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Biofilm production and antibiotic susceptibility profiles of Staphylococcus strains isolates from urinary catheter at the university hospital center of Tlemcen (Algeria)

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ABSTRACT

Staphylococci are a major cause of infections associated with urinary catheterization and other medical devices. The ability of Biofilm production is an important step in the pathogenesis of these *Staphylococci* and depends on the expression of the *ica*ADBC operon leading to the synthesis of a polysaccharide intercellular adhesion.
In this study , 44 staphylococci isolates obtained from urinary catheter at the university hospital center (UHC) of Tlemcen (North-West Algeria) were analyzed for detecting the presence or absence of the intercellular adhesion *ica*A and *ica*D genes by polymerase chain reaction, phenotypic biofilm production was examined by tissue culture plate (TCP) and Congo red agar (CRA) methods. 17of 44 isolates were shown to carry ica -specific DNA, 18 produced slime on CRA plates but only 8 produced biofilm spontaneously on the polystyrene surfaces. Staphylococci strains isolated from urinary catheter showed high levels of resistance to penicillin (98%) and gentamicine (75%). The data reported indicate an important role of *ica* genes, phenotypic variability of biofilm production and antibiotic multiresistance as virulence factors in staphylococcal from urinary catheters.
Keywords: Staphylococcus; Urinary catheter; Biofilm; ica operon; slime; TCP.

I. INTRODUCTION

Staphylococcus, a commensal microorganism routinely found on the human skin and in the hospital environment, has become the most important cause of nosocomial infections in recent years **(Chokr et al.,2007)** . They are generally associated with chronic infections related to implanted medical devices such as urinary catheterization **(Espinasse et al.,2010).**

However, bacterial colonization of implanted foreign material can cause chronic infections which are difficult to treat, lead to longer hospitalization time, and can result in much higher treatment costs **(Gad et al.,2009)** . The major virulence factor associated with this organism's ability to cause infections is dependent on adherence to medical devices and formation of a biofilm **(Cerca et al.,2005)**.

The biofilm consists of a multiple layers of sessile cells that adhere to the implant surface as well as to each other. Once a biofilm is formed, it can be very difficult to treat clinically the associated infection. This is due to the fact that the bacteria within the biofilm are well protected from the host immune response as well as antibiotic agents **(Hoyle and Costerton, 1990; Cramtonet al., 1999).** In fact, biofilm formation proceeds in two phases: primary attachment of staphylococcal cells on a biomaterial **(Macka et al., 2004)** followed by cell-cell adhesion, forming the multiple layers of the biofilm. This latter process is associated with the polysaccharide intercellular adhesin (PIA)(**Cramtonet al., 1999)** .

The synthesis of PIA is mediated by the products of the chromosomal ica gene (intercellular adhesion), which are organized in an operon structure. This operon contains the icaADBC genes, in addition to the icaRgene which exerts a regulatory function and is transcribed in

the opposite direction. Once this operon is activated, four proteins are transcribed, IcaA, IcaD, IcaB and IcaC, which are necessary for the synthesis of PIA **(Cafiso et al.,2004)**. PIA is synthesized from UDP-N-acetylglucosamine by N-acetylglucosaminyltransferase, which is encoded by the ica locus, particularly icaA. The expression of this gene alone induces low enzymatic activity and the production of low amounts of polysaccharide. However, the simultaneous expression of icaA and icaD promotes a significant increase in N-acetylglucosaminyltransferase, with a consequent increase in the amount of polysaccharide, forming oligomers of 10-20 b-1,6-Nacetylglucosamine residues **(Dobinski et al.,2002; Gotz,2002; Oliveira et al.,2010)**. IcaB is the deacetylase responsible for the deacetylation of mature PIA. In addition, the transmembrane protein IcaC seems to be involved in externalization and elongation of the growing polysaccharide **(Diemond-Hernández et al .,2010)** .

