Current Research in Microbiology and Biotechnology
Vol. 5, No. 2 (2017): 1031-1037
Research Article
Open Access

ISSN: 2320-2246

Detection of biofilm formation and *icaA* gene among Iraqi clinical isolates of both methicillin resistant and susceptible *Staphylococcus aureus*

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Received: 18 February 2017 Accepted: 11 March 2017 Online: 14 March 2017

ABSTRACT

Antibiotic resistance and biofilm production ability make *Staphylococcus aureus* a serious life threatening bacteria. Biofilm forming ability is a main cause of *Staphylococcus* pathogenicity since it provides resistance to both antibiotics and host immune response. The main biofilm extracellular substances are polysaccharide intercellular adhesion (PIA), cell surface protein, and extracellular DNA. PIA is coded by *ica* operon which consist of four genes A, D, B and C. in this study 40 Iraqi clinical isolates of *staphylococcus aureus* was screened for in-vitro biofilm formation and *icaA* gene presence. The ability of isolates to form in-vitro biofilm was detected in two different screening methods, Congo red agar method, and Microtiter plate method. Additionally, in this study, the correlation between *icaA* locus detected by PCR and biofilm formation by Microtiter plate method was examined. Among the forty isolates as screened by Microtiter plate method, 26 isolate (65%) were biofilm producing bacteria (8 MRSA and 18 MSSA). While 14 isolate (35%) were biofilm non-producing bacteria (5 MRSA and 9 MSSA). Only 15 isolate (38%), 4 MRSA and 11MSSA, were biofilm forming bacteria as detected with Congo red agar method. The result of PCR revealed that only 16 isolate (40%) out of 40 possesses the *icaA* gene. Among the 16 *icaA* positive isolate, 6 were MRSA and 10 were MSSA. A good correlation was observed between Microtiter plate method and *icaA* + detected by PCR, suggesting that this in-vitro screening method is more reliable than Congo red agar method. Additionally, the outcome of this study revealed that in-vitro biofilm formation in case of MRSA is *icaA* dependent, and it is *icaA* independent in case of MSSA.

Keywords: *Staphylococcus aureus*, MRSA, MSSA, Congo red, Microtitter plate, icaA gene, Biofilm, PCR Detection.

1. INTRODUCTION

Staphylococcus aureus is a gram positive bacteria that is wildly involved in nosocomial infections, especially infections that associated with the use of contaminated medical devices [1]. The pathogenicity of these bacteria is attributed to the possession of both antibiotic resistant genes and virulence genes that code for virulence factors. One of the virulence factors that *staphylococcus* harbor is the ability to form biofilm, which is a layer of aggregated cells immersed in an extracellular matrix that assist cells to adhere to each other and to adhere to any available moist solid surface, like host tissue. The ability of *staphylococcus* to enclose themselves inside the extracellular matrix protects them from the host immune defense, antimicrobial agents and antibiotics because this matrix is acting as a protective shield and a way for bacteria to survive against these factors [2-5].

Since it has been found that 80% of implant-associated infection is caused by *Staphylococcus spp* [6], many studies have been conducted on this bacteria to understand the bacterial mechanism behind biofilm formation, understanding the Quorum sensing system that regulates the expression of genes involved in bacterial pathogenicity, and understanding the chemical nature of the extracellular matrix. Nucleic

