Antibiotic resistance pattern and biofilm formation in clinical *Staphylococcus aureus* isolates

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Abstract

In this study a total of one hundred and twenty samples were collected from patients who were admitted to West Erbil Emergency, Emergency, and Rizgary teaching Hospitals during the period from 1 March 2015 to 20 May 2015. Samples collected from different clinical sources: 50% from burns, 32% from surgical wounds, 10% from dental carries, and 8% from urine samples. Isolates were identified using cultural, morphological, biochemical tests, and confirmed by VITEK2 compact system. Fifty isolates were identified as *Staphylococcus aureus.* Antibiotic Sensitivity test by disk diffusion method was done for all *S. aureus* isolates against 20 commonly used antibiotics and the resistance percentage was as the following: 100% for AMC, AP, AX, and PG, 92% for ME, 68% for CAZ, 64% for TM, 62% for T, 60% for E, 50% for CRO, 48% for CTX, KF, and S, 46% for L, 42% for RA, 40% for DA, 36% for CIP, 34% for C, and 4% for GM and while all isolates were sensitive to vancomycin. All isolates were tested for β-lactamase production, and all of them showed a positive result. PCR technique was used for the detection of *mecA* gene in *S. aureus* isolates and the results showed that 62% of isolates were *mecA* positive, while 38% of the isolates were *mecA* negative. All *S. aureus* isolates were tested for their *in vitro* ability for biofilm formation using two methods: Congo red agar method, and microtiter plate assay. The percentages of biofilm production were 94%, and 100% respectively. PCR technique was also used to detect the presence of *icaAD*gene in *S. aureus* isolates and the results showed that 94% of the isolates harbor this gene, while 6% were *icaAD* negative.

Keywords: *Staphylococcus aureus,* antibiotics resistance ,and biofilm formation

Introduction

Staphylococcus aureus is a major cause of nosocomial infections and remain a versatile and dangerous pathogen in human. The frequency of both community acquired and hospital acquired staphylococcal infections have increased steadily (Lowy, 1998). *S. aureus* is coagulase-positive, often hemolyse blood and produce a variety of extracellular enzymes and toxins(Brooks *et al*., 2001).In humans, infection with *S. aureus* may cause suppuration, abscess formation, a variety of pyogenic

infections and even fatal septicemia. It can also cause food intoxication due to elaboration of heat-stable enterotoxin (Collee *et al*., 1996; Kloos & Bannerman, 1999). Antimicrobial resistance is an increasing threat afflicting hospitals worldwide (Graffunder and Venezia, 2002). Antimicrobial drug resistance in hospitals is driven by failures of hospital hygiene, selective pressures created by overuse of antibiotics, and mobile genetic elements that can encode bacterial resistance mechanisms (Weinstein, 2001).Methicillin-resistant *S. aureus* (MRSA) isolates were once confined largely to hospitals, other health care environments, and patients frequenting these facilities. Since the mid-1990s, however, there has been an explosion in the number of MRSA infections reported in populations lacking risk factors for exposure to the health care system (David and Daum, 2010) . Biofilms are aggregates of unicellular microorganism forming multicellular structures that adhere to surfaces (Watnick&Kolter, 2000). Pathogenic bacteria and fungi can form biofilm on inert surfaces of implanted devices such as catheters, prosthetic heart valves and joint replacement (Li *et al*., 2003).S. *aureus* is capable of biofilm formation, which increases its persistence and boosts its levels of antimicrobial resistance. Genetic analyses of S. aureus have shown that the progression of biofilm development consists of initial cell- to- surface interaction followed by cell-to-cell interaction (Cross *et al*., 2001). A great variety of Staphylococcus aureus and Staphylococcus epidermidis strains carry the ica cluster, and some of them constitute biofilm. Loss of the ica locus results in an incapacity to produce polysaccharidic intercellular adhesin and to develop biofilms (Cramton *et al*., 2001). Staphylococcal infections produced by ica carriers can be more problematic due to the presence of methicillin and mupirocin resistance genes. The rapid detection of the ica locus in hospital staphylococcal isolates, together with the simultaneous detection of antibiotic resistance genes, will allow the development of prevention methods to reduce the bacterial capacity to invade the in-dwelling medical devices (López *et al*., 2002) .Therefore, this paper concerned with isolation and identification of *S.aureus* from different clinical specimen and study both antibiotic resistance pattern and biofilm formation in *S.aureus* isolates .

Materials and Methods

Samples collection

Samples were collected from 120 patients who were admitted to West Erbil Emergency, Emergency, and Rizgary teaching Hospitals during the period from 1 March 2015 to 20 May 2015. The age of patients ranged from one to 45 years. Samples were taken from different sites: burns, surgical wounds, dental carries and urine. The samples were obtained by rubbing the inflamed or discharged wound, burn, or decayed teeth by a sterile disposable swabs with normal saline to keep samples fresh while transporting it to the laboratory for further processing. Urine samples were collected by taking a loop full from the urine sample and streak it directly on the culture media.

Identification of the isolates

Identification of these isolates was carriedout using microscopical, morphological,biochemical tests and VITEK2 compact system (Forbes *et al*., 2007; Winn and Koneman, 2006; Goldman and Green, 2009).

