

Detection of *icaA*, *icaD* genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients

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Abstract

Background: Staphylococci are a common cause of catheter-associated urinary tract infections. The present study evaluated biofilm forming capacity and the presence of both *icaA* and *icaD* genes among staphylococci strains isolated from patients undergoing ureteral catheterization.

Methodology: Different bacterial strains were isolated from urine and stents segments collected from 100 patients. Strains were identified by traditional microbiological methods. Stents were examined for biofilm using a scanning electron microscope (SEM). Staphylococcal isolates were tested for their ability to produce biofilm using the tissue culture plate assay method (TCP). The presence of *icaA* and *icaD* genes was determined by PCR technique.

Results: Fifty-three staphylococcal strains were isolated and identified from 284 samples (18.7%). Forty-six staphylococcal strains were isolated from stent segment cultures while only seven strains were isolated from urine samples at the day of stent removal. *S. aureus* represented 6.3%, and *S. epidermidis* represented 12.3%. Out of the 18 *S. aureus* strains, 15 (83.3%) were biofilm producers and out of 35 *S. epidermidis* strains, 31 (88.6%) were biofilm producers. Staphylococcal strains were further classified as high (56.6%), moderate (30.2%) and non biofilm producers (13.2%). All biofilm producing strains were positive for *icaA* and *icaD* genes, and all biofilm negative strains were negative for both genes.

Conclusion: Staphylococci isolated from catheter segments showed a higher extent of biofilm production than that isolated from urine samples. All biofilm producing staphylococci were positive for *icaA* and *icaD* genes, which indicates the important role of *ica* genes as virulence markers in staphylococcal infections associated with urinary catheterization.

Keywords: Staphylococci, biofilm, *icaA*, *icaD*

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Introduction

Staphylococci are most often associated with chronic infection of implanted medical devices. The increased use of indwelling medical devices has had considerable impact on the role of staphylococci in clinical medicine. The predominant species isolated in these infections are *Staphylococcus epidermidis* and *Staphylococcus aureus*. It was found that the major pathogenic factor is the ability to form biofilm on polymeric surfaces to which it adheres and colonizes artificial materials [1]. Biofilms are a population of multilayered cells growing on a surface and enclosed in exopolysaccharide matrix. Biofilm formations are considered to be a two-step process in which the bacteria first adhere to a surface, followed by multiplication to form a multilayered biofilm.

Microbial biofilms are considered the major problem posed to catheterized patients because they cause chronic infections which are difficult to treat, lead to longer hospitalization time, and can result in much higher treatment costs [2]. Biofilm formation is regulated by expression of polysaccharide intracellular adhesion (PIA), which mediates cell to cell adhesion and is the gene product of *icaADBC* [3]. The intercellular adhesion (*ica*) locus consisting of the genes *icaADBC* encodes proteins mediating the synthesis of PIA and polysaccharide/adhesin PS/A in staphylococci species [4].

Among *ica* genes, *icaA* and *icaD* have been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis* [5]. The *icaA* gene encodes N-acetylglucosaminyltransferase,

the enzyme involved in the synthesis of N-acetylglucosamine oligomers from UDP-N-acetylglucosamine. Further, *icaD* has been reported to play a role in the maximal expression of N-acetylglucosaminyltransferase, leading to the phenotypic expression of the capsular polysaccharide [6]. The aim of our study was to determine the biofilm-forming capacity of microorganisms isolated from urinary tract catheterized patients and the occurrence of *icaA* and *icaD* genes in biofilm-producing strains in a collection of staphylococcal isolates.

Materials and methods

Patients, specimens, and strains

Patients

Our institution did not require informed consent from patients. All patients were selected by Dr. Hassan Abolella (Professor of Urology, Department of Urology at Assuit University Hospital) during the study period from December 2007 to June 2008. However, all samples were collected after we obtained informed consent from each patient, following a discussion with each about our study in order to facilitate our work. Eight patients of our study did not return to undergo stent removal in the hospital. This was the reason that the number of urine samples and stent segment was 92 instead of 100 on the day of stent removal.

