4	36.3	7	63.7	7	77.8	2	22.2
Cefotaxime 30 µg	4	36.3	7	63.7	9	100	0	0
Ciprofloxacin 5 µg	6	54.6	5	45.4	7	77.8	2	22.2
Ampicillin 10 µg	4	36.3	7	63.7	5	55.6	4	44.4
Vancomycin	11	100	7	0	9	100	0	0

DISCUSSION

Most *S. epidermidis* related infections are acquired in hospitals and are associated with the use of medical devices. In this study, we detected *S. epidermidis* in 13.3% of patients with CRBSIs. The rate of our detection of *S. epidermidis* strains in CRBSIs was nearly similar (14.7%) to the study of Lepainteur *et al.* [15], but different from the study of Seisdedos Elcuaz *et al.*, who reported a very high rate of detection (38%) [16].

Using the modified TCP method, 55% of our strains were biofilm producers; 30% were classified as strongly adherent and 25% as moderate adherent. Approximate results were reported by Gad *et al.* who have found that 51.4% of *S. epidermidis* strains were strong biofilm producers, 37.1% were moderate biofilm producers, and 11.4% were biofilm non-producers[17]. Nasr *et al.* compared the different phenotypic methods for biofilm production and reported that the TCP method remains the best tool for biofilm screening[18].

Using the CRA method, 45% of our isolates were biofilm producers. Approximate results were reported by Chaieb *et al.* who found that 50% of their *S. epidermidis* isolates were biofilm producers by the CRA method [19], while Oliveira and Cunha, found this percentage to be as high as 73% using the same method [3].

In the present study, the CRA method shows 81.8 % sensitivity, 100% specificity, and 90% accuracy when compared to modified TCP as a gold standard method. Jain and Agarwal reported that the sensitivity and specificity of the CRA assay were 90.63% and 90.6% respectively [20]. In addition to its high sensitivity and specificity, Fitzpatrick et al. stated that the CRA test is easier and faster to perform than other phenotypic tests in the identification of biofilm positive isolates [21].

Detection of *icaA* and *icaD* genes by PCR showed that *icaA* genes were detected in 20% of the isolates, while *ica D* genes were detected in 30% of them. Out of the eleven biofilm-producing strains detected by the modified TCP method, four strains (36.3 %) expressed *icaA* gene and six strains (54.5%) expressed *icaD* gene. The *Ica* genes were not detected in biofilm non-producers. Detection of *icaD* gene was more sensitive (54.5%) than the *icaA* gene (36.4%) and both of them showed specificity 100%. These results were in accordance with those of Robert *et al.* who detected *icaD* and *icaA* genes in 28.1% and 34.4% of their strains respectively [22], but in discordance with those of Prasad *et al.* who found that 76.9% of their biofilm-producing isolates and 10.3% of their biofilm non-producing isolates were *ica* positive [23]. Nasr *et al.* stated that the presence of *icaA* and *icaD* genes does not always correlate with the phenotypic biofilm formation [18].

The biofilm forming ability of some isolates in the absence of *icaA* and *icaD* genes highlights the importance of further genetic investigations of *ica* independent biofilm formation mechanisms. Some researchers attributed the differences between the results of phenotypic and genotypic biofilm detection methods to the fact that *icaA* and *icaD* expression are subject to environmental conditions such as environment anaerobiosis, low iron concentrations which strongly increases biofilm formation and the use of glucose or NaCl or combination of both which enhances biofilm producing capacity of staphylococcal isolates irrespective of the presence or absence of *ica* operon[24-25]. Presence of other genes controlling biofilm production as *bhp*, *ccrA*, *ccrB* and *MecA* genes may be another cause [26-27]. Tang *et al.* reported that the *aap* gene plays a key role in the process of biofilm formation in *S. epidermidis* and that the ability of the biofilm-formation is enhanced the when *aap* and *ica* genes coexist [28].

All *icaA* positive strains in this study were also positive for *icaD* gene. Similarly, the study of Zhou *et al.* concluded that there was a greater correlation

between the presence of both *icaA* and *icaD* and the slime production than the single expression of *icaA* or *icaD* and the presence of slime [29]. Cafiso et al. showed that the *icaD* gene was always expressed in *S. epidermidis*, but that phenotypic biofilm production only occurred when *icaA* was expressed simultaneously [30]. This relationship might be explained by the fact that the *icaD* gene alone does not induce transferase activity and *icaA* induces little activity; however, the combined expression of *icaA* and *icaD* produces large amounts of polysaccharide intercellular adhesion and biofilm formation [31].

