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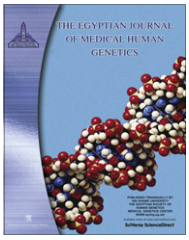

ORIGINAL ARTICLE

Biofilm formation and presence of *icaAD* gene in clinical isolates of staphylococci

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Abstract In view of the significant negative impact of biofilm mediated infection on patient health and the necessity of a reliable phenotypic method for detecting biofilm producers, this study aimed to determine biofilm producing ability and presence of *icaAD* gene in clinical staphylococcal isolates as well as to assess the reliability of two phenotypic methods used for detection of biofilm. A total of 50 staphylococcal strains were isolated from 124 clinical specimen (94 intravascular catheters and 30 blood samples) collected from in-patients at Pediatric Hospital of Ain Shams University. Two phenotypic methods were used for detection of biofilm production; qualitative Congo red agar (CRA) and quantitative Microtiter plate (MTP). PCR was used to determine the presence of *icaAD* gene. Biofilm production was detected in 23(46%) isolates by CRA and MTP, however, both methods correlated only in 10(20%) of isolates. The *icaAD* gene was detected in 16(32%) staphylococcal isolates. Correlating phenotypic methods with *icaAD* gene detection, only 8(50%) of the *icaAD* positive staphylococci were positive by MTP, while 5(31%) were positive by CRA method. Unexpectedly, 15(30%) and 18 (36%) of the isolates were *icaAD* negative while MTP and CRA positive, respectively.

In conclusion, despite the presence of *icaAD* gene, it does not always correlate with in vitro biofilm formation. The biofilm-forming ability of some isolates in absence of *icaAD* gene highlights the importance of further genetic investigations of *ica* independent biofilm formation mechanisms. Comparing phenotypic methods, MTP remains a better tool for biofilm screening.

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1. Introduction

Biofilm-mediated infections in the hospital environment have a significant negative impact on patient health and place an enormous burden on the resources of the health services [1]. *Staphylococcus aureus* (SA) together with *Staphylococcus epidermidis* are a common cause of biofilm-mediated life-threatening infections associated with intravenous catheters, artificial heart valves, and prosthetic joints [2]. The ability of nosocomial pathogens to form biofilms is of significant clinical interest, as biofilm formation makes the organisms more resistant to antibiotics and host defenses [2,3] and thus influences the subsequent outcome of an infection [4]. Furthermore, the colonized devices may become the focus of infection and can cause localized and generalized infections [5].

The ability of bacteria to aggregate and form biofilm is strictly related to the capacity of producing an extracellular mucoid substance: the slime, whose main component is of polysaccharide nature and consists of glycosaminoglycans [6]. Development of biofilm is considered to be a two step process; first, the bacteria adhere to a surface mediated by a capsular antigen, namely capsulare polysaccharide/adhesin (PS/A), second, the bacteria multiply to form a multilayered biofilm, associated with production of polysaccharide intercellular adhesin (PIA) which mediates cell to cell adhesion [7].

It has been shown that both SA and *S. epidermidis* contain the intercellular adhesion (*ica*) operon responsible for slime production. This operon contains the *icaADBC* genes, in addition to the *icaR* gene which exerts a regulatory function. Among *ica* genes, the *icaA* and *icaD* have been reported to a play a significant role in biofilm formation. The *icaA* gene encodes *N*-acetylglucosaminyltransferase, the enzyme involved in PIA synthesis. Further, *icaD* has been reported to a play a critical role in the maximal expression of *N*-acetylglucosaminyltransferase, leading to the full phenotypic expression of the capsular polysaccharide [4,8].

Early detection and management of biofilm-forming staphylococci can be one of the essential steps towards prevention and management of device-associated nosocomial infections. Thus there is a need to evaluate a simple reliable phenotypic method for detection of biofilm producers [5].

The purpose of the present study was to determine the biofilm producing ability and the presence of the *icaAD* gene in *Staphylococci* isolated from blood and intravascular catheters of in-patients at Pediatric Hospital of Ain Shams University as well as to assess the reliability of two phenotypic methods used for detection of biofilm; Congo red agar method and microtiter plate method.