Specimens

Two hundred and eighty four clinical samples (urine samples and stent segments) were collected from 100 in-patients undergoing ureteral catheterization at the Department of Urology at Assuit University Hospital. One hundred urine samples were collected from patients before stent insertion and 92 urine samples were collected at the day of stent removal. Ninety-two catheter segments were collected from the 92 patients undergoing ureteral stent removal. Urine samples were streaked onto the surfaces of mannitol salt agar, MacConkey agar, and blood agar and incubated at 37°C for 24 hours [7]. Each catheter sample was placed in 10 ml of trypticase soy broth (TSB, Difco), sonicated for one minute, and then vortexed for 15 seconds. Exactly 0.1 ml of the sonicated broth was surface-plated by using a wire loop on trypticase soy agar (TSA) (with 5% sheep blood) and MacConkey agar. Organisms were then identified by routine microbiological techniques [8], and API Staph system (Biomerieux, France) was used to screen all

coagulase negative staphylococci, following the instructions of the manufacturer. Different biochemical activities were performed for identification of the isolated strains according to the standard biochemical methods described by Koneman *et al.* (9) and Collee *et al.* (10).

Strains

In the present study, 292 strains were recovered and identified from 284 clinical samples. Out of 292 isolates, 53 were staphylococci, which were used in our study. The organisms were stored in trypticase soy broth (TSB), to which 15% glycerol was added at -20°C.

Scanning Electron Microscopy (SEM)

Catheter segments were fixed in 2.5% (vol/vol) glutaraldehyde in Dulbecco PBS (PH 7.2) for 1.5 hours, rinsed with PBS, and then dehydrated through an ethanol series. Samples were dried and gold-palladium coated. SEM examinations were made on a JSM-840 SEM (JEOL Ltd., Tokyo, Japan) [11].

Detection of biofilm formation by tissue culture plate method (TCP)

The TCP assay is most widely used and was considered as standard test for detection of biofilm formation. All isolates were screened for their ability to form biofilm by the TCP method as described by Christensen *et al.* [12] with a modification in duration of incubation which was extended to 24 hours, according to O'Toole and Kolter [13].

Isolates from fresh agar plates were inoculated in trypticase soy broth with 1% glucose and incubated for 24 hours at 37°C in stationary condition and diluted (1 in 100) with fresh medium. Individual wells of sterile, polystyrene, flat-bottom tissue culture plates were filled with 0.2 ml aliquots of the diluted cultures, and only broth served as control to check sterility and non-specific binding of media.

The tissue culture plates were incubated for 24 hours at 37°C. After incubation, the content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating planktonic bacteria; then 25µl of 1% solution of crystal violet was added to each well (this dye stains the cells but not the polystyrene) plates. The plates were incubated at room temperature for 15 minutes, rinsed thoroughly and repeatedly with water. Adherent cells, which usually formed biofilm

on all side wells, were uniformly stained with crystal violet. Crystal violet-stained biofilm was solubilized in 200 µl of 95 % ethanol (to extract the violet color), of which 125 µl were transferred to a new polystyrene microtiter dish, which was then read. Optical densities (OD) of stained adherent bacteria were determined with a micro ELISA auto reader (model 680, Bio rad), and the wavelength of values was considered as an index of bacteria adhering to surface and forming biofilms. Experiments for each strain were performed in triplicate and repeated three times. To compensate for background absorbance, OD readings of wells with ethanol were used as blank and subtracted from all tests' values. Biofilm production is considered high, moderate, or weak 570 nm (OD_{570 nm}) as shown in Table 1.

Table 1. Classification of bacterial adherence by TCP method.

Biofilm formation	Adherence	Mean OD values
Non/weak	Non/weak	< 0.120
Moderate	Moderately	0.120 - 0.240
High	Strong	> 0.240

PCR for amplification of *icaA* and *icaD* sequences

Bacterial DNA extraction

After overnight culture on brain-heart infusion agar plates, one or two colonies were suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100°C for 20 minutes. From this suspension, a 5 µl aliquot was directly used as a template for PCR amplification.