acids that result from regulated auto-cell lysis [7], Curli proteins which are an aggregated Amyloid protein, especially in *E.coli* [8], cytoplasmic proteins in the case of *S. aureus* [9] and polysaccharide are the main components of the extracellular matrix excreted by biofilm producing bacteria [10,11]. The component of extracellular matrix may vary depending on the type, the age of microorganism and the environment surrounding them [12]. Advanced study on the chemical nature of the extracellular matrix in *S. epidermidis* and *S. aureus* found that polysaccharide intercellular adhesion (PIA), which is poly-β (1-6)-N-acetylglucosamine, is the main polysaccharide component of the extracellular matrix [13]. The PIA is coded by *ica* operon (made up of four genes A, D, B, and C) [14] which is part of accessory genes that is regulated by the Quorum sensing system. Since the *ica* operon is part of accessory genes, not all staphylococcus isolates will have it. Accordingly, the biofilm mechanism within staphylococcus classified into *ica*-dependent biofilm pathway [15] and *ica*-independent biofilm pathway [16]. The *ica*- dependent biofilm pathway is depending on PIA as a virulence factor that mediates cell-cell adhesion and cell-solid surface adhesion [17]. A deletion of *ica* locus in *ica*-dependent biofilm strains can diminish their ability to form biofilm in-vitro [18]. While the *ica*-independent biofilm pathway, is depending on cell surface and cytoplasmic proteins and extracellular DNA to mediate the adhesion [19].

Since that staphylococcus infection is prevalent, this bacterium has become a serious clinical problem. Therefore, 40 isolates of *staphylococcus aureus* which include 13 methicillin resistant *Staphylococcus aureus* (MRSA) and 27 methicillin sensitive *Staphylococcus aureus* (MSSA) form Iraqi patients have been tested for their ability to form biofilm in-vitro and screened for the presence of *icaA* locus by polymerase chain reaction. Additionally, the conducted study aimed to find out if there is any correlation between biofilm formation ability of the tested isolates and there resistance to methicillin in one hand and if there is any correlation between biofilm formation ability and the presence of *icaA* locus on the other hand. Because there is no in-vitro biofilm detection method with high accuracy and in order to determine the reliable one, the two well-known detection methods, the Microtiter plate method, and Congo red Agar method, have been used. Since that biofilm production may be affected by environment factor and media composition, four different media was used to figure out whether media compositions have any effect on biofilm formation or not with maintaining the same pH value (pH 7), same incubation temperature and time, 37 °c and 24 hr., respectively.

2. MATERIALS AND METHODS

2.1 Bacterial isolates

Forty strains of *Staphylococcus aureus* were isolated from Iraqi people. Most of these isolates were obtained from noise and some from blood and wounds. *Staphylococcus aureus* isolates were identified by different biochemical tests oxidase, coagulase, and catalase [20]. Additionally, the *Staphylococcus aureus* isolates were confirmed by PCR for the presence of *nuc* gene. While Methicillin resistant *staphylococcus aureus* (MRSA) was identified by PCR for *mec* gene presence.

2.2 Effect of media on biofilm production.

Four different broth media were used to determine the best media for biofilm production. The tested media was nutrient broth, nutrient broth supplemented with 1% glucose, Brain heart infusion broth, and Brain heart infusion broth supplemented with 1% glucose. An overnight growth of isolates in nutrient broth was diluted 1:100 in the four different tested media and incubated in 96-well Microtiter plate for 24 hour at 37 °C. The next day, the bacterial growth was poured out and the plate was washed with distilled water then stained with 0.1% crystal violet for 10 minutes. After the 10 minute, the stain was removed and the plate was left to dry for 1 hour. The stained wells were treated with 30% acetic acid to solubilize the attached stain. The optical density of the solubilized crystal violet was measured by ELISA auto reader at 590 nm.

2.3 Assay for biofilm formation

2.3.1 Microtiter plate

A cheap standard assay for biofilm formation is the Microtiter Plate [21, 22]. Overnight cultures of *staphylococcus* isolates grown on nutrient broth media were diluted 1:100 into brain heart infusion broth supplemented with 1% glucose for biofilm assay**.** 200 µl of the diluted cultures was transferred into 96 Microtiter plate, a duplicate was used for each isolates. The diluted cultures were incubated for 24 hour at 37 ºC. After the 24 hour of incubation period, the bacterial growths were poured out and the Microtiter plate was washed with distilled water to remove any non-adherent cell, then the plate was left to dry. Each well was stained with 200 µl of 0.1% crystal violet to stain the layer of cells that had attached to the wells bottom. The excess stain was poured out and washed with distilled water and the plate was left to dry for approximately two hours.