2. Materials and methods

2.1. Specimens

One hundred and twenty-four clinical samples (94 intravascular catheters and 30 blood sample) were collected from patients hospitalized at Pediatric Hospital of Ain Shams University during the period from June 2009 to August 2010. Samples were processed and cultured and isolates were identified according to Mermel et al. [9] and Cheesbrough [10].

2.2. Bacterial isolates

A total of 50 non-repetitive staphylococcal strains were isolated and identified from 124 clinical samples. Identification of SA and coagulase negative staphylococci (CoNS) was based on colony morphology, Gram stain, catalase and coagulase tests. The isolates were stored in brain heart infusion (BHI) broth, to which 15% sterile glycerol was added, at −20 °C.

2.3. Biofilm formation

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

2.3.1. Congo red agar (CRA) method

According to Freeman et al. [11], the CRA medium was prepared with 37 g/l BHI broth, 50 g/l sucrose, 10 g/l agar, and 0.8 g/l Congo red. Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121 °C for 15 min separately from other medium constituents, and was then added when the agar had cooled to 55 °C. Plates were inoculated and incubated at 37 °C for 24 h. The plates were inspected for the color of the colonies at 24 and 48 h. A positive result was indicated by black colonies whereas non-producing strains developed red colonies. The Congo red dye directly interacts with certain polysaccharides, forming colored complexes or more likely some metabolic changes of the dye to form a secondary product could play a more important part in the formation of dark colonies [5,8].

For colonies color evaluation, a four-color reference scale was used according to Satorres and Alcaráz [12]: black and bordeaux almost black were classified as biofilm-producers, while bordeaux and red as non-biofilm-producing strains. This method was performed in triplicate.

2.3.2. Microtiter plate (MTP) method

This method was carried out to according to Stepanovic et al. [13]. Staphylococcal strains were grown overnight at 37 °C in BHI supplemented with 2% sucrose. The culture was adjusted to to 0.5 McFarland then diluted 1:100 in fresh medium. Individual wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plates (TPP – Switzerland) were inoculated with 200 µl of the diluted culture then incubated. After 24 h incubation at 37 °C, the contents of each well were removed by decantation and each well was washed three times with 300 µl of phosphate buffered saline (to remove free-floating "planktonic" bacteria). Biofilms formed by adherent "sessile" organisms were heat-fixed by exposing them to hot air at 60 °C for 60 min, then 150 µl crystal violet (2%) stain was added to each well. After 15 min, the excess stain was rinsed off by decantation and the plate was washed and left to dry.

Quantitative analysis of biofilm production was performed by adding 150 µl 95% ethanol to each well, and after 30 min, the optical densities (OD) of stained adherent bacterial films were read using a microtiter-plate reader (Tecan Sunrise Remote, Austria) at 570 nm. Each assay was performed in triplicate. As a negative control, uninoculated medium was used to determine background OD. The average OD values were

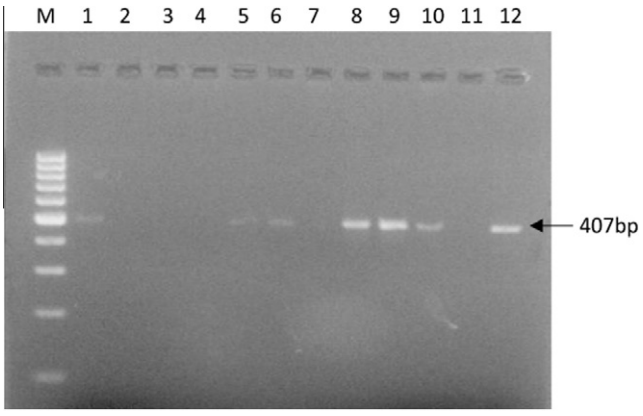

Figure 1 Gel electrophoresis indicating the presence pf *icaAD* gene (407 bp). M: molecular size marker.

calculated for all tested strains and negative controls, the cut-off value (ODc) was established. It is defined as a three standard deviations (SD) above the mean OD of the negative control: ODc = average OD of negative control + (3 × SD of negative control). ODc value was calculated for each microtiter plate separately. When a negative value was obtained, it was presented as zero, while any positive value indicated biofilm production. For interpretation of the results, strains were divided into the following categories:

1. OD ⩽ ODc = Non biofilm producer (0).
2. ODc < OD ⩽ 2 × ODc = Weak biofilm producer (+ or 1).
3. 2 × ODc < OD ⩽ 4 × ODc = Moderate biofilm producer (+ + or 2).
4. 4 × ODc < OD = Strong biofilm producer (+ + + or 3).