The sequences of *icaA* and *icaD* were taken from the GenBank sequence database of the National Center for Biotechnology Information. Primers specific for *icaA* and *icaD* were picked on the gene sequences by the Primer3 program. The primers were synthesized by Koma Biotech Inc. (Kore).

For the detection of *icaA*, 5'-TCTCTTGCAGGAGCAATCAA was used as a forward primer and 5'-TCAGGCACTAACATCCAGCA was used as a reverse primer. The two primers include a 188-bp region. For detection of *icaD*, 5'-ATGGTCAAGCCCAGACAGAG was used as a forward primer and 5'-CGTGTTTTCAACATTTAATGCAA was used as a reverse primer. The two primers include a 198-bp region. PCR was performed in a DNA thermal cycler (UNO II Thermocycler; Biometra GmbH, Göttingen, Germany). The reaction volume was 25 µl containing

2.5 µL of each the forward and reverse primers (1 µM each), together with 150 ng (5µl) of the extracted DNA, 10 µl of EzWay™ PCR Master Mix and 5 µl of distilled water.

A thermal step program for both *icaA* and *icaD* was used, including the following parameters: incubation at 94°C for 5 minutes, followed by 50 cycles at 94°C for 30 seconds (denaturation), 55.5°C for 30 seconds (annealing), 72°C for 30 seconds (extension), and 72°C for 1 minute after 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 stained with ethidium bromide). The Gene Ruler 100 bp DNA ladder (Koma Biotech Inc., Kore) was used as a DNA size marker [6].

Results

Seventy-six patients (76%) before stent insertion and 80 patients (86.95%) on the day of stent removal had positive urine cultures, and 84 (91.3%) patients had positive stent cultures.

A total of 292 bacterial isolates were recovered from 284 clinical samples collected from 100 patients. As shown in Table 2, *Klebsella spp.* were the most prevalent microorganism (21.9%) followed by *Pseudomonas spp.* (18.8%) and *Staphylococci spp.* (18.2%). Out of 292 isolates, 53 staphylococcal strains were identified (18.2%). *Staphylococcus aureus* represented 6.2% (18 strains) and *Staphylococcus epidermidis* represented 12% (35 strains).

Table 2. Prevalence of different microorganisms isolated from different patients.

Microorganisms	No.	%*
Staphylococci spp.:	<u>53</u>	18.2
<i>S. aureus</i>	18	6.2
<i>S. epidermidis</i>	35	12
<i>E. coli</i>	52	17.8
Klebsella spp.:	<u>64</u>	21.9
<i>K. pneumoniae</i>	40	13.7
<i>K. oxytoca</i>	24	8.2
Pseudomonas spp.	55	18.8
Proteus spp.	<u>33</u>	11.3
<i>p. vulgaris</i>	17	5.8
<i>p. mirabilis</i>	16	5.5
<i>Providencia rettgeri</i>	14	4.8
<i>Citrobacter freundii</i>	14	4.8
<i>Serratia marcescens</i>	7	2.4

*Percents were correlated to the total number of isolates (292).

Polymicrobial bacteriuria represented 22.9% of positive cultures. All staphylococcal strains were polymicrobial with *klebseilla pneumoniae*, *E. coli*, *Pseudomonas spp.*, *Proteus spp.*, *Providencia rettgeri*, and *Serratia marcescens*. For *S. aureus*, five strains were found mixed with *K. pneumoniae*, six strains with *Pseudomonas spp.*, four strains with *Proteus spp.*, one strain with *Providencia rettgeri*, and two strains with *Serratia marcescens*. For *S. epidermidis*, seven strains were found mixed with *E. coli*, 10 strains with *K. pneumoniae*, 12 strains with *Pseudomonas spp.*, three strains with *Proteus spp.* and three strains with *Serratia marcescens*.