The expression of the ica operon and as a result, the formation of biofilms seems to be highly variable among staphylococci. Thus, biofilm expression is influenced by environmental signals and can be induced in response to external stress and subinhibitory concentrations of certain antibiotic **(Ziebuhr et al.,1997; Mempel et al.,1994; Cho et al.,2002)**.

The differentiation of staphylococci with respect to its biofilm phenotype might help to elucidate the impact of staphylococci in diagnosis of infections related to biomedical devices**.** These observations can be useful in the prevention of device related infections **(Mathur et al.,2006)** .

Several studies have been published on the detection of the gene Ica among the staphylococcal strains **(Gad et al.,2009; Cho et al.,2002; Touati et al.,2007; Chaieb et al.,2005; Wang et al.,2010; Duran et al.,2010)** . However despite the increasing interest in the subject in recent years, the collect of data from Algerian hospital an institution is relatively difficult, hence, the low number of related studies.

The objective of the present study is to characterize Staphylococci strains isolates from urinary catheter at university hospital of Tlemcen in terms of antibiotic susceptibility, biofilm formation and presence of the icaA and icaD genes.

II. METHODS AND MATERIAL

1. Bacterial strains

The strains selected in this study were isolated from urethral catheterization obtained from the Intensive Care Unit, urology and neurology services at the University Hospital Center (CHU) of Tlemcen (North-West Algeria). Urinary catheters studied were in latex without antibiotics; they were transported at 4°c, and analyzed immediately at the laboratory.

2. Identification

Microbiological analysis after removal of the catheter was performed by the technique of "Brun-Buisson"**(Brun-Buisson, 1994).** This technique consists in rinsing the lumen of the catheter with saline water and to vortex its extremity intravascular before culturing on Chapman agar plates allowing selection of staphylococci. Moreover, all isolates were identified by classic microbiological methods including: colonial morphology, Gram staining, catalase test, coagulase test and the Api-Staph test (BioMérieux®).

3. Antibiotic sensitivity test

Antimicrobial susceptibility testing was performed in accordance with the guidelines established by the antibiogram committee of the French Microbiology Society (**CASFM, 2010) using** 17 antibiotic discs including :

Penicilin**(10ug)**, Oxacillin **(5ug),** Cefoxitin **(30ug)**, Gentamicin **(10ug)**, Tobramycin **(10ug)** Amikacine **(30ug)**, Vancomycine **(30ug),** Rifampim**(30ug)**, Fosfomycin**(50ug)**, Fusidic Acid **(10ug)**, Clindamycin **(2ug)**, Pristinamycin **(15ug)**, Erythromycin**(15ug),** Ofloxacin**(5ug),** Tetracycline**(30ug),** Chloramphenicol **(30ug),** Triméthoprime /sulfaméthoxazole **(25ug).**

4. Detection of Biofilm Formation

4.1 Tissue culture plate method (TCP)

Quantitative determinations of biofilm formation in 96-well tissue-culture plates (Sigma, UK) were performed based on the method of Christensen *et al.* **(Christensen et al.,1985)** with a modification in duration of incubation which was extended to 48 hours. Therefore, we had evaluated biofilm production in three different media, BHIB, BHIB with 2% sucrose, and BHIB with 1% glucose.

Bacteria were grown overnight in respective media, cultures were then diluted 1:100 and incubated in a microtiter polystyrene plate at 37°C. Microtiter wells were washed 3 times with distilled water, dried in an inverted position, and stained with 0.5% (w: v) crystal violet solution **(Mathur et al.,2006)**. The adherent cells were resuspended in 95% ethanol solution and the absorbance was measured at 540 nm by using a micro ELISA autoreader (model 680, Biorad, UK). The isolates were classified into three categories: a) non adherent, optical density lower than 0.120; b) weakly adherent, optical density higher than 0.120 or equal to or lower than 0.240; c) strongly adherent, optical density higher than 0.240.