2.4. Polymerase chain reaction (PCR) for amplification of icaAD gene

DNA was extracted from all staphylococcal isolates using bacteria DNA preparation kit (Jena Bioscience, Germany) according to manufacturer's instructions. Amplification of the *icaAD* gene was done according to Yazdani et al. [7] using specific primers: 5′ TATTCAATTTACAGTCGCAC 3′ and 5′ GATTCTCTCCCTCT-CTGCCA 3′, yielding a PCR product of 407 base pairs (bp). These primers were designed from the published gene bank sequences (locus AF086783). The reaction mixture (25 µl) contained 2.5 mM MgCl2, 1 U of Taq DNA polymerase, 100 µM of each dNTPs, 1 µM of each primers and 200 ng of DNA sample.

DNA amplification was carried out in a thermocycler (Gene Amp PCR System 9700, Applied Bio system, Singapore) with the following thermocycler profile: an initial denaturation step (2 min at 94 °C) followed by 30 cycles of amplification (denaturation at 94 °C for 30s, annealing at 58 °C for 30 s and elongation at 72 °C for 30 s) terminated with a 3 min incubation step at 72 °C. After amplification, 10 µl of PCR product was analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized under UV transillumination. The Gene Ruler 100 bp DNA ladder was used as a DNA size marker (Fig. 1).

2.5. Statistical analysis

Data were presented as count and percent. The sensitivities, specificities, positive predictive values (PPV) and negative predictive values (NPV) of the phenotypic methods for BF production were calculated as described by Ilstrup [14].

3. Results

A total of 50 staphylococcal isolates were recovered from 124 clinical specimens (intravascular catheters and blood samples) of which 44 (88%) were SA and 6 (12%) were CoNS. Twenty-five strains (50%) were isolated from each of intravascular catheters (21SA and 4CoNS) and blood (23 SA and 2 CoNS).

The results of biofilm production by staphylococci by MTP (quantitative) method and CRA (qualitative) method are demonstrated in Table 1. In MTP method, biofilm production was detected in 23 (46%) of the 50 staphylococcal isolates with different intensities; 13 (26%) isolates were strong producers, 6 (12%) isolates were moderate and 4 (8%) isolates were weak biofilm producers, whereas 27 (54%) were non biofilm producers. By CRA method, 23 (46%) were also positive for biofilm; however, CRA method showed little correlation with MTP assay where only 10 (20%) of the isolates were positive by both the MTP and CRA methods (Table 2).

As regards staphylococci isolated from intravascular catheters, 14 (56%) were biofilm producers by CRA while 8 (32%) were positive by MTP.

The *icaAD* gene was detected in 16 (32%) staphylococcal isolates while 34 (68%) did not possess such gene. Correlating the phenotypic biofilm production methods with *icaAD* gene detection; of the 16 *icaAD* positive staphylococcal isolates, only 8 (50%) were positive by MTP, while only 5 (31%) were positive by CRA method. Unexpectedly, 15 (30%) and 18 (36%) of all the isolates were *icaAD* negative and produced biofilm by MTP and CRA methods, respectively. When compared to PCR, the sensitivity and specificity of MTP method

Table 1 Results of biofilm production in staphylococcal isolates according to MTP and CRA methods.

Isolates (*n*)	No. (%) of isolates						
	MTP					CRA	
	No	Weak	Moderate	Strong	Total +	–	+
SA (44)	23(52.3)	3(6.8)	5(11.4)	13(29.5)	21(47.7)	24(54.5)	20(45.5)
CoNS (6)	4(66.6)	1(16.6)	1(16.6)	0 (0)	2(33.3)	3(50)	3(50)
Total (50)	27(54)	4 (8)	6 (12)	13(26)	**23(46)**	27(54)	**23(46)**

Table 2 CRA versus MTP methods for detection of biofilm production by staphylococcal isolates.

	MTP	
	+	−
CRA		
+ (23)	10	13
− (27)	13	14
Total (50)	23	27

in biofilm detection were 50% and 55.88%, respectively, while those of CRA method were 31.25% and 47.05%, respectively (Table 3).