Detection of biofilm producing strains

Stents examined by scanning electron microscope showed two types of bacteria, a dense mass of biofilm, and a high level of encrustation (Figure 1). To explain the presence of biofilm mass and encrustation, biofilm producing ability was tested by TCP method for all isolates (Table 3). Table 3 shows that most of isolates were biofilm producers, which explains the presence of a dense mass of biofilm produced by two microorganisms on both the surface and the lumen of the catheter. Urease enzyme production was tested for all isolates. It was found that most of isolates were urease positive, which increased urine pH and produced an alkaline condition resulting in precipitation of Ca and Magnesium phosphate (Table 4).

isolates among different samples. All staphylococcal strains were isolated from urine samples on the day of stent removal (three strains of *S. aureus* and four strains of *S. epidermidis*) and stent segments (15 strains of *S. aureus* and 31 strains of *S. epidermidis*). Staphylococci were not isolated from urine samples before stent insertion. In addition, staphylococci were isolated from stent segments cultures in an incidence higher than that from urine samples on the day of stent removal. Table 5 also shows that staphylococci were not isolated from the urine sample and catheter segment of the same patient, which indicates that the sensitivity of urine cultures to stent colonization is low, and negative urine cultures do not rule out a colonized stent.

Staphylococcal strains of different origin were further classified according to the extent of biofilm production to high, moderate and non/weak biofilm producers. *S. aureus* and *S. epidermidis* strains isolated from urine samples were non/weak biofilm producers, but those isolated from catheter segments were moderate and high biofilm producers. Out of eighteen *S. aureus* isolates, twelve (66.7%) were strong biofilm producers, three (16.7%) were moderate biofilm producers, and three (16.7%) were considered as non or weak biofilm producers. On the other hand, out of 35 *S. epidermidis*, 18 (51.4%) were strong biofilm producers, 13

Table 3. The incidence of biofilm production among the isolated microorganisms using microtiter plate method.

Microorganisms	No. of microorganisms	Number of biofilm producing organisms	
		No.	%*
<i>S. aureus</i>	18	15	83.3
<i>S. epidermidis</i>	35	31	88.6
<i>E. coli</i>	52	40	76.9
<i>Klebseilla spp.</i>	64	54	84.8
<i>Pseudomonas spp.</i>	55	50	90.9
<i>Proteus spp.</i>	33	28	84.4
<i>Provedencia rettgeri</i>	14	12	85.7
<i>Citrbacter freundii</i>	14	12	85.7
<i>serratia marcescens</i>	7	5	71.4
Total	292	247	84.6

*Percents were correlated to the number of each isolate.

A higher incidence of Staphylococci was isolated from catheter segments than from urine samples. Table 5 shows the distribution of staphylococcal

(37.1%) were moderate biofilm producers, and four (11.4%) were considered as weak or non biofilm producers (Table 6 and Figure 2).

Table 4. Incidence of urease enzyme production by biofilm producing microorganisms.

Microorganisms	No. of microorganisms producing biofilm	No. of urease positive organisms	
		No.	%*
Staphylococci:	46	7	15.2
<i>S. aureus</i>	15	4	26.7
<i>S. epidermidis</i>	31	3	9.7
<i>E.coli</i>	40	0	0
<i>Klebseilla Spp.</i>	54	23	42.9
<i>Proteus Spp.</i>	28	26	92.9
<i>Pseudomonas Spp.</i>	50	39	78
<i>Providencia rettgeri</i>	12	6	50
<i>Citrbacter freundii</i>	12	5	41.7
<i>Serratia marcescens</i>	5	3	60
Total	247	109	44.12

*percents were correlated to the number of microorganisms producing biofilm.

Table 5. Distribution of staphylococcal strains isolated from different urine samples and stent segments.

Samples	<i>S. aureus</i> (n=18)		<i>S. epidermidis</i> (n=35)	
	No.	%	No.	%
Urine samples before stent insertion	0	0	0	0
Urine samples at the day of stent removal only	3	16.7	4	11.4
Catheter segment only	15	83.3	31	88.6
Urine sample and stent segment of the same patient	0	0	0	0

Table 6. Screening of the extent of biofilm formation by the isolated staphylococci by tissue culture plate assay (TCP).