Antimicrobial susceptibility test (Disk diffusion method)

This test was performed according to Schwalbe *et al.,* (2007) and Ferraro *et al.,* (2006).Antibiotic impregnated discs with required concentration were dispensed on the surface of Mueller-Hinton agar medium that has been spread with a pure bacterial suspension of 10^5 CFU/ml. After incubation, inhibition zones were measured and translated into predetermined categories as susceptible, intermediate, or resistant.

β- lactamase production

Iodometric method was used to determine the ability of bacteria to produce β-lactamase, 0.1ml of penicillin G solution (6000 µg/ml) placed in a sterile tube and a loop full of well isolated bacteria was transferred to the tube. The tubes were left at room temperature for 30 minutes, then 2 drops of starch solution and 1 drop of iodine solution was added to each tube. Blue color appeared immediately as a result of reaction between starch and iodine. The tubes were shacked well and left at room temperature for 10 minutes. Appearance of white color indicated the ability of an organism to produce β-lactamase (Mustafa, 2015).

Genomic DNA extraction

A Presto™ Mini gDNA Bacteria Kit was used for genomic DNA extraction from *S. aureus* isolates. A loop full of bacteria were incubated over night in a tube containing LB broth. The kit's instructions was followed carefully to obtain a good DNA extracts.

Detection of *mecA* gene in *S. aureus* clinical isolates

The standard PCR assay was performed using the DNA amplification instrument Mastercycler gradient (Eppendorf, Germany) to detect *mecA* gene. The *mecA*-specific primer pairs used for amplification of 533 base pair (bp) fragment are: Forward, 5'-AAAATCGATGGTAAAGGTTGGC-3'and Reverse, 5'-AGTTCTGGAGTACCGGATTTGC-3' (Bühlmann *et al*., 2008).A volume of 20µl deionized distilled water (ddH_2O), 1.3 µl Reverse primer, 1.3µl Forward primer and 2.5 µl of extracted DNA (template) was added to the ready to use PCR reagent tube (Bioneer, South Korea) which contains the following (for the 20µl reaction): 1U *Top* DNA polymerase , 250 µM of each: dNTP (dATP, dCTP, dGTP, dTTP),10 mMTris-HCl (pH 9.0),30 mMKCl,1.5 mM $MgCl_2$, Stabilizer and tracking dye.The thermal cycling protocol for PCR was comprised as described by Pournajaf *et al.* (2014) :1.Initial denaturation at 95^0C for 3 minute. 2. Thirty three cycles of:Denaturation at 94^0C for 1 minute, annealing at 53^0C for 30 seconds, elongation at 72^0C for 1 minutes and final extension at 72^0C for 6 minutes.

Detection of PCR products:

About 5µl of the amplified products were visualized by electrophoresis in 1.5% agarose gels stained with ethidium bromide under UV transilluminator and photographed.The amplicon (PCR product) generated from *S. aureus* gene sequences by this PCR method was a DNA fragment of 533 bp length. Therefore a positive PCR test should yield a 533 bp DNA fragment which appeared as an intense band on an ethidium bromide stained agarose gel. The molecular size of the band was verified by comparing its migration to that of a DNA marker (100bp DNA ladder) run on the same gel. A negative PCR product did not produce any visible band on the gel.

Detection of biofilm formation

1.Congo red agar (CRA) method

Plates of CRA were inoculated and incubated at 37^0C 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 nonproducing 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 (Jain and Agarwa, 2009; Arciola *et al*., 2001).

2. Microtiter plate (MTP) method

S. aureus isolates were incubated in nutrient broth at 37^0C for 24 hours; grown colonies were diluted in 1:200 and incubated in microtiter plates (96-flat bottom well microtiter plates). After 24 hours the wells were washed with PBS buffer two up to three times and left in the room temperature for drying. In the next step 0.4% crystal violet solution was used as stain for 10 minutes. The plates then washed off using sterilized distilled water and kept for air-dry. The bound bacteria were quantified by addition of ethanol 70%. Finally the absorbance at 490nm was determined; an OD of 490nm >0.12 was regarded as a biofilm positive sample (Namvar *et al.,* 2013).

Polymerase chain reaction (PCR) for amplification of *icaAD* gene

The extracted genomic DNA from all isolates was tested to detect the presence of *icaAD* gene. The amplification of the *icaAD* gene was done according to Yazdani*et al.,* (2006) using specific primers:Forward, 5'- TATTCAATTTACAGTCGCAC-3' and Reverse, 5'-GATTCTCTCCCTCTCTGC-3' yielding a PCR product of 407 base pairs (bp). A volume of 20µl deionized distilled water (ddH_2O), 1.3 µl Reverse primer, 1.3µl Forward primer and 2.5 µl of extracted DNA (template) was added to the ready to use PCR reagent tube (Bioneer, South Korea) which contains the following (for the 20µl reaction): *Top* DNA polymerase 1 U, each: dNTP (dATP, dCTP, dGTP, dTTP) 250 µM, Tris-HCl (pH 9.0) 10 mM, KCl 30 mM, $MgCl_2$ 1.5 mM Stabilizer and tracking dye.The thermal cycling protocol for PCR was comprised as described by Nasr *et al.,* (2012) as following: Initial denaturation step(2 minutes at 94^0C), 30 cycles of: denaturation at 94^0C for 30 seconds, annealing at 58^0C for 30 seconds, elongation at 72^0C for 30 seconds, final extension at 72^0C for 3 minutes.

Detection of PCR products:

After amplification , 5µl of PCR product was analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized under UV trans-illuminator. The gene ruler 100bp DNA ladder was used as a DNA size marker. A positive PCR product yielded a band of 407 bp, while a negative one did not produce any visible band on the gel.

