Phenotypic and Genotypic Detection of Biofilm Formation in Staphylococcus epidermidis Isolates from Retrieved Orthopaedic Implants and Prostheses

Noha Tharwat Abou El-Khier1*, Samah Sabry El-Kazzaz1 and Abd Elrahman Elganainy2

1Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Egypt. 2Orthopaedic Surgery Department, Faculty of Medicine, Mansoura University, Egypt.

Authors’ contributions

This work was carried out in collaboration between all authors. Authors NTAE-K and AEE designed the study. Author NTAE-K performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors NTAE-K and SSE-K managed the analyses of the study. Authors NTAE-K, AEE and SSE-K managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Most of orthopaedic implant and prosthesis infections are actually biofilm-correlated infections that are highly resistant to antibiotic treatment and to the host immune responses. Staphylococcus epidermidis is considered one of the principal etiologic agents of orthopedic implant infection and prosthetic joint infection (PJI). Moreover, it has strong implant-adhering ability, and its biofilm-forming ability is considered as a serious pathogenic factor. Early detection and management of biofilm-forming S. epidermidis can be one of the essential steps towards the prevention and management of orthopaedic implant and prosthesis infections.

Aim of the Study: To evaluate the biofilm developing ability of the S. epidermidis isolates

*Corresponding author: E-mail: nohat75@yahoo.com;
INTRODUCTION

Biofilms are medically important, accounting for over 80% of microbial infections in the body, including prostheses and internal fixation devices [1]. In routine orthopedic surgery, several different foreign materials are regularly implanted, e.g. bone cement, polyethylene compounds, and different metal alloys. These orthopedic biomaterials are foreign bodies that provide surfaces for bacteria to adhere to and subsequently form biofilms [2,3]. Biofilm offers protective barrier to organisms, resulting in resistance to antimicrobial agents [4] and host immune responses [5,6]. It is difficult for the immune system and antibiotics to eradicate the bacterial cells embedded in the biofilm. These facts make the treatment of biofilm-related infections difficult and could lead to persisting inflammation and tissue damage. Moreover, removal of the infected prosthesis or implant is the only solution to cure the infection [3]. The bacteria in biofilms usually elicit less inflammatory response than the planktonic bacterial cells which makes it more difficult to isolate, so the diagnosis of these infections requires techniques different from those used in conventional microbiology laboratories [7,8,9].

S. epidermidis, although an important commensal, has emerged as the most significant pathogen in infections related to implanted foreign body materials, especially the prosthetic joints infections (PJIs) [10]. These infections are often long-lasting, difficult to treat, and involve biofilm formation [11].

S. epidermidis biofilm formation depends on the production of an intercellular polysaccharide adhesin termed PIA (or PNSG) which is a sugar polymer consisting of a 6-1,6-linked glucosaminoglycan backbone substituted with different side groups [12,13]. It is synthesized in vitro from Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) by the enzyme N-acetylglucosaminyl transferase, encoded by the ica operon comprising the icaA, icaD, icaB, and icaC genes, and the regulator gene icaR [14,15]. Sole expression of icaA induces only low enzymatic activity, but co-expression of icaA with icaD significantly increases the activity and is related to the phenotypic expression of the capsular polysaccharide [15,16].

Although, the ica locus that encodes the production of polysaccharide intercellular adhesins is required for biofilm formation, there is no strict association between the presence of the icaADBC operon and in vitro biofilm formation [17]. The presence of the entire cluster does not
always correlate with biofilm production and, conversely, isolates lacking the *ica* operon have been found to be able to form the biofilm [18,19]. Therefore, the aim of the present study was to evaluate the efficiency and specificity of different methods in estimating the biofilm development ability of *S. epidermidis* isolates obtained from retrieved orthopedic implants and prostheses, including the phenotypic analyses of Microtiter plate assay (MtP) and Congo red agar (CRA) test, as well as the genotypic technique of the *icaA* and *icaD* gene amplification by PCR.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates

Thirty nine isolates of *S. epidermidis* were analyzed to study the biofilm production. The isolates were originated from orthopedic implants (plates, screws, intramedullary devices and wires or pins used in external fixators) and prostheses (hip, knee prosthesis) retrieved from 100 patients suffering from orthopedic implants infections and PJIs in the department of Orthopedic Surgery at Mansoura University Hospital by sonication protocol as described previously [20]. The explanted implant or prosthesis was placed in an autoclaved 1-Liter wide-mouthed container, filled with 400 mL of sterile Ringer solution. The container was vortexed and sonicated at room temperature. Fifty milliliters of the sonicate fluid were concentrated 100-fold by centrifugation at 2,000 ×g for 20 min and resuspended, then 0.1-mL aliquots of the concentrated sonicate fluid were plated onto sheep blood agar plates and were incubated at 35-37°C in 5-7% carbon dioxide aerobically for 48 hours. All isolates were identified by colony morphology (opaque, raised, smooth, grayish white, non-haemolytic). Gram staining (Gram-positive cocci arranged in irregular grapelike clusters), conventional biochemical tests (catalase positive, coagulase negative) and commercial identification system (API-STAPH; bioMérieux, Fance). The biofilm-producer *S. epidermidis* ATCC 35984 and the biofilm negative strain *S. epidermidis* ATCC 12228 were used as reference strains.

2.2 Phenotypic Detection of Biofilm Formation

Two phenotypic methods were used for detecting the biofilm production of the staphylococcal isolates; one qualitative (Congo red agar method) and another quantitative (Microtiter plate method).

2.2.1 Biofilm production assay on Congo red agar (CRA) [16,21]

The isolates were cultured on CRA plates, prepared by adding 0.8 g of Congo red stain (Sigma, USA) and 36 g of sucrose to 1 L of BHI broth (both from Oxoid, UK). After 24 h incubation at 37°C, plates were further incubated for 12 h at room temperature. Isolates with red colonies were considered to be non-slime producing, and those with black colonies were considered to be slime-producing or biofilm-producers.

2.2.2 Microtiter plate assay (MtP) [22,23]

Organisms isolated from fresh agar plates were inoculated in 10 mL trypticase broth (TSB), supplemented with 1% glucose. Broths were incubated at 37°C for 24 h and then were diluted 1:100 with fresh medium. Aliquot (200 μL) of the diluted cultures was poured into the individual wells of sterile flat-bottomed 96-well polystyrene tissue culture plates (Sigma-Aldrich, USA); while 200 μL of TSB supplemented with 1% glucose was used as the negative control. The positive control organism (*S. epidermidis* ATCC 35984) was also incubated, diluted and added to tissue culture. The plates were incubated for 24 hours at 37°C and the contents of each well were removed by gentle tapping. Washing was then performed three times for each well with sterile phosphate-buffered saline (PBS, pH 7.2). After that, the fixation step was done by air drying. Subsequently, the adherent biofilm layer was stained by crystal violet for 15 minutes at room temperature, followed by the washing three times with sterile PBS. Then the plates were air dried and resolubilized with ethanol (95% v/v) for 30 minutes. Finally, the optical density (OD<sub>570 nm</sub>) of each well was measured, and average OD values of negative controls and samples were calculated separately. Optical density cut-off value (OD<sub>c</sub>) = average OD of negative control + 3× standard deviation (SD) of negative control.

Interpretation of results was described as follows:

1. OD ≤ OD<sub>c</sub>= Non biofilm producer (N).
2. OD<sub>c</sub> < OD ≤ 2×OD<sub>c</sub> = Weak biofilm producer (WP).
3. 2×OD<sub>c</sub> < OD ≤ 4×OD<sub>c</sub> = Moderate biofilm producer (MP).
4. 4×OD<sub>c</sub> < OD = Strong biofilm producer (SP).
Table 1. Nucleotide sequence of the primers used in this study and their target genes

| Primer code | Primer code and sequence (5'→3') | Target gene | Product size (bp) | Reference |
|-------------|---------------------------------|-------------|------------------|-----------|
| ICAA-F      | 5'-CCTAACTAAGCGAAAGGTAG-3'      | icaA        | 188              | [24]      |
| ICAA-R      | 5'-AAGATATAGCGATAAGTGC-3'       |             |                  |           |
| ICAD-F      | 5'-AAACGTAAGACGTGG-3'           | icaD        | 198              |           |
| ICAD-R      | 5'-GGCAATATGATCAGATAC-3'        |             |                  |           |

2.3 PCR Detection of icaA and icaD Genes

2.3.1 Strain storage

A single colony of each bacterial strain was inoculated in 8 ml of TSB. After incubation for 24 h at 37°C, the broth culture was fractioned into 1-ml aliquots, which were stored at -80°C.