For biofilm quantification, each stained well was treated with 200 µl of 30% acetic acid for 10 minute to solubilize the dye. The solubilized crystal violet was transferred into a new Microtiter plate. The optical density of each well was measured by micro ELISA auto reader at wavelength 590 nm, 200 µl of 30% acetic acid was used as a blank and to determine the background. A duplicate was performed and the average optical density and standard deviation were calculated. In this study, the investigated isolates at OD_{590} were classified into three categories, $OD_{590} < 0.4$ a non- biofilm forming cell, $0.4 \leq OD_{590} > 0.8$ a weak biofilm forming cell, $OD_{590} \geq 0.8$ a strong biofilm forming cell.

2.3.2 Congo Red

An overnight growth of the forty isolates in Brain Heart Infusion Broth was streaked on Congo Red Agar to test

their ability to form biofilm. The composition of media was as following, 37 gm/L Brain Heart Infusion Broth, 5% Sucrose, 10 gm/L Agar, 2% sucrose, 1.5 % NaCl, and 0.08% Congo Red [23, 24, 25]. The pH of Congo red media was adjusted to 7. The Congo red stain was prepared individually and autoclaved at 121 ºC for 15 min. After cooling (55ºc), the stain was added to the sterile media. The investigated isolates were incubated for 24 hour at 37 ºC. The ability of bacteria to form biofilm is attributed to their ability to excrete slim into their surroundings. If the isolates are able to excrete slim, the color of colonies will be black but if not it will be red or Bordeaux. Isolates that formed almost black, black, and very black colonies were considered as biofilm forming isolates (positive), while isolates that formed red or Bordeaux colonies were considered as biofilm non-forming isolates (negative) [26, 27].

2.4 Detection of *ica*A loci

PCR for detection of icaA loci

DNA was extracted from 1 ml of overnight culture using Promega DNA extraction kit supplemented with 30 µg/ml lysozyme enzyme. 1µl of 100ng DNA was used as template for PCR. *ica*A loci presence among the extracted DNA of isolates was determined by polymerase chain reaction by using forward (5'-AAACTTGGTGCGGTTACAGG-3') primer and revers (5'-TCTGGGCTTGACCATGTTG-3') primer [28], that will yield 750 bp fragment. 25 µl of PCR reaction contains 1x master mix buffer, 10 pmol/µl forward and reverse primers, 200 ng/µl DNA and water. The PCR reaction was carried out with the following parameters: initial denaturation at 94 °C for 5min, second denaturation at 94 °C for 1 min, annealing at 54 °C for 1min, extension at 72 °C for 50 sec. 30 cycle of amplification was applied. To analyze the PCR products, 10 µl of PCR mixture was loaded to 2% agarose in the presence of 100 b.p DNA ladder. After performing gel electrophoresis, the gel was exposed to U.V by using U.V Transilluminator.

3. RESULTS AND DISCUSSION

3.1. Media composition affects biofilm production

The ability of five isolates (three were MRSA 1, 2, &4 and two were MSSA 3 &5) out of forty to form biofilm was tested in four different media to determine which media is best for biofilm production and to figure out whether media composition has any effect on biofilm formation or has not. The four different types of media were nutrient broth, nutrient broth + 1% glucose, Brain Heart Infusion Broth, and Brain Heart Infusion Broth + 1% glucose. The biofilm formation was determined in 96-well Microtiter plate with the four different media and as described earlier in materials and methods. Figure 1 shows the OD of the solubilized crystal violet at 590 nm and at the four different types of the investigated media. The outcome of this test (figure1.) revealed that the highest biofilm formation of the five tested isolates was observed in Brain Heart Infusion Broth supplemented with 1% glucose $0.3 \leq OD_{590} \geq 0.55$, While the lowest was observed in nutrient broth without any addition of glucose ($OD_{590} \leq 0.3$). The obtained result is in accordance to a study conducted on *Listeria monocytogenes* where the ability of the mentioned bacteria to form biofilm was increased by the addition of trehalose or glucose, as a sole source for carbon, to their growth media [29].