Results and Discussion

Isolation and Identification of *S. aureus*

Fifty isolates were identified among 120 different clinical specimens including: 32% wound swab, 50% burn swab, 8% urine and 10% from dental carries. The identification of the isolates were carried out using conventional method based on cultural characteristics, cell morphology, Gram stain reaction biochemical properties and VITEK2 compact system. All isolates were able to grow on mannitol salt agar (selective media for Staphylococcus). *S. aureus*has the ability to change the color of the media from Pink – Orange to yellow, because it can ferment the mannitol which present in the medium that leads to change in the color (Morello*et al*., 2003). circular, smooth, yellow to golden colonies raised on blood agar with various degrees of hemolysis (mostly beta hemolysis). Prepared smears of *S. aureus*isolates appeared as purple single, diplo, and grape like Gram positive cocci under light microscope. All isolates were positive for catalase, coagulase and DNase. All isolates were identified as *S. aureus* by VITEK2 compact system with over 85% probability percentage.

Antibiotic resistance pattern of *S. aureus* isolates

Antibiotic Sensitivity test by disk diffusion method for 50 isolates of *S. aureus* was done against 20 commonly used antibiotics (AMC, AP, AX, C, CAZ, CIP, CRO, CTX, DA, E, GM, KF, L, ME, PG, RA, S, T, TM, VA). The resistance percentage of *S. aureus* isolates varied for different antibiotics used in this study as shown in table (1).The results revealed that the resistance was 100% for AMC, AP, AX, and PG and 92% for ME. This supports the 100% positive result of Beta-lactamase test.Resistance percentage for other antibiotics were 68% for CAZ, 64% for TM, 62% for T, 60% for E, 50% for CRO, 48% for CTX, KF, and S, 46% for L, 42% for RA, 40% for DA, 36% for CIP, 34% for C, and 4% for GM while all isolates were sensitive for vancomycin).Our results are in agreement with that of Al-Jebouri and Mdish (2013) that found that *S. aureus* isolates from patients with urinary tract infections were highly resistant to ampicillin and amoxicillin. The results of Al-Ugaili *et al.* (2014) showed that (77%) of isolates were oxacillin-resistant *Staphylococcus aureus* and exhibited multiple resistances to other tested antibiotics wich is close to our results.AL-Marjani *et al.* (2015) reported that the resistance patterns of *S. aureus* were: for levofloxacin (20 %), for norfloxacin (16 %), for ofloxacin (18 %), for ciprofloxacin (16 %), for lomofloxacin (14%) and for nalidixic acid (50 %), while the results of Al-Azzawi and Flayyih (2014) revealed that (8.10%) of *S. aureus* isolates were amikacin resistant, (100%) of isolates were amoxicillin resistant ,(86.48 %) of isolates were ampicillin resistant ,(54.05 %) were resistant to (cephotaxim, erythromycin, tetracycline), (21.62%) ofisolates were methecillin resistant (MRSA) ,

and (10.81%) were vancomycin resistant. Nurjadi *et al.,* (2014) found that *S. aureus* isolates from Africa were: 54% resistant for trimethoprim, 21% for sulfamethoxazole and 19% for trimethoprim/sulfamethoxazole, while Juayang *et al*., (2014) reported that a total of 94 cases from 2010 to 2012 were diagnosed to have *S. aureus* infection using conventional bacteriologic methods. From these cases, 38 (40.6%) were identified as MRSA and 37 (39.4%) were inducible clindamycin resistant. In England, surveillance of surgical site infections has been running since 1997. During the 5 year period between January 2003 and December 2007, at least one causative microorganism was reported for 77% of surgical site infections. The most common organism was *S. aureus* (accounting for 38% of surgical site infections), of which 64% were MRSA . However, between October 2008 and September 2009, the proportion of *S. aureus* isolates (accounting for 31% of surgical site infections) that were methicillin resistant decreased to 32%. This decrease in surgical site infections due to MRSA in England appears to mirror the decline in MRSA bacteremia (Johnson, 2011).The antimicrobial agents are losing their efficacy because of the spread of resistant organisms due to indiscriminate use of antibiotics, lack of awareness, patient non compliance and unhygienic condition. It is the need of the time that antibiotic policies should be formulated and implemented to resist and overcome this emerging problem. Every effort should be made to prevent spreed of resistant organisms. There are multiple factors, which contribute to the global spread of resistance. Decreasing unnecessary antibiotic use, with narrow spectrum agents, improving compliance with therapy, decrease in use of antibiotic in animal and agriculture, and improving infection control all have a role in confronting this problem (Gupta *et al.,* 1993).

Table.1 Resistance percentage of *S. aureus* to antibiotics.