2.3.2 Bacterial DNA extraction

Bacteria were harvested by centrifuging 100 µl of each broth culture. Cells were resuspended in 45 µl of H2O and supplied with 5 µl of lysostaphin solution. The samples were incubated at 37°C for 10 min; then 5 µl of proteinase K solution and 150 µl of 0.1 M Tris-HCl (pH 7.5) were added, and incubation proceeded for a further 10 min. Samples were then heated for 5 min at 100°C [16].

2.3.3 PCR method for amplification of icaA and icaD sequences

The amplification reactions were prepared in a 25 µl volume containing the following: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 mM MgCl2, 100 µM each of dATP, dCTP, dGTP, and dTTP, each of the oligonucleotide primers specific for icaA and icaD, respectively (see above Table 1 for the sequences) at the concentration of 1 µM, 1 U of Taq polymerase, and 200 ng template DNA [17]. The thermal amplification program included the following steps: an initial denaturation at 94°C for 5 min; 50 cycles of amplification with 94°C for 30 s (denaturation), 55.5°C for 30 s (annealing), 72°C for 30 s (extension); and then final extension at 72°C for 1 min.

For checking the PCR products, 10 µl of the amplification products were electrophoresed on agarose gel along with molecular weight marker (100 bp DNA ladder) (Fermentas, India), and the presence or absence of any resulting bands was evaluated under ultraviolet transillumination.

2.4 Statistical Analysis

Data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 17.0. Descriptive statistics were calculated in the form of Frequency (Number/percent). Inter-group comparison of categorical data was performed using chi square test (X²-value). The sensitivity, specificity, PPV, NPV and accuracy of CRA and MIP were calculated. Agreement between MIP assay and the presence of ica genes was measured using the kappa coefficient. P value <0.05 was considered statistically significant.

Sensitivity: proportion of PCR-positive isolates that tested positive by the other phenotypic methods; specificity: proportion of PCR-negative isolates that tested negative by the phenotypic methods [25,26].

3. RESULTS

3.1 Isolates

In total, 39 S. epidermidis isolates were obtained from the retrieved orthopedic implants and prostheses of 100 patients suffering from orthopedic implants infections and PJs.

3.2 Detection of Biofilm Production by CRA

In this analysis, biofilm formation was detected in 17 (43.6%) out of the 39 S. epidermidis isolates, which were evidenced by their rough black colonies. The remaining 22 isolates were not biofilm producers since they formed smooth red colonies. In addition, with the CRA method, both the positive and negative reference strains were correctly identified.

3.3 Detection of Biofilm Production by MIP Assay

By MIP assay, 20 out of 39 S. epidermidis isolates (51.3%) were found to be biofilm producers with different grades: 12 (30.8%) were SPs; 5 (12.8%) were MPs; and 3 (7.7%) were WPs. The two reference strains, ATCC 35984...
and ATCC 12228, were correctly identified as positive and negative, respectively, for the biofilm formation.

### 3.4 PCR Detection of icaA and icaD

The icaA and icaD genes were detected concomitantly in 15 (38.5%) of the 39 isolates, e.g. all the icaA positive isolates were also icaD positive, giving the 188-bp and 198-bp bands, respectively. The biofilm producing reference strain ATCC 35984 showed both the icaA and icaD genes. The non-slime-producing reference strain ATCC 12228 was negative for both genes.

### 3.5 Comparison of Biofilm Detection Methods

Among 17 isolates that were biofilm producers by CRA test, 15 were also positive by MtP assay, including 11 of SPs and 4 MPs; while only 2 isolates were NPs (Table 4).

The sensitivity and specificity of CRA and MtP tests were calculated using the presence of ica genes by PCR (concomitant presence of icaA and icaD) as a parameter. The CRA had a sensitivity of 93.3% and specificity of 87.5% (Table 2); while the MtP assay represented 100% sensitivity and 79.2% specificity (Table 2).

There was a substantial agreement between MtP assay and the concomitant presence of icaAD genes (kappa 0.74). It was also noticed that all the SP isolates were also positive for ica genes. Perfect agreement (kappa 0.83) was observed between SP isolates and the concomitant presence of icaAD genes (p<0.0001) (Table 3).

### 4. DISCUSSION

Implant-associated infections and PJIs pose vast challenges to the medical community and adds a huge economic burden on the community. The ability of S. epidermidis to colonize implant and prosthesis surfaces and to form biofilms is the critical step in the initiation of such infection. Once encased in a biofilm, bacteria become recalcitrant to immune surveillance and to antibiotic therapy. A reliable method for their diagnosis is necessary.