Microorganism	Number of isolates	Biofilm formation (OD _{570nm})					
		High		moderate		Non/weak	
		No.	%*	No.	%*	No.	%*
<i>S. aureus</i>	18	12	66.7	3	16.7	3	16.7
<i>S. epidermidis</i>	35	18	51.4	13	37.1	4	11.4

*percents were correlated to the number of each isolate.

PCR detection of *icaA* and *icaD* genes

All strains were tested for the presence of *icaA* and *icaD* genes. All biofilm producing strains isolated from catheter segments were found to be positive for both genes, giving a 188-bp band for *icaA*, and a 198-bp band for *icaD* genes. It was also found that all strains which were positive for *icaA* were also positive for *icaD*. On the other hand, all non biofilm producing strains isolated from urine samples were negative for both genes. The

expression of *icaA* and *icaD* genes in strains isolated from catheter segments collected from patients indicates the role of *ica* genes in biofilm production and as virulence markers in staphylococcal infections associated with urinary tract catheters (Table 7 and Figure 3).

Discussion

Bacterial adhesion has long been considered as a virulence factor contributing to infections associated with catheters and other indwelling medical devices [14]. There are two possible explanations for the

Figure 1A. Scanning electron micrograph showing the surface of a ureteral stent covered with a dense mass of biofilm-containing bacteria (*S. aureus*) and crystalline layers (× 5000).

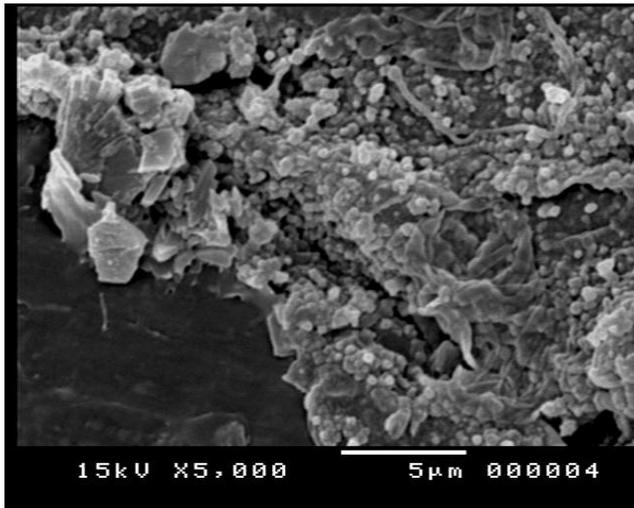


Figure 1B. Scanning electron micrograph showing the surface of a ureteral stent covered with high dense crystalline biofilm (×5000).

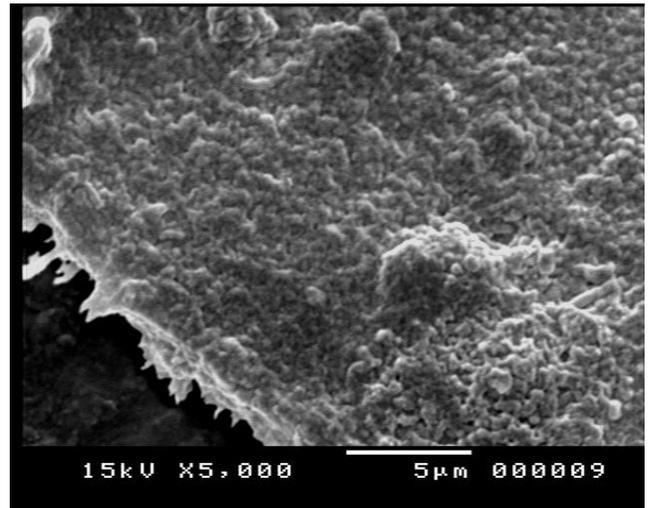


Figure 1C. Scanning electron micrograph showing the lumen of a ureteral stent covered with a big mass of biofilm-containing rods and cocci (*K. pneumoniae* and *S. aureus*) (× 5000).