Antibiotic susceptibility testing of our strains showed that biofilm producing strains are more resistant to antibiotics than biofilm non-producing strains. Hassan *et al.* also observed higher antibiotic resistance in biofilm producing bacteria than biofilm non-producers [32]. Wojtyczka *et al.* suggested that diminished susceptibility to antibiotics of biofilm-forming strains may be due to impaired penetration of the drug across the biofilm rather than to any other biochemical or genetic mechanisms [6]. Sharma *et al.* noted that more than 80% of invasive CoNS strains were resistant to multiple antibiotics and were positive for biofilm formation [33].

All our biofilm producing and biofilm non-producing strains were sensitive to vancomycin and linezolid**.** In parallel with our findings, Shah *et al.* found that all their coagulase-negative staphylococcal (CoNS) isolates were sensitive to vancomycin [34]. However, the study of Claessens et al. showed that glycopeptides were not effective in killing *S. epidermidis* embedded in biofilms, but their combination with rifampicin improve the killing efficacy *in vitro* [35]. Hellmark *et al.,* found that all their isolates of *S. epidermidis* were susceptible to linezolid [36] and de Oliveira *et al.* reported that linezolid was the most effective drug in inhibiting staphylococci in the biofilm when compared to planktonic cells [37].

In this study, biofilm non-producing isolates were more susceptible to ciprofloxacin than biofilm producing isolates. Mushtak and Narjis stated that the mechanisms of ciprofloxacin effect on the biofilm include electrostatic interfere with the adhesion of bacteria and/or glycocalyx to the substratum, activation or release of the enzymes to disrupt the exopolysaccharide (glycocalyx) in the biofilm and inhibition of the formation of new glycocalyx [38].

The biofilm producing strains in this study were more resistant to rifampicin than the biofilm non-producing strains. Although rifampicin has been proving to be one of the most effective antibiotics against *S. epidermidis* biofilm, its use as a single agent can lead to the acquisition of resistance and hence should be combined with other agents as N-acetylcysteine [39] or vancomycin [35]. Associated with the advantage of combined therapy with avoiding the emergence of antibiotic resistance, it can also cause a lower genetic expression of *icaA* genes relative to rifampicin alone [40].

The present study also revealed that the biofilm producing isolates showed more resistance to cefoxitin, erythromycin, clindamycin, sulphamethoxazole-trimethoprime and gentamycin than biofilm non-producing isolates. All the strong biofilm producing *S. epidermidis* strains of Sahal and Bilkay were multidrug resistant [8]. The observed antibiotic resistance in biofilm-producing *S. epidermidies* isolates could be simply explained by the formation of a polymer matrix around the microcolonies which cause the biofilm to grow in thickness show maximum tolerance to antibiotics [41]. Antibiotic resistance in biofilms could also be the result of an intricate mixture of intrinsic and extrinsic factors, such as the higher mutation frequency of biofilm-growing bacteria compared with planktonically growing bacteria and increased horizontal gene transmission in biofilms [42-43]. In addition, biofilm producing bacteria produce enzymes that degrade antibiotics, have antibiotic targets of low affinity and overexpress efflux pumps that have a broad range of substrate [44].

Due to the high antibiotic resistance of biofilm-producing bacteria, progress has been made on approaches that include antiadhesive strategies to prevent surface adhesion or production of bacterial adhesins, dissolution of already established biofilm, targeting of biofilm matrix for degradation and interference with the biofilm regulation [45].

We concluded that the modified TCP remains the best method for screening of biofilm production. *IcaD* gene detection is more sensitive for detection of biofilm production in *S. epidermidis* than *icaA* gene, however, the presence of *icaA* and/or *icaD* genes does not always correlate with phenotypic biofilm production. Biofilm producing *S. epidermidis* isolates are more resistant to antibiotic treatment than non-biofilm producing strains. Despite advances in the understanding of the pathogenesis of staphylococcal biofilm formation, medical devices colonized with biofilms frequently require removal and further studies are recommended for investigating new, more efficient modalities for prevention and treatment of biofilm associated infections.

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