Antimicrobial agent	Antibiotics	% of resistance	No. of resistant isolates
Amoxicillin	AMC	100	50
Amoxicillin+ clavulanic acid	AP	100	50
Ampicillin	AX	100	50
Cefotaxime	C	34	17
Ceftazidime	CAZ	68	34
Ceftriaxone	CIP	36	18
Cephalothin	CRO	50	25
Chloramphenicol	CTX	48	24
Ciprofloxacin	DA	40	20
Clindamycin	E	60	30
Erythromycin	GM	4	2
Gentamicin	KF	48	24
Lincomycin	L	46	23
Methicillin	ME	92	46
Penicillin G	PG	100	50
Rifampin	RA	42	21
Streptomycin	S	48	24
Tetracyclin	T	62	31
Trimethoprim	TM	64	32
Vancomycin	VA	0	0

Beta-lactamase production test

β-Lactamase production is common, is under plasmid control, and makes the organisms resistant to many penicillins (penicillin G, ampicillin, ticarcillin, piperacillin, and similar drugs). The plasmids are transmitted by transduction and perhaps also by conjugation (Brooks *et al.,*2013).The results of the present study showed that all *S. aureus* isolates were positive for the β-Lactamase test. Our result is in agreement with that of Mahmood (2013), and close to that of Avison and Simm (2002) and Taha (2009). Our results disagree with that of Hussein (2010) who mentioned that the percentage of β-lactamase production by Staphylococci spp. was 54.8%.Fukatsu *et al.,* (1990) reported that 81.3% of *S. aureus* isolates were β-lactamase producers in Japan. Petinaki *et al.,* (2001) reported that 79.6% of *S. aureus* isolates, 84.2% of *S. epidermidis,* 95% of CoN Staph. were β-lactamase positive. Sanaa *et al.,* (2006) found that the incidence of β-lactamase production by Gram-positive cocci was 96%, while Al-Ruaily and Khalil (2011) reported that β-lactamase production within *S. aureus* isolates was 86%. The differences in the percentage of β-lactamase production may be due to the differences in the Staphylococci spp. that was mentioned earlier in addition to the differences in the types of samples and the cities

where the isolates were collected from.An iodometric method was used to determine the ability of *S. aureus* to produce Beta-lactamase-penicillinase that breaks a bond in the beta-lactam ring of penicillins resulting in an ineffective molecule called penicilloic acid. Penicilloic acid reduces the iodine to iodide and prevents it from combining with starch, therefore a white color was appeared which indicated the ability of an organism to produce β-lactamase (Wei-wu *et al.,* 1999).

Detection of *mecA* gene in *S. aureus* isolates

All isolates were analyzed by PCR to detect the presence of *mecA* gene using forward and reverse primers described by Pournajaf *et al.,* (2013) and *Bioneer* master mix. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel and staining with ethedium bromide. The results showed that 31 isolates (62%) were harboring the *mecA* gene (533 bp), while 19 isolate (38%) were lacking the *mecA* gene(figure 1).These results shows contrast with the results of disc diffusion method for methicillin, 92% of isolates were resistant to methicillin while only 62% of it were harboring *mecA* gene. This means that the methicillin resistance mechanism in *mecA*-positive isolates was due to the production of PBP2a by *mecA* gene.

Differing levels of *mecA* gene expression of methicillin resistance, occurring every 10^4 or 10^6 cells and the absence of penicillinase plasmid, which otherwise plays an important role in the stability and phenotypic expression of the *mecA* gene (Hiramatsu, 1990) may be the cause of the differences between *mecA* gene presence and it's phenotypic expression and vice versa. Methicillin resistance is either due to expression of *mecA* gene or the synthesis of methicillinase or due to both (Khan *et al.,* 2007). In contrast to our results Shrestha *et al.,* (2002) reported that 24 (100%) isolate of methicillin resistant *S. aureus* were *mecA* positive and 10 (100%) of methicillin susceptible *S. aureus* were *mecA* negative, they found that PCR assay for the *mecA* gene was 100% sensitive and 100% specific for detecting methicillin resistance in *S. aureus.*Wielders *et al.,* (2002) mentioned that the *mecA* gene, which lies in the SCC*mec* resistance island (Hiramatsu *et al.,* 2001), is carried by 95% of the isolates that display a phenotype of methicillin resistance and was detected in all multi-resistant *S. aureus* isolates. Forty nine (100%) *S. aureus*, 72 (94.7%) *S. epidermidis,* and 14 (70%) CoN Staphylococcus isolates were *mecA* gene positive as Petikani *et al.,* (2001) reported.Al-Ruaily and Khalil (2011) in their study on detection of *mecA* gene in methicillin resistant *S. aureus* (MRSA) at prince A/Rhman Sidary Hospital, Al-Jouf, Saudi Arabia, they found that only 15 from 100 *S. aureus* isolates were methicillin resistant.The results of AK*et al.,* (2012)indicated that 94% of the 35 MRSA samples were *mecA* gene positive whereas 6% samples were *mecA* negative by PCR method, while Mahmood (2013) found that only 24 (57.14%) from total 42 *S. aureus* isolates showed *mecA* gene positive. In spite of the general agreement that PCR assay is the gold standard for detection of different genes, however molecular assays for the detection of resistance have a number of limitations. New resistance mechanisms may be missed, and in some cases the number of different genes makes generating an assay too costly to compete with phenotypic assay (Fluit *et al.,* 2001).Disk diffusion method is providing a fast,

inexpensive, and easy to perform way for antibiotic resistance profiling. It reveals not only whether an isolate is resistant to a specific agent, but also which other agents it is susceptible to.