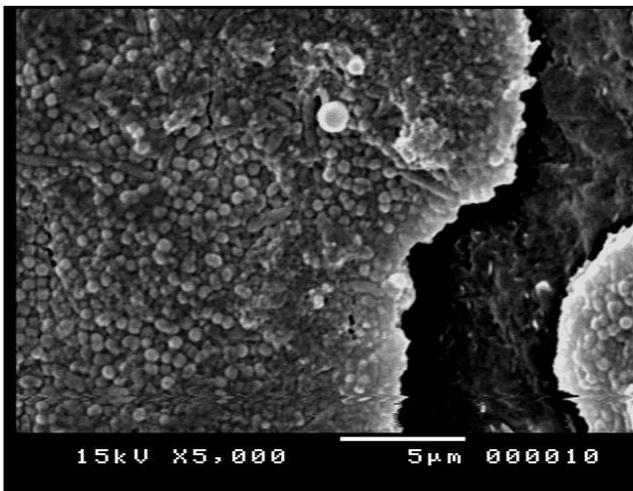


Figure 1D. Scanning electron micrograph showed the lumen of the ureteral stent (× 35) blocked with a dense mass of biofilm.

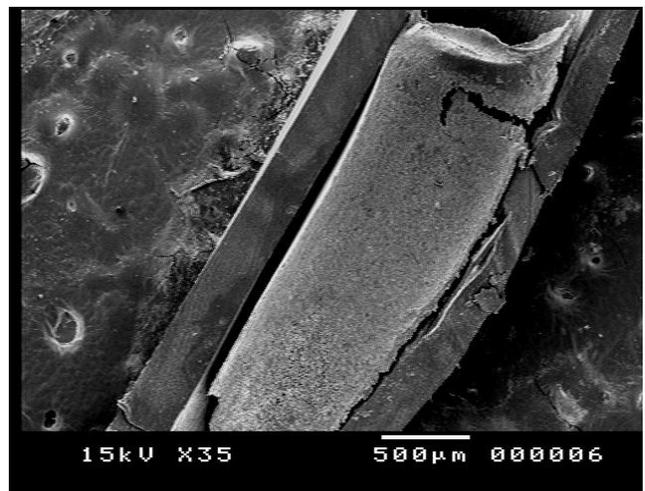


Figure 2. Screening of biofilm producers by Tissue culture plate method (TCP): high, moderate and weak/non biofilm producers.