4.2 Congo Red Agar method (CRA)

Phenotypic characterization of biofilm production was performed by culture of the staphylocoques isolates on CRA plates. This technique proposed by **Freeman *et al.*** requires the use of a specially prepared solid medium-brain heart infusion broth (BHIB) supplemented with 5% sucrose and Congo red.

The medium was composed of BHIB (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and congo red stain (0.8 gms/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C.

According to the authors, biofilm producers form black colonies on CRA, whereas non-producers form red colonies. The Congo red dye directly interacts with certain polysaccharides, forming colored complexes (**Jain and Agarwal, 2009).**

4.3 Detection of icaA and icaD loci

Extraction of bacterial DNA was made by thermal shock. After overnight culture on Luria Bertani agar plates (Bio-Rad, Marnes-la-Coquette, France), 5 colonies were suspended in 500yl of DNase- and RNase-free water (Invitrogen, England), the suspension was boiled at 100°C for 10 min in thermal block (Polystat 5, French), then centrifuged at 15000 rpm for 5min. An aliquot of 2 µL of the supernatant was used as DNA template for PCR.

The presence of *ica*A and *ica*D DNA were detected by polymerase chain reaction (PCR) using forward and reverse primers for *ica*A and *ica*D. For *ica*A, the forward primer (corresponding to nucleotides 1337–1356) had the following sequence: 5'-TCT CTT GCA GGA GCA ATC AA-3'; and the reverse primer (corresponding to nucleotides 1505–1524) had the following sequence: 5'-TCA GGC ACT AAC ATC CAG CA-3'. The primer sequences for *ica*D were: forward (nucleotides 1963–1982), 5'-ATG GTC AAG CCC AGA CAG AG- 3'; and reverse (nucleotides 2138–2160), 5'-CGT GTT TTC AAC ATT TAA TGC AA-3'. PCR amplification was carried out according to the parameters described by **Arciola *et al.***, and visualization of the amplified products by a 2% gel electrophoresis.

III. RESULT AND DISCUSSION

A. Results

1. Characterization of Staphylococci Isolates from Urinary Catheter

A total of 44 strains have been obtained from urinary catheter used more than 48 hours at the University Hospital of Tlemcen. After biochemical identifications, all 44 strains were staphylococci species and included: *21 S.epidermidis*, 11 *S.saprophyticus*, 11 *S.aureus, and 1 S.hominis*.

2. Antibiotic Sensitivity Test

Antibiotic susceptibility testing showed that majority of Staphylococci strains was resistant to more than nine antibiotics and were found to be susceptible to four

major antibiotics: Rifampim **,** Fosfomycin **,** Clindamycin and Chloramphenicol**.**

Moreover, no strains resistant to vancomycin and pristinamycin were found. The total percentage of resistance against each antibiotic is represented in Figure 1.

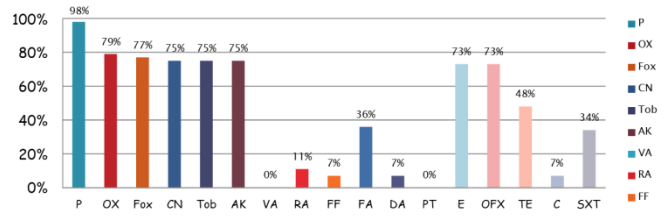

Figure 1 : Antibiotic resistance of Staphylococcus strains isolates from urinary catheter.

3. Detection of biofilm production

3.1. Detection of slime-producing Staphylococci Strains.

Phenotypic production of slime by all strains under study was assessed by culture on CRA plates. Slime producing strains appear as black colonies, and non-slime-producing strains appear as red colonies (Figure2).

Among the clinical isolates, 18 of 44 (41%) staphylococci strains were slime producers it included 6/11 S.saprophyticus, 5/11 S.aureus, 1/1 S.hominis and 6/21 S.epidermidis. The remaining 26 strains were non-slime producers.