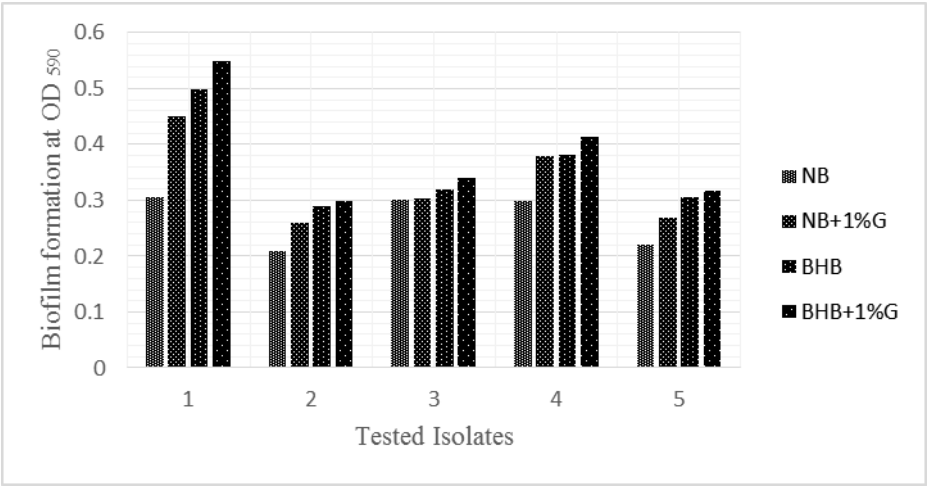

Figure 1: Biofilm formation among five tested isolates at four different types of media NB, NB+1% Glucose, BHIB, & BHIB + 1% Glucose. The layer of adherent cells, as described in materials and methods, was stained with crystal violet and measured at OD_{590}

3.2 Detection of biofilm formation

Since that BHIBB+1% glucose media gave the highest biofilm production, it has been used as a typical media for biofilm formation in Microtiter plate method. among the forty tested isolates, 26 (65%) were positive (8 MRSA and 18 MSSA) for biofilm production while 14 isolates (35%) were negative (5 MRSA and 9 MSSA) for biofilm production. Among the biofilm producing isolates, 3 (11.5%) were strong (two are MSSA) and 23 (88.5%) were moderate (figure 2&3, Table.1). 15 isolates, 4 MRSA isolates and 11 MSSA isolates, (38%) were biofilm producer in Congo Red Agar method,

while 25isolates (62%) were non-biofilm producer (table 1). The biofilm producing isolates were almost black, black, & very black colonies, while the non-biofilm producing isolates were Bordeaux colonies (figure 4). The result obtained from these two different methods was not similar. No correlation was occurred between Congo Red Agar and the Microtiter plate.

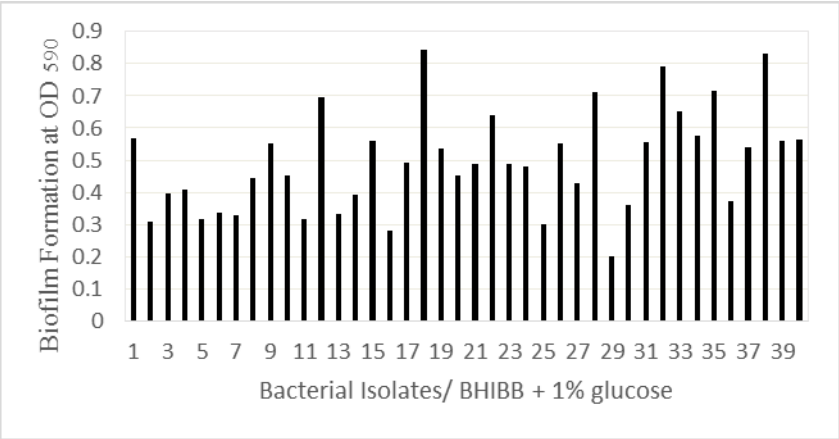

Figure 2: Solubilized crystal violet at OD_{590} in Microtiter plate assay.