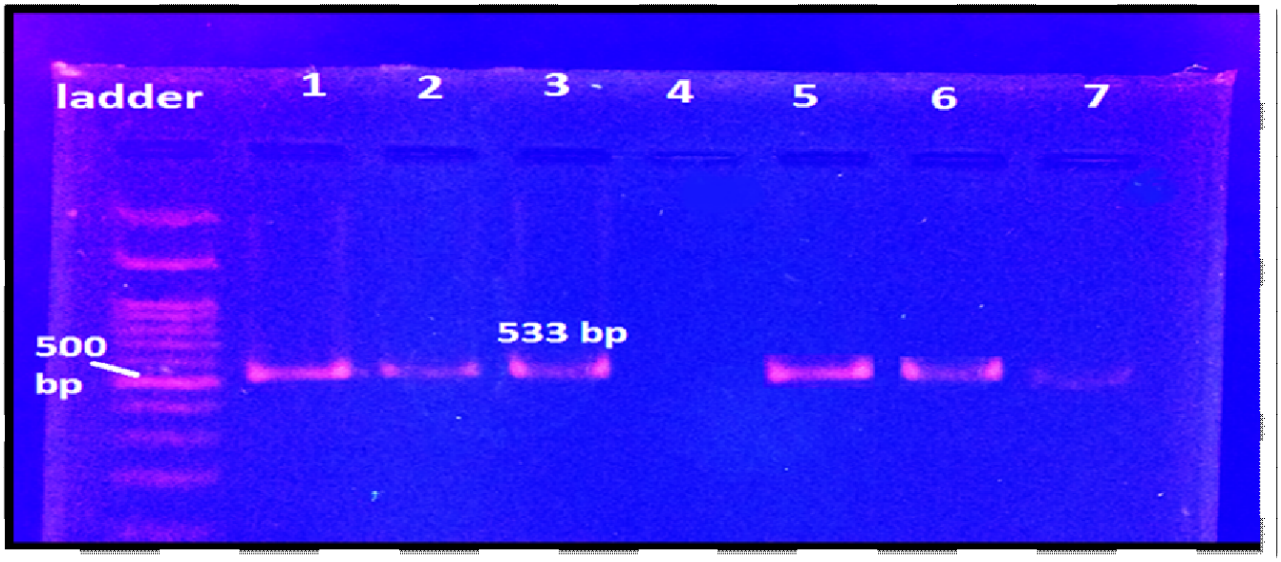

Figure.1 PCR amplification of *mecA* gene in seven isolates of *S. aureus*.PCR amplification of *mecA* gene for isolate number 1, 2, 3, 5, 6, and 7 were *mecA*-positive while isolate number 4 was *mecA*- negative.

Biofilm formation

1.Congo red agar method (Slime layer production)

Forty seven isolates (94%) of *S. aureus* gave positive results for slime production on Congo red agar. Pigmented colonies were considered as slime producing isolates, whereas non-pigmented colonies (red) were classified as non-slime producing isolates (figure 2). Congo red binds to exopolysaccharides that leads to the production of black colonies. Eighteen isolates (36%) were strong biofilms formers that appear as very black colonies, nineteen isolate (38%) were moderate biofilm formers that appear as black colonies (sometimes with a clear zone around the colonies), ten isolates (20%) were weak biofilm formers that appear as a dark red colonies (maroon), and three isolates (6%) were considered as non-biofilm producers that appear as pale red colonies (close to pink) as shown in table (2). Congo red stain was chosen because it has been used as a stain for showing the presence of the exopolysaccharide (Freeman *et al.,* 1989). Congo red binds to exopolysaccharide lead to produce black colonies. The exopolysaccharide may vary in chemical and physical properties, but it is primarily composed of polysaccharide. Some of these polysaccharide are neutral or polyanionic. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates confirms the anionic property (Sutherland, 2001). This property is important because it allows association of divalent cations such as calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm (Lens *et al.,* 2003). Congo red agar method show increasing number of positive cases by increasing time of incubation this result is in agreement

with that of (Oliveria *et al.,* 2007) and this is due to that not all staphylococcus spp. are heavy biofilm producer, some isolate are moderate and weak (Eftekhar and Dadaei, 2010), and longest time of incubation give largest chance to these isolate to produce the stain.

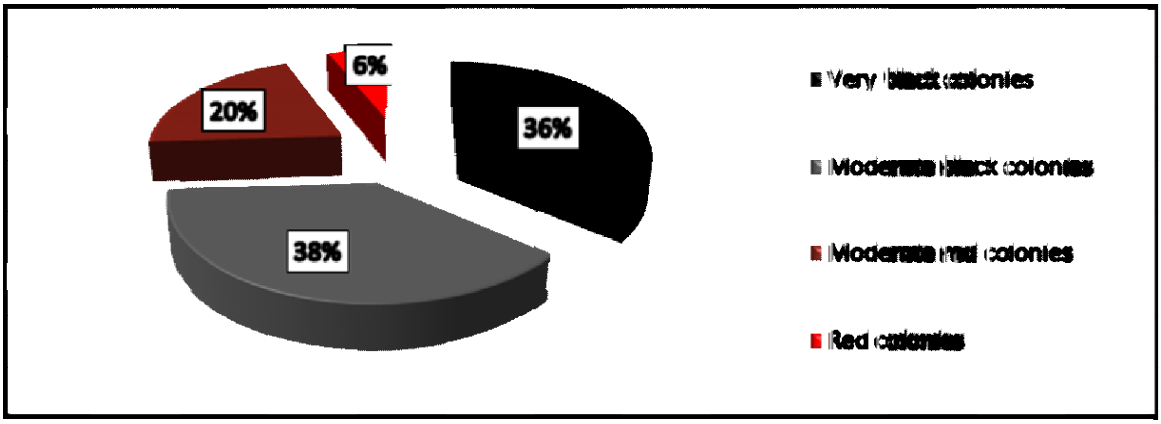

Figure (2): Percentage of biofilm formation on Congo red agar.