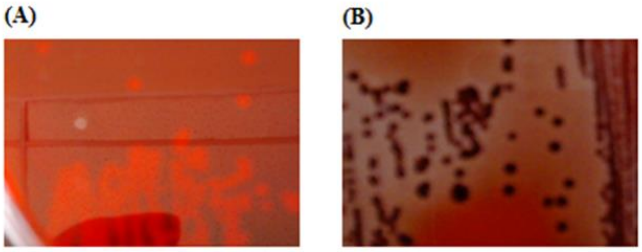

Figure 2: CRA plate test**. (A):** Non slime producing strains / **(B):** slime-producing strains

3.2. Study of biofilm production by the tissue culture plate method (TCP)

Quantitative determinations of biofilm formation were made by measuring adherence of broth cultures to 96-well tissue culture plates as outlined in Materials and Methods. Under standard growth conditions in BHI broth only 8 of 44 (18%) isolates were found to be biofilm forming.

To evaluate the impact of environmental growth conditions on biofilm formation by clinical isolates we performed biofilm assays using growth media supplemented with 1% glucose or 2% sucrose as described previously. This resulted in an increase in the numbers of isolates capable of biofilm formation with 15 of 44 (34%) of isolates producing biofilm in the presence of one of these media supplements. Thus, the overall rate of biofilm-forming strains raised from 18 to 34% after stimulation (Figure3 and4).

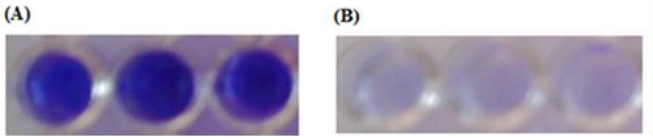

Figure3: Screening of biofilm producers by Tissue culture plate method (TCP).
(A) Biofilm producers / **(B)** non biofilm producers

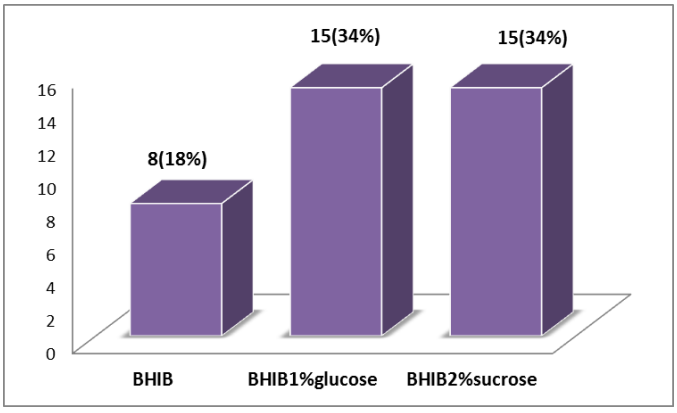

Figure 4: Biofilm formation of Staphylococcus strains on BHIB, BHIB 1% glucose and BHIB 2% sucrose.

3.3. PCR detection of ica A and ica D loci

The PCR technique was applied to the 44 staphylococcal strains. The *ica*A and *ica*D genes were detected concomitantly in 17 (38, 5 %) of the 44 staphylococci isolates giving a 188-bp band for the *ica*A gene and a 198-bp band for the *ica*D gene (Figure5). Furthermore, 2 Staphylococci strains presented only the loci *ica*D.