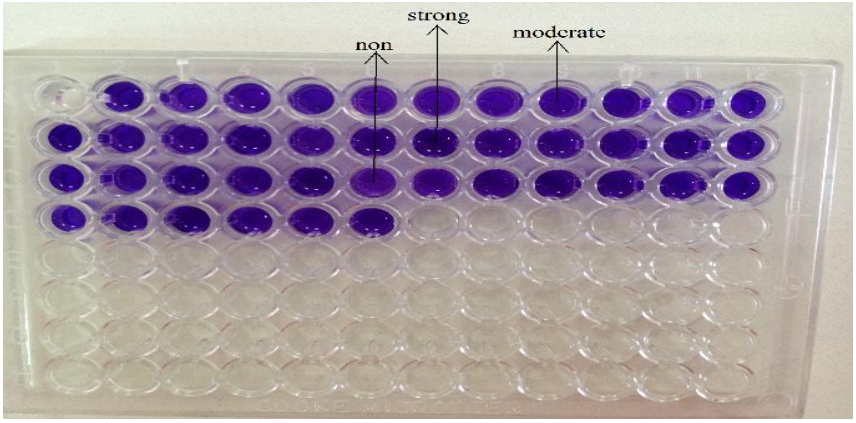

Figure 3: Microtiter plate method for biofilm screening. Three categories (non, moderate, & strong) were discriminated with crystal violet stain.

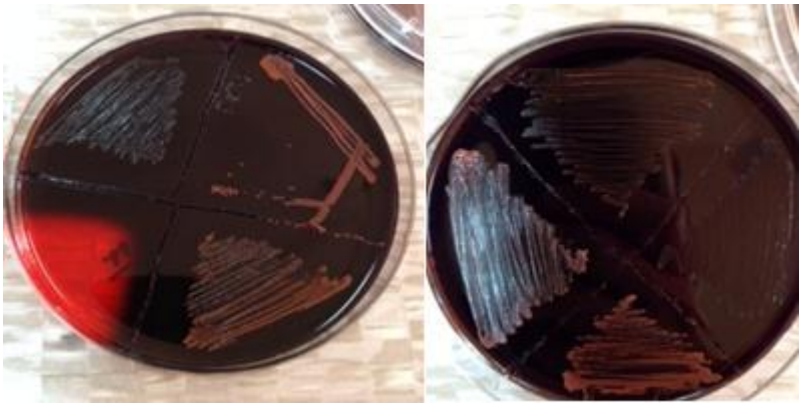

Figure 4: Congo Red Method for detection of slim and biofilm production of the tested isolates. Black, and almost black is biofilm producing isolate, while Bordeaux is non- biofilm producing isolate.

3.3 PCR for *icaA* detection and the correlation between *icaA* and biofilm formation.

Among the forty isolates of staphylococcus, only 16 isolates (40%) have the *icaA* gene while 24 isolates (60%) do not have it (figure 5, table 1). Among the 16 *icaA* positive isolates, only four isolates (25%) formed biofilm on Congo Red Agar while 11 isolates (68.7%) were biofilm forming bacteria in microtiter plate (table 1). On the other hand, Within the 24 *icaA* negative isolates, 12 isolates (50%) were biofilm forming bacteria on Congo Red Agar and Microtiter plate (table 1).