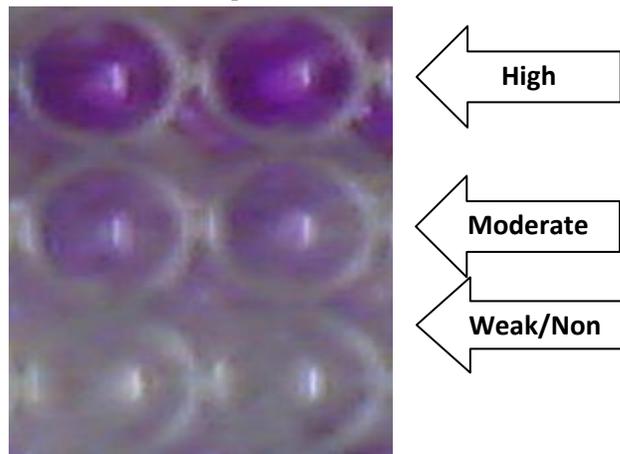
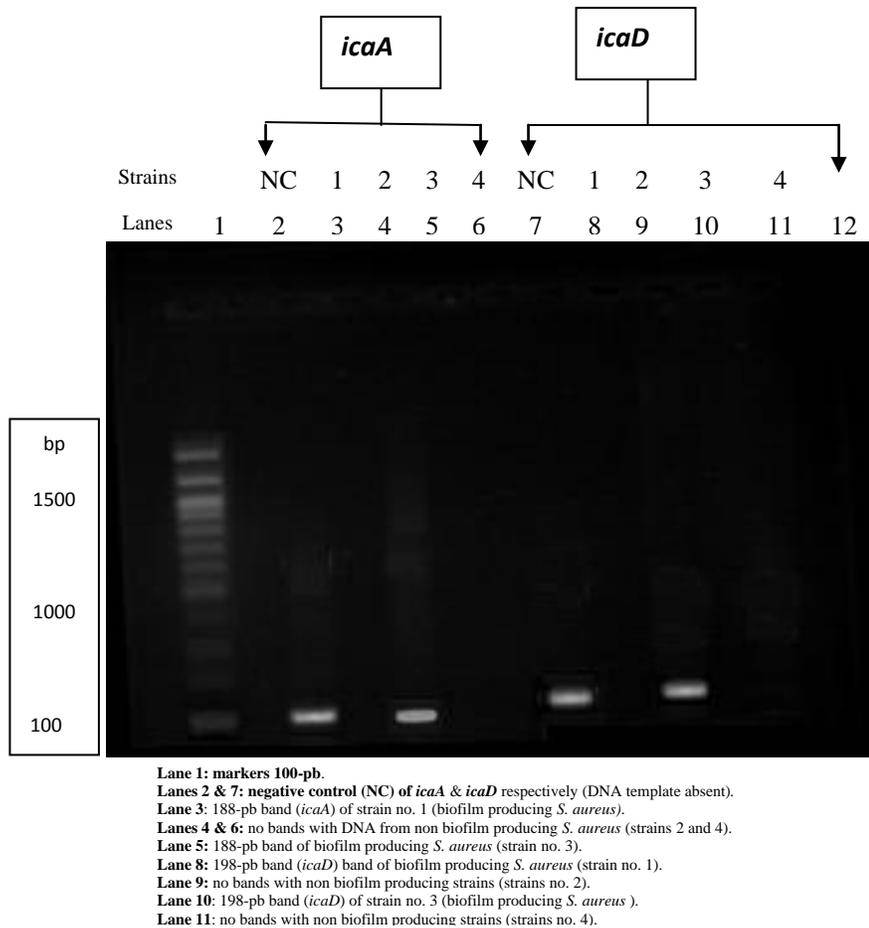


Table 7. Relationships among biofilm production (TCP assay), the presence of *icaA* and *icaD* genes and sample origin.

Microorganisms	Number of biofilm positive strains				Total positive		Strains positive for <i>icaA</i> and <i>icaD</i>	
	Catheter segments		Urine samples at the day of stent removal					
	No.	%*	No.	%*	No.	%*	No.	%*
<i>S. aureus</i> (n=18)	15	83.3	0	0	15	83.3	15	83.3
<i>S. epidermidis</i> (n=35)	31	88.6	0	0	31	88.6	31	88.6
Total (n=53)	46	86.8	0	0	46	86.8	46	86.8

*Percents were correlated to the number of each isolate.

Figure 3. PCR detection of *icaA* and *icaD* genes in 4 *Staphylococcus aureus* strains (1 and 3 were biofilm producers and 2 and 4 were non-biofilm producers).



ability of staphylococcal species to colonize artificial materials. The first is the bacterial production of polysaccharide slime. The second is the presence of adhesins for the host matrix proteins that are adsorbed onto the biomaterial surface [15]. The ability of staphylococci to form biofilms helps the bacterium to resist host immune response and is considered responsible for chronic or persistent infections as biofilm protects microorganisms from opsonophagocytosis and antimicrobial agents [16, 17].

In this study, it was found that *Klebseilla spp.* was the most prevalent microorganism (21.9%), followed by *Pseudomonas spp.* (18.8%), *staphylococci spp.* (18.2%) and *E. coli* (17.8%). These results are close to those obtained by Reid *et al.* [18], who reported that *Klebseilla* species were the most prevalent microorganism (33.3%) isolated from 42 hospitalized patients with urinary tract catheters, followed by *E. coli* (26.2%), and coagulase negative staphylococci (11.9%). Similar results were obtained in a surveillance study carried out by Savas *et al.* [19] to determine microorganisms responsible for urinary tract infection (UTI), who found that *Klebseilla spp.* (21.9%), *E. coli* (18.8%), and *Pseudomonas spp.* (17.8%) were the most prevalent microorganisms isolated from urinary tract catheterized patients. On the other hand, they found that coagulase negative staphylococci were isolated only from urine samples of non catheterized patients, which differs from our results. Our results showed that polymicrobial bacteriuria represented 21.9% of positive cultures, which are close to results obtained by Ko *et al.* [20], who reported that polymicrobial bacteriuria represented 21.1%.