4. Discussion

The ability of staphylococci to form biofilms helps the bacterium to resist host immune response and is considered responsible for chronic as biofilm protects microorganisms from opsonophagocytosis and antimicrobial agents [15]. In view of the large number of infections caused by biofilm producing bacterial, a reliable method for their diagnosis is necessary [16].

The MTP assay is most widely used and was considered as standard test for detection of biofilm formation [17,18]. This method has been reported to be most sensitive, accurate and reproducible screening method for determination of biofilm production by clinical isolates of staphylococci and has the advantage of being a quantitative tool for comparing the adherence of different strains [17,19]. Together with its capacity to examine a large number of isolates simultaneously, was the reason for its use in our study.

In the current study, of the 50 staphylococcal isolates from blood and intravascular catheters, biofilm production was found in 46% with different intensities by MTP method and 26% of isolates were strong producers, 12% were moderate and 8% were weak biofilm producers while in 54% no biofilm was formed. In the study of Mathur et al. [17] 57.8% of staphylococcal clinical isolates displayed a biofilm-positive phenotype and 14.47% and 39.4% exhibited high and moderate biofilm formation, respectively, while in 46% of isolates weak or no biofilm was detected.

Yazdani et al. [7] also reported that 52% of SA isolated from wound infection was positive biofilm forming, while Jain and Agarwal [5] demonstrated that 78% of SA and 52.7% of CoNS isolated from blood and intravascular catheters were biofilm positive. A higher rate of biofilm formation was demonstrated by Gad et al. [18] where 83.3% of SA and 88.6% of *S. epidermidis* isolated from urinary tract catheterized patients produced biofilm by the MTP assay.

As regards CRA method, 46% staphylococcal isolates in this study produced biofilms, which agrees with the findings of Satorres and Alcaráz [12] where 41.3% of the staphylococci isolated from blood and intravenous catheters were biofilm producers. In the study of Arslan and Özkarde [20], CRA method demonstrated positive results in 38.5% of staphylococci isolated from clinical specimens.

Concerning staphylococci isolated from intravascular catheters, 56% were biofilm producers by CRA while where 32% were positive by MTP. In the study of Satorres and Alcaráz [12] 47% of staphylococci from intravascular catheters were biofilm positive by CRA method while Jain and Agarwal [5] reported a higher rate where 64% and 68% of staphylococcal intravascular isolates were biofilm producers by CRA method and MTP methods, respectively. The colonized device represents a major risk as it may become the focus of localized and generalized infections, besides, intraluminal colonization has been reported as the major source for the migration of organisms leading to bloodstream infections [5,21].

When comparing the results of both CRA with MTP method, although 46% of the total staphylococcal isolates were positive by both methods, yet they correlated only in 10 (20%) isolates. A low correspondence between both methods was also demonstrated by Marthur et al., [17] where screening on CRA did not correlate with MTP results except for 8/152 (5.2%) of staphylococcal isolates. On the other hand, better correlation between both methods were reported by other investigators where all staphylococci positive by one test were also positive by the other in the study of Cafiso et al. [22]. Yazdani et al. [7] also reported that 2 of 27 SA strains produced black colonies on CRA were biofilm negative on MTP.

Earlier studies proposed CRA method as an alternative to MTP method for screening staphylococcal isolates for biofilm production [23,24] being rather easy to perform, less time taking, sensitive and specific. However, our findings confirm what was concluded by Mathur et al., [17] that CRA method can not recommended for detection of biofilm formation by staphylococcal clinical isolates.

Several studies have shown that formation of biofilm in staphylococci causing catheter associated and nosocomial infections is associated with the presence of both *icaA* and *icaD* genes [8,12,18,25]. Coexpression of these genes is necessary for the full phenotypic expression of biofilm in clinical staphylococcal isolates [8,16,22]. PCR was used in this study for *ica* genes detection as a reference for the phenotypic method based on several studies [8,16,18,22].

Table 3 Statistical evaluation of the phenotypic biofilm production methods compared to detection of *icaAD* genes in staphylococcal isolates.