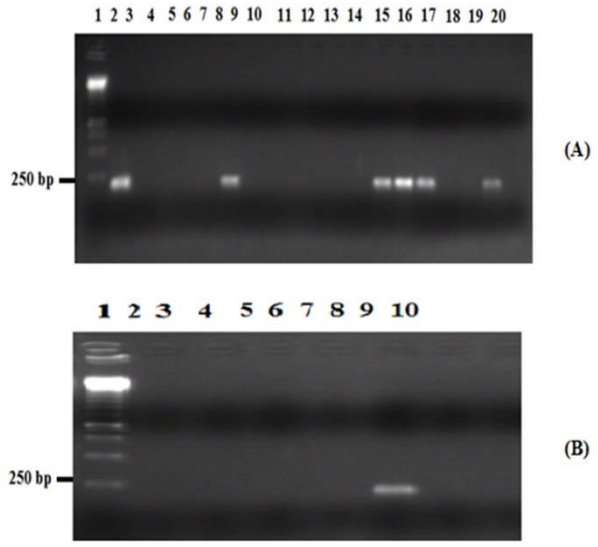

Figure 5: PCR detection of *icaA* and *icaD* genes. (A) PCR results with primers for *icaA.* Lane 1, 250-bp DNA molecular size marker; lanes 2–20, PCR amplicons obtained with DNA of Staphylococcus strains: 2 , S1 ; 3, S78 ; 4 , S51 ; 5 , S54; 6, S59; 7 , S60; 8 , S62; 9 , S64; 10, S69; 11, S73; 12, S77; 13, S79; 14 , S80; 15 , S84 ; 16 , S58;17 , S89; 18 , S91; 19 , S94 ; 20, negative control. (B) PCR results with primers for *icaD.* Lane 1, 250-bp DNA molecular size marker; lanes 2–10, PCR amplicons obtained with DNA of Staphylococcus strains: 2 , S96; 3 , S104; 4 , S105; 5 , S106; 6 , S107; 7 , S108; 8 , S109; 9, S110; 10, negative control.

4. Relationships between presence of the ica operon, slime production and TCP method

16 of the 17 *ica* A+ and *ica* D+ positive strains were slime producing and 8 produced a visible biofilm on the polystyrene surfaces under standard growth conditions. After stimulation by sugar supplementations 7 of 9 of the formerly *ica* A+/*ica* D+ positive and biofilm-negative formed a visible biofilm on polystyrene tissue culture plates. However, two ica-positive isolates remained biofilm negative even after exposure to these biofilm-inducing growth conditions. Two strains was *ica*A-/*ica*D- and biofilm negative was a producer of slime and the two strains *ica* A-/*ica*D+ was slime negative and biofilm negative.

All 23 *ica*A- /*ica*D-negative strains were unable to produce slime on CRA and biofilm on polystyrene tissue culture plates. The results obtained with all the strains are summarized in Table 1.

Table 1: relationships between the presence of the ica operon and biofilm production

Strain	Species	Production of Slime	Biofilm			Presence of icaA /icaD
			BHIB	**BHIB** 1%glu	**BHIB** 2%sac	
S106	***S.saprophyticus***	-	-	-	-	**icaA-/icaD-**
S107	***S.saprophyticus***	+	+	+	+	**icaA+/icaD+**
S110	***S.saprophyticus***	+	+	+	+	**icaA+/icaD+**
S104	***S.saprophyticus***	+	-	-	-	**icaA-/icaD-**
S60	***S.saprophyticus***	+	-	+	+	**icaA+/icaD+**
S84	***S.saprophyticus***	+	+	+	+	**icaA+/icaD+**
S78	***S.saprophyticus***	-	-	-	-	**icaA-/icaD-**
S86	***S.saprophyticus***	-	-	-	-	**icaA-/icaD-**
S66	***S.saprophyticus***	-	-	-	-	**icaA-/icaD-**
S52	***S.saprophyticus***	-	-	-	-	**icaA-/icaD-**
S1	***S.saprophyticus***	+	+	+	+	**icaA+/icaD+**
S105	***S.aureus***	-	-	-	-	**icaA-/icaD+**
S89	***S.aureus***	-	-	-	-	**icaA-/icaD+**
S62	***S.aureus***	-	-	-	-	**icaA-/icaD-**
S80	***S.aureus***	+	-	+	+	**icaA+/icaD+**
S65	***S.aureus***	+	+	+	+	**icaA+/icaD+**
S54	***S.aureus***	-	-	-	-	**icaA-/icaD-**
S79	***S.aureus***	-	-	-	-	**icaA -/icaD-**
S77	***S.aureus***	-	-	-	-	**icaA-/icaD-**
S57	***S.aureus***	+	-	+	+	**icaA+/icaD+**
S100	***S.aureus***	+	+	+	+	**icaA+/icaD+**
S83	***S.aureus***	+	-	-	-	**icaA+/icaD+**
S108	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S109	***S.epidermidis***	+	-	+	+	**icaA+/icaD+**
S103	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S91	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S92	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S95	***S.epidermidis***	+	-	+	+	**icaA+/icaD+**
S94	***S.epidermidis***	+	-	+	+	**icaA+/icaD+**
S7	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S96	***S.epidermidis***	-	-	-	-	**icaA -/icaD-**
S74	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S51	***S.epidermidis***	-	-	-	-	**icaA -/icaD-**