#### Table 2. Statistical evaluation of the phenotypic analyses compared to the detection of icaA/icaD genes in the S. epidermidis isolates

| Phenotypic analysis | icaA/icaD detection | χ² | P   | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Accuracy (%) |
|---------------------|----------------------|----|-----|----------------|----------------|---------|---------|--------------|
|                     | Negative             | No | %   | No | %   | Total | % | No | %   | No | %   | Total | % | No | %   | No | %   | Total | % |
| CRA                 | Negative             | 21 | 87.5 | 1 | 6.7 | 22 | 56.4 | 24.5 | <0.0001 | 93.3 | 87.5 | 82.4 | 95.5 | 89.7 |
|                     | Positive             | 3  | 12.5 | 14 | 63.3 | 17 | 43.6 |      |          |      |      |      |      |      |
|                     | Total                | 24 | 100.0 | 15 | 100.0 | 39 | 100.0 |      |          |      |      |      |      |      |
| MtP                 | Negative             | 19 | 79.2 | 0 | 0.0 | 19 | 48.7 | 23.2 | <0.0001 | 100  | 79.2 | 75  | 100  | 87.17 |
|                     | Positive             | 5  | 20.8 | 15 | 100.0 | 20 | 51.3 |      |          |      |      |      |      |      |
|                     | Total                | 24 | 100.0 | 15 | 100.0 | 39 | 100.0 |      |          |      |      |      |      |      |

#### Table 3. Comparison of MtP and PCR detection of icaA/icaD genes in the S. epidermidis isolates

| MtP | icaA/icaD detection | P |
|-----|---------------------|--|
|     | Negative             | No | % | No | % | Total | % | No | % | No | % | Total | % | No | % | No | % |
|     | 19                   | 79.2 | 0 | 0.0 | 19 | 48.7 | 0.001 |
|     | 3                    | 12.5 | 0 | 0.0 | 3  | 7.7  | 0.5  |
|     | 2                    | 8.3  | 3 | 20.0 | 5  | 12.8 | 0.38 |
|     | 0                    | 0.0  | 12 | 80.0 | 12 | 30.8 | <0.0001 |
|     | Total                | 24  | 100.0 | 15 | 100.0 | 39 | 100.0 |      |          |      |      |      |      |      |

#### Table 4. Qualitative (CRA) method versus quantitative (MtP) method for detection of biofilm forming S. epidermidis isolates

| CRA  | MtP                | Positive (20) | Negative (19) |
|------|--------------------|---------------|---------------|
|      | Strong positive    | Moderate positive | Weak positive |
|      | 11                 | 4             | 0             | 2             |
|      | Positive (17)      |               |               |               |
|      | Negative (22)      | 1             | 1             | 3             | 17            |
|      | Total (39)         | 12            | 5             | 3             | 19            |
Although there are several methods for biofilm detection [22,27,28], but there is still no standard protocol. The most widely used assay for evaluation of biofilm formation is the MIP [29].

In this study, the CRA test as a qualitative method detected 43.6% isolates (17/39) as biofilm producers, which was quite similar to Ruzicka et al. [30] who detected biofilm production in 64 out of 147 (43.5%) isolates by CRA method. Our findings also agree with the findings of Satorres and Alcaráz [31] and Arslan and Özkarde [32] that biofilm formation was detected with CRA test in 41.3% and 38.5% of staphylococci isolates, respectively.

On the other hand, low effectiveness of CRA method in evaluation of biofilm production was reported by Mathur et al. [33], de Silva et al. [19] and Wojtuczka et al. [34] in strains isolated from blood, skin of neonates and hospital environment. It has been reported that the variations in composition of Congo red agar and the incubation parameters could affect the accuracy of biofilm detection [35].

As regard MIP assay, it was not only identified the biofilm producing ability, but also ranked the isolates quantitatively. The detection of biofilm formation in 20 out of the 39 (51.3%) isolates demonstrated it more sensitive than the CRA test. The ranking of 30.8% as SPs, 12.8% as MPs, 7.7% as WPs and 48.7% as NPs were similar from study in India that out of 152 isolates, 53.9% were biofilm producers identified by MIP method [33].

In general, the results of our study are similar to those reported in the previous literature, that the biofilm-producing strains ranged from 31% to 89% in different S. epidermidis populations [32,36].