Table (2): Number and percentage of isolates for detection of biofilm by Congo red agar method.

Slime production	No. of isolates	% of isolates
Very black colonies (strong biofilm)	**18**	**36**
Moderate black colonies (moderate biofilm)	**19**	**38**
Moderate red colonies (weak biofilm)	**10**	**20**
Red colonies (non-biofilm former)	**3**	**6**
Total	**50**	**100**

Arciola *et al.,* (2002) reported that 57.5% of *S. epidermidis* strains were slime-forming on CRA while Mariana *et al.,* (2009) reported that 78% of MRSA strains exhibited black pigmentation. Zmanta r*et al.,* (2010) showed that 56.5% of the clinical *S. aureus*isolates were slime producers developing almost black or very black colonies on CRA plate. Oliveria and Cunha (2010) reported that 73% of their CoN Staph. were positive for biofilm formation by this method. Fiadh (2011) mentioned that biofilm formation by CoN staph was 29% after 24h. of incubation and increased to 41% after 48h. of incubation, while biofilm formation by this method remained 25% after 24 and 48 hours of incubation regarding the coagulase positive Staph. Nasr *et al.,* (2012) reported that 45.5% of their *S. aureus* isolates were biofilm formers on CRA plates, while Namvar*et al,* (2013) found that 65% of their *S. aureus* isolates formed biofilms by CRA method. Kaiser *et al.,* (2013) reported that under aerobic incubation at 35 °C for 24 h, 39 (51.3%) *S. epidermidis* strains that were carrying the *icaAB* genes produced biofilm, showing colonies with colors ranging from brown to black. Rewatkar and Wadher (2013) reported that out of 60 isolates, CRA method detected 54 as high biofilm producer. Mustafa (2015) found that 100% of *A. sobria* gave

positive results for slime layer in Congo red agar method. The Congo red agar (CRA) method has many advantages like providing a rich nutritional materials that induce the slime layer production as it consist of brain- heart infusion agar and 5% sucrose, and by this method the colonies remain viable on the medium and we can choose the slime producer ones and keep it for further analysis.

2. Microtiter plate (MTP) assay (Tissue culture plate assay "TCP")

The ability of *S. aureus*isolates to produce biofilms were evaluated quantitatively by using microtiter plate assay. A pre-sterilized, flat bottom, 96-well, polystyrene microtiter plates were used and then the absorbance was determined by an ELISA reader at 490nm, absorbance reading represented the degree of biofilm thickness that adhere to the surface of the microtiter wells. All isolates were positive for biofilm production by this method.A reading greater than 0.12 was regarded as biofilm positive sample (Namvar*et al.,* 2013). The mean absorbance values was calculated to obtain more accurate results, as shown in table (3).

Table (3): Mean of absorbance values at 490 nm for *S. aureus* isolates.

Isolate number	Mean of O.D	Isolate number	Mean of O.D	Isolate number	Mean of O.D
1	0.43	18	0.90	35	0.92
2	0.34	19	0.41	36	0.60
3	0.33	20	0.51	37	0.90
4	0.35	21	0.60	38	0.81
5	0.26	22	0.52	39	0.93
6	0.35	23	0.90	40	1.60
7	0.31	24	0.50	41	0.70
8	0.25	25	0.53	42	0.65
9	0.20	26	0.61	43	0.50
10	0.27	27	0.70	44	0.44
11	0.24	28	0.74	45	0.60
12	0.30	29	0.70	46	0.63
13	0.32	30	0.91	47	0.75
14	0.52	31	0.82	48	0.90
15	0.49	32	0.90	49	0.65
16	0.58	33	0.93	50	0.54
17	0.40	34	0.80		

Of the *S. aureus* strains, 57.1% displayed a biofilm-positive phenotype under optimized conditions in the MTP test as Knobloch*et al.,* (2002) reported. Mathur*et al.,* (2006) reported that 88(57.8%) displayed a biofilm-positive phenotype under the optimized conditions in the MTP method. While 81% of CoNStaphylococcus was