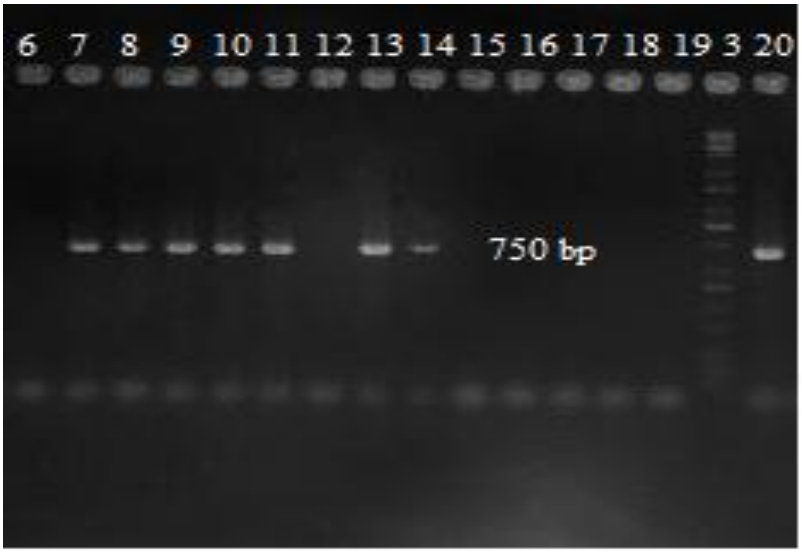

Figure 5: Lane 3: 10000 b.p DNA Ladder, Lane 7-11, 13-14 & 20: positive result, lane 6, 12, &15-19: negative result.

Table 1: Microtiter plate and Congo Red Agar for biofilm formation and *icaA* detection by PCR.

Isolate	Strain type	Congo red agar	Microtiter plate/ OD_{590}	icaA
1	MRSA	Almost black	0.568	-
2	MRSA	Bordeaux	0.309	+
3	MSSA	Bordeaux	0.395	+
4	MRSA	Bordeaux	0.407	+
5	MSSA	Bordeaux	0.315	-
6	MRSA	Bordeaux	0.336	-
7	MRSA	Bordeaux	0.329	-
8	MRSA	Bordeaux	0.446	+
9	MSSA	Bordeaux	0.552	+
10	MSSA	Bordeaux	0.451	+
11	MSSA	Bordeaux	0.316	+
12	MRSA	Very black	0.696	+
13	MSSA	Bordeaux	0.334	-
14	MSSA	Almost black	0.391	+
15	MRSA	Bordeaux	0.56	+
16	MRSA	Bordeaux	0.282	-
17	MRSA	Almost black	0.493	-
18	MSSA	Very black	0.844	-
19	MSSA	Bordeaux	0.534	-
20	MSSA	Bordeaux	0.451	-
21	MSSA	Bordeaux	0.49	-
22	MRSA	Almost black	0.639	-
23	MSSA	Bordeaux	0.49	+
24	MRSA	Bordeaux	0.481	+
25	MSSA	Bordeaux	0.3	+
26	MSSA	Almost black	0.55	-
27	MRSA	Bordeaux	0.43	+
28	MSSA	Almost black	0.71	-
29	MSSA	Bordeaux	0.2	-
30	MSSA	Bordeaux	0.36	-
31	MSSA	Almost black	0.554	+
32	MSSA	black	0.793	-
33	MSSA	Almost black	0.65	-
34	MSSA	Almost black	0.575	-
35	MSSA	Very black	0.716	-
36	MSSA	Almost black	0.373	-
37	MSSA	Bordeaux	0.538	+
38	MSSA	black	0.83	-
39	MSSA	Bordeaux	0.5	-
40	MSSA	Bordeaux	0.564	-

One of the objectives of this study was to test the effect of culture media composition on biofilm production of *S. aureus* isolates in vitro**.** This was achieved by comparing the ability of five *S. aureus* isolates to form biofilm in four different media (NB, NB+1% glucose, BHIB, and BHIB + 1% glucose) by Microtiter plate assay. The five tested isolates gave the highest biofilm formation in BHIB+ 1% glucose comparing with the rest tested media. This indicates that BHIB is better than NB in enhancing the in-vitro biofilm production. Additionally, results showed that carbon play an important role in the formation of biofilm and indicates that media composition does effect biofilm production. Our obtained result is in an agreement with previous studies which showed that the absence or limitation of glucose decreases the ability of *Citrobacter* to form biofilm in-vitro and a high biofilm formation in *E. coli* was obtained when glucose was added to media [30]. Biofilm enhancements by glucose telling us that glucose is effecting some factors that regulate biofilm production, *icaR* in case of *ica*-dependent biofilm production or *rbf* in case of *ica*-independent biofilm production.