Ziebuhr et al. [37] have reported that 87% of S. epidermidis clinical isolates from catheter infections are slime forming, but their strains were isolated from blood cultures, not directly from implants and prostheses. Therefore, the isolation resource of the bacteria, as well as the variations in composition of media and the incubation conditions, could affect the proportion of biofilm producers in the S. epidermidis population.

In the present study, the concomitant detection of icaA and icaD genes in 15 of the 39 (38.5%) isolates was similar to El Farrann et al. [38] that detected ica operon in 19 out of 58 screened S. epidermidis (32.8%). Several studies have shown that the formation of biofilms by S. epidermidis is associated with the presence of the icaA and icaD genes [16,37,38,39]. Sole expression of icaA induces only low enzymatic activity, but co expression of icaA with icaD leads to a significant increase in activity and is related to phenotypic expression of the capsular polysaccharide [15].

However, the detection of icaAD genes by the PCR analysis in a non biofilm producer evidenced by the CRA test (red colonies) and the absence of icaAD in 7 biofilm producers confirmed by MIP and/or CRA methods demonstrated the possibility that biofilm formation was not always linked to these genes. In the study of Oliveira and Cunha [40], one CoNS was classified as strongly adherent by MIP assay but did not carry any of the ica genes. Some investigators reported the presence of certain genes in ica-negative biofilm-forming staphylococci, the accumulation associated protein (aap) [41] and Bap homolog protein (bhp) genes [42]. These genes were found to induce an alternative PIA-independent mechanism of biofilm formation. On the other hand, Qin et al. [43] studied two biofilm-positive/ica-negative strains of S. epidermidis and they suggested a novel molecular mechanism in the biofilm formation by these two clinical isolates. They assumed that the biofilm-positive/ica-negative strain represents a newly emergent subpopulation of clinical strains, arising from selection by antibiotics in the nosocomial milieu, especially that epidemiological data show a tendency towards an increasing proportion of this subpopulation in staphylococci-associated infections. The reason for the absence of biofilm production in some icaA and icaD positive isolates in the present study may be the lack of icaC. These data suggest that the phenotypic change may be caused by deletion or insertion of the ica operon which inactivates the ica genes. Ziebuhr et al. found that, in S. epidermidis, the insertion of a 1,332-bp sequence element, known as IS256, causes inactivation of icaA gene in non-slime-forming variants of slime-forming S. epidermidis strains [44].

In our study, the 17 S. epidermidis isolates identified as biofilm producers by the CRA method had the probability of 82.4% to be actually positive for biofilm production (PPV). On the other hand, 95.5% of the isolates identified as negative by CRA test were actually negative
Although it had a higher specificity (87.5%) than the MtP assay, it had lower sensitivity (93.3%) and NPV (95.5%). For this reason, Knobloch et al. [45] did not recommend the CRA method for biofilm detection in their study.

The higher sensitivity (100%) and relatively lower specificity (79.2%) of MtP assay could be explained as this technique is used for direct detection of polysaccharide production and the spectrophotometric measurements provide quantitative information on the ability of bacterial strains to rapidly grow while adhering to the substratum. However, it can be less accurate in determining their specific ability to secrete PIA [46].

Comparing the phenotypic methods (CRA and MtP) for detection of biofilm production with the molecular detection of icaAD genes, the results indicated that the MtP test should be the first choice because this test showed the best sensitivity (100%), identified all of the biofilm positive strains, it also, had a good agreement with the concomitant presence of icaAD gene (kappa 0.74). Moreover, perfect agreement (kappa 0.83) was observed between SP isolates and the concomitant presence of icaAD genes (all the SP isolates were also positive for ica genes). Similar results were also reported by other researchers [46,47,48].

Our study had some limitations, such as, small sample size; the identification of isolates only based on the phenotypic characterization without verification by molecular methods; and the shortage of study on the role of genes other than ica operon. Further studies about the mechanism of the ica with more cases will clarify the pathogenesis of biofilm in implants and prosthesis.

5. CONCLUSION

In conclusion, molecular detection of the icaAD genes is associated with biofilm production. Also, the absence of ica genes did not exclude the ability of the strains to develop biofilm in vitro. So both approaches (genetic and phenotypic) are required for optimum evaluation of the biofilm producing ability of clinical S. epidermidis populations. Moreover, MtP assay is recommended as a general screening method for detection of biofilm on retrieved orthopaedic implants and prostheses because of its easy application, reliable results with excellent sensitivity, as compared to CRA methods.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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