positive for biofilm formation by this method as Oliveria and Cunha (2010) mentioned. Hassan *et al.,* (2011) considered MTP method to be superior to TM and CRA. From the total of 110 clinical isolates, TCP method detected 22.7% as high, 41% moderate and 36.3% as weak or non-biofilm producers.Fiadh (2011) mentioned that biofilm formation in Coagulase negative staphylococci by MTP method was 59% and increased to 76% with glucose addition to the medium. While Biofilm formation by Coagulase positive staphylococci was 75% and increased to 87% when glucose was added to the medium. Nasr *et al.,* (2012) reported that 46% of their 50 staphylococcal isolates were biofilm formers by this method with different intensities. Sixty eight percent of *S. aureus*isolates were biofilm formers as Ghellai*et al.,* (2014) mentioned, while Namvar*et al.,* (2013) showed that 58% of their *S. aureus* were biofilm formers by this method. Mustafa (2015) found that 2.38% of *A. sobria* were strong biofilm formers by MTP method, 73.80% were moderate, and 23.80% were weak or non-biofilm formers.The biofilm mode of life, besides providing community level resistance, can also promote cellular level resistance. Biofilms have a greatly enhanced mutation rate (up to 100 times higher than planktonic cells) which inevitably leads to faster development of antibiotic resistant mutants (Fridman*et al.,* 2014). Moreover, the close proximity of various microbial organisms within biofilm aggregates and the abundance of exogenous DNA (eDNA) likely facilitate horizontal gene transfer and acquisition and spread of resistance determinants. Indeed, it has been shown that biofilms may constitute specific foci of genetic adaptation and evolution, leading to the selection of subpopulations with a greater ability to acquire antibiotic resistance (Conibear*et al.,* 2009) and the horizontal acquisition of exogenous DNA (Macía*et al.,* 2005). Biofilms promote the acquisition and exchange of integron gene cassettes, many of which encode antibiotic resistance (Koenig *et al.,* 2011). Biofilms in animal digestive systems, aquatic environments, the rhizosphere and phyllosphere also promote conjugation and natural transformation (Taylor *et al.,* 2011). Basal rates of bacterial evolution are thus accelerated in biofilms, especially when exposed to sub-inhibitory concentrations of antibiotics (Gillings and Stokes, 2012). Because the barrier effect of the biofilm matrix can significantly decrease the penetration of drugs, the resulting sub-inhibitory concentration of antibiotics in parts of the biofilm creates favorable conditions for selection of resistant phenotypes, without the cells being exposed to lethal levels of the antibiotic. Furthermore, exposure to sub-inhibitory antibiotic concentrations induces increased rates of mutation, recombination and lateral transfer (Gillings and Stokes, 2012).

Detection of *icaAD* gene among *S. aureus* isolates using PCR technique

The ability of *S. aureus* to form biofilms helps the bacterium to survive in hostile environment within the host, and is considered responsible of chronic and persistent infections (Costerton *et al.,* 1999; Götz, 2002). Several studies have shown that formation of slime and biofilm in *S. aureus*and *S. epidermidis* causing catheter associated and nosocomial infections is associated with the presence of *icaA*and *icaD*genes (Arciola *et al.,* 2001; Vogel *et al.,* 2000). Co-expression of these genes is necessary for the full phenotypic expression of biofilm in clinical Staphylococcal

isolates (Arciola *et al.,* 2001; Satorres and Alcaraz, 2007; Gad *et al.,* 2009; Vogel *et al.,* 2000). In the present study, all *S. aureus* isolates were tested for the presence of *icaAD gene* by the use of forward and reverse primers as described by Nasr *et al.*, (2012) and *Bioneer* master mix. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel and staining with ethedium bromide,(figure 3). The results showed that all but three isolates (94%) were harboring the *icaAD* gene and a 407 bp band was obtained.

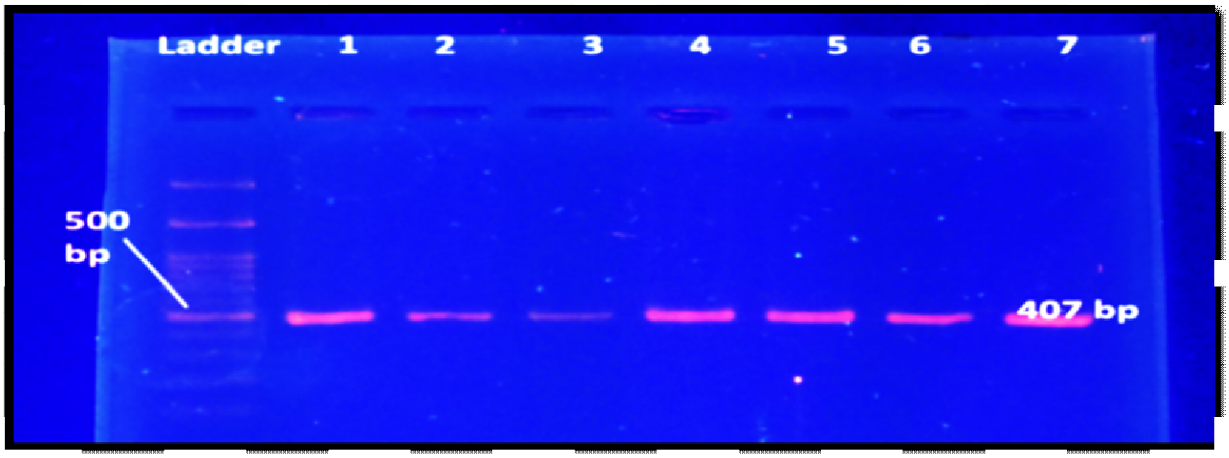

Figure (3): Gel electrophoresis for amplification of *icaAD* gene.

