**ORIGINAL ARTICLE**

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

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**Key words:** Staphylococcus epidermidis, catheter-related infections, biofilm production, icaA gene, icaD gene, antibiotic susceptibility testing

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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 54.3% respectively. All of them showed specificity 100%. Biofilm producing isolates were more resistant to ceftoxime, 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.

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**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. *S. epidermidis* is one of the major biofilm-producing bacteria and it works by attaching itself to several surfaces. 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. 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.

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. 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 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.

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. Post *et al.* identified biofilm formation and antibiotic resistance as associated with poor clinical outcome in *S. epidermidis* device-related infections.

The aim of this study was to detect biofilm producing *S. epidermidis* from patients with catheter-related bloodstream 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.

The isolates were classified into three categories, non-adherent (OD ≤ 0.111), moderately adherent (OD >0.111 to ≤ 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.

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.

Genotypic detection of biofilm formation:

Genomic DNA was extracted by the boiling method as described by Pérez-Roth et al., 2001. 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:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Nucleotide position</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>IcaA forward primer</td>
<td>5’TCTTGGAGAGGGAATCAA-3’</td>
<td>1337 - 1356</td>
<td>188 bp</td>
</tr>
<tr>
<td>IcaA reverse primer</td>
<td>5’TCAAGCCACTAATCCAGCA-3’</td>
<td>1505 - 1524</td>
<td></td>
</tr>
<tr>
<td>IcaD forward primer</td>
<td>5’ATGTTGGCTAACGCGAG-3’</td>
<td>1963 - 1982</td>
<td>198 bp</td>
</tr>
<tr>
<td>IcaD reverse primer</td>
<td>5’CGTGTTCATTTAATGCAA-3’</td>
<td>2138 - 2160</td>
<td></td>
</tr>
</tbody>
</table>
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 MgCl2. 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-Triethoprim 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).](image-url)
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 number 1, 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):

<table>
<thead>
<tr>
<th>Method</th>
<th>MTCP</th>
<th>CRA</th>
<th>IcaA PCR</th>
<th>IcaD PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>11 (55%)</td>
<td>9 (45%)</td>
<td>4 (20%)</td>
<td>6 (30%)</td>
</tr>
<tr>
<td>Negative</td>
<td>9 (45%)</td>
<td>11 (55%)</td>
<td>16 (80%)</td>
<td>14 (70%)</td>
</tr>
</tbody>
</table>

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:

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRA</td>
<td>81.8%</td>
<td>100%</td>
<td>100%</td>
<td>81.8%</td>
<td>90%</td>
</tr>
<tr>
<td>icaA PCR</td>
<td>36.4%</td>
<td>100%</td>
<td>100%</td>
<td>56.3%</td>
<td>65%</td>
</tr>
<tr>
<td>icaD PCR</td>
<td>54.5%</td>
<td>100%</td>
<td>100%</td>
<td>64.3%</td>
<td>80%</td>
</tr>
</tbody>
</table>

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.
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., but different from the study of Seisdedos Elcuaz et al., who reported a high rate of detection (38%) in S. epidermidis in CRBSIs.

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. Nasr et al. compared the different phenotypic methods for biofilm production and reported that the TCP method remains the best tool for biofilm screening.

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, while Oliveira and Cunha, found this percentage to be as high as 73% using the same method.

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. 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.

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 strains of S. epidermidis, while in discordance with those of Prasad et al. who found that 76.9% of their biofilm-producing isolates were ica positive. Nasr et al. stated that the presence of icaA and icaD genes does not always correlate with the phenotypic biofilm formation.

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 environmental 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.

Presence of other genes controlling biofilm production as bhp, ccrA, ccrB and MecA genes may be another cause. 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.

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. Cañizo 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. 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.

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. 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. Sharma et al. noted that more than 80% of invasive CoNS strains were resistant to multiple antibiotics and were positive for biofilm formation.

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. 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. Hellmark et al. found that all their isolates of S. epidermidis were susceptible to linezolid and de Oliveira et al. reported that linezolid was the most effective drug in inhibiting staphylococci in the biofilm when compared to planktonic cells.

In this study, biofilm non-producing isolates were more susceptible to ciprofloxacin than biofilm producing isolates. Mushtak and Narijs 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.

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 or vancomycin. 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.

The present study also revealed that the biofilm producing isolates showed more resistance to cefoxitin, erythromycin, clindamycin, sulphanmethoxazole-trimethoprim and gentamycin than biofilm non-producing isolates. All the strong biofilm producing S. epidermidis strains of Sahal and Bilkay were multidrug resistant. The observed antibiotic resistance in biofilm-producing S. epidermidis 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. 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. 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.

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.

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.

REFERENCES