S59	***S.epidermidis***	+	-	-	-	**icaA -/icaD-**
S87	***S.epidermidis***	-	-	-	-	**icaA -/icaD-**
S72	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S58	***S.epidermidis***	+	+	+	+	**icaA+ /icaD+**
S69	***S.epidermidis***	-	-	+	+	**icaA+ /icaD+**
S73	***S.epidermidis***	+	+	+	+	**icaA +/icaD +**
S64	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S68	***S.epidermidis***	-	-	-	-	**icaA -/icaD-**
S71	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S90	***S.epidermidis***	-	-	-	-	**icaA-/icaD-**
S99	***S.hominis***	+	-	-	-	**icaA+ /icaD+**

B. Discussion

In the last two decades, with the increased use of indwelling medical devices, nosocomial infections caused by Gram-positive bacteria, in particular staphylococci have become more prevalent as a cause of hospital-acquired infection **(Fitzpatrick et al.,2002).**

The major pathogenic factor is the ability to produce an extracellular slime and constitute a biofilm, making clinical treatment extremely difficult. The biofilm development process requires polysaccharidic intercellular adhesin, which is synthesized by the enzymes encoded by the intercellular adhesion cluster (*ica*) **(Martín-López et al., 2002)**.

Detection of biofilm production in staphylococcal strains isolated from indwelling medical devices is important. It helps to know virulence factors for bacterial pathogenicity. Therefore, in the present study, we have isolate 44 staphylococci strains from urinary catheter in order to test the occurrence of slime genes, biofilm production and slime production in staphylococci by PCR, TCP method and Congo red agar method respectively.

The results revealed *S. epidermidis* as the most frequently isolated species corresponding to 48% of all strains isolated. Other staphylococci species were also identified, including *S. saprophyticus*, *S.aureus* and *S.hominis*. These results are close to those obtained by **Diemond-Hernández *et al.***

It has been noticed in several studies that the *S. epidermidis* is the most frequently isolated species. That is that *S. epidermidis* makes up a significant part of the normal bacterial flora of the human skin and mucous membranes. It is probably easily introduced as a contaminant during the surgical implantation of the polymeric device (**Otto, 2008).**

In this study, *ica* A and *ica* D were detected concomitantly in 17 of the 44 staphylococci strains isolated from urinary catheter and the *ica* D gene alone in 2 staphylococci strains.
These results are close to those obtained by **Cafiso *et al.*** who also investigated the presence of genes involved in biofilm production. In that study, 35% of the isolates were positive for the *ica* A and *ica* D genes and some isolates only carried the *ica*D gene.

In the TCP assay with BHIB used as standard growth media, 8 of 17 *ica*A/D positive strains exhibited a biofilm. This was in agreement with observations of other investigators **(Cho et al.,2002; Mathur et al.,2006; Johannes et al.,2002)** in which only few or no biofilm producing isolates could be detected using this medium. Surprisingly, supplementation of BHIB media with different sugars (BHIB $_{2\%suc}$, BHIB $_{1\%glu}$) increased biofilm formation significantly, and 34% of the investigated isolates formed biofilm in at least one of the used media. Furthermore, two isolates of staphylococci *ica* D+ /*ica* A- did not form biofilm in both medium.