Scanning electron microscope (SEM) was used to examine catheters for the presence of encrustation and bacterial biofilm. Scanning electron micrographs showed biofilm mass formed on the surface and the lumen of the catheters containing two types of microorganisms. The surface of catheters showed high levels of encrustation, which may be due to urease production by the existing microorganisms, which increases urine pH resulting in calcium and magnesium phosphate precipitation. Many researchers used SEM to examine urinary catheters for crystalline biofilms [21-24].

Our findings indicate that the sensitivity of urine cultures to stent colonization is low, which supports results obtained by Kehinde *et al.* [25], who found that on the day of stent removal, 17% of patients had

positive urine cultures, while 42% of stents cultures were positive.

Christensen *et al.* [12] reported that optical densities of bacterial biofilms adherent to plastic tissue culture plates (TCP) serve as a quantitative model for the study of the adherence of coagulase negative staphylococci to medical devices and act as a reliable quantitative tool for comparing the adherence of different strains. They also found that classifying strains into three adherence groups indicated the distance between the adherence coordinates and the origin. Furthermore, they found that coagulase negative staphylococci isolated from catheters associated with sepsis were found to be more strongly adherent than those isolated from blood culture contaminant and skin strains, which agrees with our results as strains isolated from catheters were more strongly adherent than those isolated from urine samples. Mathur *et al.* [26] reported also that the TCP method is an accurate and reproducible method for screening and determination of biofilm production. Therefore, we used this method in our study. Mather's group also showed that increasing the incubation period from 18 hours to 24 hours could lead to a better discrimination between moderate and non biofilm producing staphylococci. Under conditions of using TSB media supplemented with 1% glucose and an 18-hour incubation period, 80 staphylococci strains showed biofilm production, but after a 24-hour incubation period, the number of biofilm producing strains increased to 82. In our study, we used the same conditions (TSB with 1% glucose and a 24h incubation period) since the use of sugar supplementations is essential for biofilm formation and since extended incubation time affects biofilm formation in staphylococci.

In the present study, all biofilm and non biofilm producing staphylococci strains (*S. aureus* and *S. epidermidis*) were subjected to PCR for determining *icaA* and *icaD* genes to identify and confirm biofilm producing strains. It was found that all biofilm producing staphylococci strains were positive for *icaA* and *icaD* genes. These results are in agreement with those of De Silva *et al.* [27] and Mack *et al.* [28]. Arciola *et al.* [6] reported that all *S. aureus* and *S. epidermidis* biofilm positive strains isolated from intravenous catheters were positive for *icaA* and *icaD* genes and that these genes are required for full slime synthesis, which is in agreement with our results. These findings are consistent with those of other studies that showed a high incidence of slime

producing staphylococci in isolates from clinically significant medical device-associated infections of different origins [29-33].

Biofilm production is an important pathogenic factor which facilitates adherence of microorganisms to medical devices and protects them from the host immune system and antimicrobial therapy. Results revealed that both *icaA* and *icaD* genes were either present or absent and no single strain had shown the presence of one gene. These results confirm the fact that both genes are part of one operon and so the entire operon was either present or absent. In addition, our results showed that both genes (*icaA* and *icaD*) were present in all biofilm producing strains, indicating the important role of *ica* genes as virulence markers in staphylococcal infections. In conclusion, there is a high prevalence of biofilm production among microorganisms isolated from catheterized patients, the sensitivity of urine cultures to stent colonization is low, and staphylococci isolated from catheter segments showed a higher extent of biofilm production than those isolated from urine samples. All biofilm producing staphylococci were positive for *icaA* and *icaD* genes. It is important to diagnose and to give prophylactic antibiotics just before and during the surgical procedure to eliminate planktonic bacteria before they can form a biofilm.

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