Method	*ica*+	*ica*−	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MTP						
+	8	15	50	55.88	34.78	70.37
−	8	19				
CRA						
+	5	18	31.25	47.05	21.73	59.25
−	11	16				

The *icaAD* gene was detected in 16 (32%) staphylococcal isolates in the present study. Satorres and Alcaráz [12] also reported that of all staphylococci isolated from blood and intravascular catheters, 42.2% were positive for *icaA* and *icaD* genes, while *ica*-operon was identified in 45% of *S. epidermidis* isolates from catheter-related and other nosocomial infections in the study of Cafiso et al. [22]. On the other hand, other studies showed that the *ica* gene was detected in all SA isolated from patients suffering of bacteremia with prosthetic joints [26] and wound infection [7].

Correlating the phenotypic biofilm production methods with presence of *icaAD* gene, 50% of the *icaAD* positive staphylococcal isolates were positive by MTP, while 31% formed biofilm by CRA method. A good correspondence between the phenotypic characterization methods and *ica* genes presence was reported by Aricola et al. [8] and Gad et al. [18] where all staphylococcal biofilm producing strains were positive for *icaA* and *icaD*. In addition, in the study of Satorres and Alcaráz, [12], only one out of 65 staphylococci was found to be biofilm negative by CRA while possessing the *icaA* and *icaD* genes.

However, in accordance with our findings, other studies demonstrated that the presence of the *ica* genes did not always correlate with biofilm production. De Silva et al. [27] reported that only 59% of *S. epidermidis* strains positive for the *ica* operon were biofilm producers by CRA method. In correlating also to MTP method, Cafiso et al. [22] demonstrated that 83.3% of the *ica*-positive isolates produced biofilm by both methods, while Yazdani et al. [7] reported that only 54% and 52% of *ica*-positive strains were also positive by CRA and MTP methods, respectively.

Some authors attributed the absence of biofilm production in some staphylococcal isolates despite the presence of the *ica* operon to the insertion of a 1332-bp sequence element, known as IS256, in *icaA* causing its inactivation [23,28,29]. However, the transposition of IS256 into the *ica* operon has been found to be a reversible process as after repeated passages of the PIA-negative insertional mutants, the biofilm-forming phenotype could be restored [30]. Cafiso et al. [22] also proposed that the product of *icaR* gene (a regulator gene which seems to function as a repressor) [4] could influence transcription of the ica operon. Nevertheless, irrespective of *ica* genes expression, *ica*-positive isolates should be considered to be potential biofilm producers [16].

Unexpectedly, 30% and 36% of the study isolates were *icaAD* negative and produced biofilm by MTP and CRA methods, respectively. Although, Arciola et al. [31] reported that (*icaA*/*icaD* +)/MTP− strains represented 8%, however (*icaA*/*icaD*−)/MTP+ strains were 16%. In the study of Oliveira and Cunha Mde [16], one CoNS was classified as strongly adherent by MTP assay but did not carry any of the *ica* genes.

In an attempt to explain these unexpected findings, some investigators reported the presence of certain genes in *ica*-negative biofilm-forming staphylococci, the accumulation-associated protein (*aap*) [32] and Bap homolog protein (*bhp*) genes [33]. These genes were found to induce an alternative PIA-independent mechanism of biofilm formation. However, Qin et al. [34] studied two biofilm-positive/*ica*-negative strains of *S. epidermidis* and they did not detect these two genes. These authors suggested a novel molecular mechanism mediating biofilm formation in 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.

Compared to PCR, sensitivity and specificity of MTP method in biofilm detection were 50% and 55.88%, respectively, while of CRA method were 31.25% and 47.05%, respectively. A much better finding was reported by Oliveira and Cunha Mde [16], where the sensitivity and specificity of the phenotypic methods when compared to *ica* genes detection were 97.6% and 94.4% for MTP and 89% and 100% for CRA method. However, these authors concluded that CRA might be imprecise in the identification of positive isolates when compared to molecular analysis of the genes involved in biofilm production.

In conclusion, though about half of staphylococci isolated from blood and intravascular catheters were capable of forming biofilm; however, the presence of *icaAD* gene was not always associated with in vitro formation of biofilm. On the other hand, the biofilm-forming ability of some strains in the absence of *icaAD* gene highlights the importance of further genetic investigations of *ica* independent biofilm formation mechanisms. Comparing the phenotypic biofilm detection methods, CRA, although easier and faster to perform, still MTP remains a better tool for screening of biofilm formation.

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