According to our results and those found in the literature, the co-expression of *ica*A with *ica*D increases the enzyme responsible for N-acetylglucosaminyl transferase activity considerably and is related to phenotypic expression of the capsular polysaccharide. **(Chaieb et al., 2005; Arciola et al., 2001; Gerke et al., 1998).**

Moreover, the expression of the *ica* operon and as a result, the formation of biofilms seems to be highly variable among staphylococci **(Ziebuhr et al.,1997; Mempel et al.,1994)**. Thus, biofilm expression is influenced by environmental signals and can be induced in response to external stress and subinhibitory concentrations of certain antibiotics (**Cho et al., 2002)**. **Cramton *et al.,*** suggested that anaerobiosis strongly increases biofilm expression. The expression of biofilm

is also regulated by iron, with maximum expression occurring at low concentrations (**Chaieb et al.,2005**). However, two staphylococci strains *ica* A+/*ica* D+ remained biofilm negative even under PIA-expression-stimulating growth conditions. The detection of no biofilm, despite the presence of *ica*, could be due to several reasons such as inactivation of the *ica* operon by insertion of an IS256 element in the *ica*C gene **(Ziebuhr et al.,1999)** , the action of the *Ica*R repressor **(Conlon et al.,2002)** , or post-transcriptional regulation **(Dobinsky .,2003)** .

The Comparison of the CRA test and the results obtained by PCR revealed that among the 17 *ica*A+ and *ica*D+ positive strains 16 were slime producing. In fact, these results correspond to those obtained by **Aricola *et al.,*** and **El- Mahallawy *et al*** ., In their study, a strong correlation was found between *ica* gene positivity and the ability to produce slime by CRA test (P <0.001) compared to the TCP method.

Two isolates of staphylococci were slime + /biofilm- and *ica* A-/*ica*D-. **Chokr *et al*** reported this phenomenon and suggested that in this strains variability in the *ica* locus sequence exists allowing production of a polysaccharide which reacts with the anti-PIA serum.

Furthermore, Staphylococci Strains isolates from urinary catheter showed high levels of resistance to different classes of antibiotics except for vancomycine and pristinamycin. They were significantly resistant to the penicillin (98%), oxacilline (79%), gentamicine (75%) and ofloxacine (73%). These results are close to those obtained by **Touati *et al*** who reported that Staphylococci isolates from catheter-related infection are significantly resistant to oxacilline (76,8%), gentamicine (46,4%) and ofloxacine (75%).

The high frequency of antibiotic resistance among strains isolates for urinary catheter is associated with high pressure antibiotic selection. The multicellular organization bacterium in biofilm gives them the advantage to acquire new genes. The biofilm is a perfect environment for exchange resistance plasmids (**Touati *et al.,* 2007)** since it includes both the greater probability of contact between cells and the negligible effect of shear forces **(Donlan,2001).**

Gilbert *et al* reported that biofilm producers were to be 10-1000 times less susceptible towards antibiotics than are the equivalent cells growing planktonically. Biofilm hampered penetration of antimicrobial and the concentrations required to eradicate biofilm producing bacteria are higher than those required to eradicate strains that did not produce biofilm **(Seif El-Din et al.,2011)**.

IV. CONCLUSION

The presence of *ica* A and *ica* D genes in most of staphylococci strains allows the production of biofilm. The latter facilitates the development of infections by compromising the immune system of the patient and contributing to the failure of antibiotic therapy. This may result in recurrent infections and the emergence of multiresistant pathogens. Therefore, the analysis of the presence and expression of *ica* genes can clarify the different adhesion mechanisms in the pathogenesis of infections associated with medical devices and it could also be of value in the development of new preventive and therapeutic measures to eradicate biofilm in hospitals.

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