Biofilm formation ability of *S. aureus* isolates were tested by two different assay methods, Microtiter plat, and Congo red agar. Biofilm formation tested by the two mentioned methods was compared with each other. By Microtiter plate assay, 65% of *S. aureus* were biofilm positive (2 MSSA and 1 MRSA) and 35% were negative. while 38% of isolated were positive for biofilm and 62% were negative in Congo red agar. If we compare between these two methods, we can high light the differences that might be the reason behind result differences. In case of Microtiter, BHIB+1% glucose was used, and biofilm formed on a plastic surface. Although BHA was used in Congo red agar method, but it supplemented with many other additions (see materials and methods) and the biofilm formed on the surface of the media. Therefore, growth condition like media composition and surface type might be the reason behind result differences between Congo red method and Microtiter plate.

Depending on our observation, Microtiter plate is more reliable screening method than Congo red Agar. As detected by Microtiter plate method, the highest biofilm forming ability was belong to methicillin resistant isolate, this result emphasized that biofilm is a virulence profile of *S.aureus*. The high ability of this isolate to form biofilm protected it from the effect of methicillin because it acts as a protective shield that obstructs the penetration of antibiotic. This result is agreeing with Gilbert et al. (2002) [31] who reported that the susceptibility of bacteria to antibiotics will decrees 10-1000 times as it has ability to form biofilm. Surprisingly and as detected by Microtiter method, the second highest biofilm forming ability with OD equals 0.83 at 590 nm was owned to methicillin sensitive *S. aureus* isolate. This result was unexpected because with such high ability to form biofilm, the bacteria should be protected from the effect of Methicillin since it will act as a protective shield.

Other objective of this study was to detect the presence of one of biofilm associated gene, the *icaA* gene, among our MRSA and MSSA isolates. A study done by Cramton *et al* (1999) [32] and had been confirmed by Arciola *et al* (2001) [15] showed that most of *Staphylococcus aureus* and *epidermidis* have *icaA* gene, which enhance their adherence ability and biofilm formation. Our study found that *icaA* gene was detected only in 16 isolates (6 isolates are MRSA and 10 isolates are MSAA) out of 40 isolates which agree with a study conducted by Cafiso *et al (2004)* [33] in which only 35% of investigated Staphylococcus aureus had the *icaA* gene. Our conducted study found that not all the *icaA* carrying isolates (*icaA+)* were able to form biofilm in-vitro, 7 (5 MRSA and 2 MSSA) out of 16 *icaA*+ isolates were positive for biofilm production in Congo red agar method while 10 *icaA*+ isolates (4 MRSA and 6 MSSA) were biofilm positive in microtiter plate method, these results indicate that biofilm formation among *icaA*+ staphylococcus aureus is depending on growth conditions that might have a negative effect on *icaA* gene regulation factors. Our observation presumed a good correlation between Microtiter plate method and *icaA* + detected by PCR compared with Congo red. An opposite observation was established by El-Mahallawy *et al.* (2009) [34] who found a week correlation between *icaA* gene positivity by PCR and Microtiter plate compared with Congo red agar method. The remaining 24 isolates were *icaA*-. Although they don't carry the *icaA* gene, some of them were able to form biofilm in-vitro, this observation suggested that these isolates have other genes involve in biofilm formation in-vitro rather than *icaA* gene (ica- independent biofilm forming bacteria). The obtained data form this study suggests that the ability of MRSA isolates to form biofilm in-vitro requires the presence of *icaA* gene and it is affected by growth conditions. In case of MSSA isolates, the obtained result suggests that the ability to form biofilm in-vitro is *ica*- independent.

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