The results of the current study is close to that of Yazdani *et al.,* (2006) that mentioned that all of their isolates carried the *icaAD* gene. Our results disagree with that of Nasr *et al.,* (2012) which detected *icaAD* gene in only 32% of their Staphylococcal isolates while 68% did not possess such gene. Namvar *et al.,* (2013) detected *icaD* gene in all of their 60 isolates. Arciola *et al.,* (2001) reported that 61% of *S. aureus* strains were *icaA* and *icaD* positive. Aricola *et al.,* (2002) reported that 57.5% of all the strains were found to be *icaAD*-positive. The study of Knoblock *et al.,* (2002) found that in 128 *S. aureus* strains no *icaADBC*-negative strain was detected by a *icaA*- specific PCR, indicating that all *S. aureus* strains harbor this gene locus. Cramton *et al.,* (1999) investigated a variety of *S. aureus* strains and found that all strains tested contain the *ica* locus and that several can form biofilms in vitro. Correlating the phenotypic biofilm production methods with the presence of *icaAD gene,* all biofilm producing isolates by phenotypic methods were positive for *icaAD* and this result is the same as that of Aricola *et al.,* (2001) and Gad *et al.,* (2009) where all staphylococcal biofilm producing strains were positive for *icaA* and *icaD.* Oliveira and Cunha (2010) found that the *icaA* and *icaD* genes were detected concomitantly in 40 (40%) of the 100 CoN Staph. isolates. Fifty-seven percent of all the examined strains were found *icaA/icaD*-positive as Arciola*et al.,* (2005) reported. Arciola *et al.,* (2001) reported that all the saprophytic strains of *S. epidermidis* and *S. aureus* turned out to be negative for both *icaA* and *icaD* and also non-slime forming. Two *S. aureus* and one *S. epidermidis* strain from catheters, detected as *icaA* and *icaD* positive by PCR analysis. However, other studies demonstrated that the presence of *ica* genes did not always correlate with biofilm formation as reported by De Silva *et al.,* (2002) where only 59% of *S. epidermidis* strains positive for ica operon were biofilm producers by CRA method. Cafiso *et al.,* (2004) demonstrated

that 83.3% of the *ica*-positive isolates produced biofilm by both CRA and MTP method. According to Oliveira and Cunha (2010) and Cho *et al.,* (2002), the expression of the *ica* genes is highly variable and can be induced by variations in the culture conditions, such as an increase in the concentration of sugars or other substances that induce stress. Mathur *et al.,* (2006) also obtained better results when the glucose concentration of TSB was increased to 1% and the period of incubation was prolonged to 24 h. The addition of large amounts of sugar to a medium colonized with CoN Staph. induces a stress condition which, in turn, stimulates fermentation, thus increasing the production of PIA and consequent biofilm production (Vuong and Otto, 2002).Some researchers attributed the absence of biofilm production in some staphylococcal isolates despite the presence of *ica* operon to the insertion of a 1332-bp sequence element, known as IS256, in *icaA* causing its inactivation. Six percent of the study isolates were *icaAD* negative and produced biofilm by phenotypic methods, this may be due to the presence and expression of other genes that mediate biofilm formation like *lgrAB*, *sdrC*, and *sspBCP* as Shin *et al.,* (2013) demonstrated. The accumulation-associated protein (*aap*) gene (Rohde *et al.,* 2005) and Bap homolog protein (*bhp*) gene (Tormo*et al.,* 2005), and newly identified genes (*sdrC*, *sspBCP*) by RT-PCR. These genes were found to induce an alternative PIA-independent mechanism of biofilm formation. Ando *et al.,* (2004) investigated the presence of several virulence determinants by polymerase chain reaction assay and found eight determinants (*tst, sec, hla, hlb, fnbA, clfA, icaA,* and*agrII*) to be predominant among MRSA isolates. They reported that enhanced biofilm formation was confirmed in *hla*-, *hlb- and fnbA*-positive MRSA isolates, both individually and in combination.The *ica* operon, which encodes a polysaccharide intercellular adhesion, is currently the best understood mediator of biofilm development (Cramton*et al*., 1999); however*, ica*-independent biofilm development, biofilm-associated protein (Bap) and the *S. aureus* surface protein (SasG) have all been implicated in biofilm development and regulation (O'Neill *et al*., 2007).

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پوختە

ڕێگای بەرهەڵستی دژە زیندەگیەکان و دروست کردنی زیندە توێژال لە بەکتریای نەخۆشی ووشیە زێرینەکان

لەم لێکۆلینەوەدا سەدو بیست نمونە کۆکرایەوە لەو نەخۆشانەی کە سەردانی نەخۆشخانەی هەولێری ڕۆژئاوا، نەخۆشخانەی فریاکەوتن لە گەل نەخۆشخانەی رزگاری فێرکاری لە ماوەی نێوان 1 ئادار بۆ 20 مایۆی 2015. نمونەکان کۆکرانەوە لە سەرچاوەی نەخۆشی جیاواز کە بریتی بوو لە 50٪ لە سووتان، 32٪ لە برینی نەشتەرگەری، 10٪ لە کلۆر بوونی ددان وە 8٪ لە نمونەی میز. جیاکراوەکان دەست نیشان کران بە بەکارهێنانی سیفاتی کێلگەیی، ڕووالەتی، تێستی بایۆکیمیائی پاشان تەئکید کرا بە ئامێری ڤایتیک 2. پەنجا جیاکراوە دەست نیشانکرا وەک *Staphylococcus aureus*. تێستی دژە زیندەکی بەبەکارهێنانی ڕێگای بلاوبوونەوەی پەبکەکان بۆ هەموو جیاکراوەکان ئەنجام درا بەبەکارهێنانی بیست دژە زیندەکی کە بە بلاوەی بەکاردەهێنرێت وە ڕێژەی بەرگری بەم جۆرە بوو: 100٪ بۆ AMC، AP،AX و PG، 92٪ بۆ ME، 68٪ بۆ CAZ، 64٪ بۆ TM، 62٪ بۆ T، 60٪ بۆ E، 50٪ بۆ CRO، 48٪ بۆ CTX، KF و S، 46٪ بۆ L، 42٪ بۆ RA، 40٪ بۆ DA، 36٪ بۆ CIP، 34٪ بۆ C وە 4٪ بۆ GM، هەموو جیاکراوەکانیش هەستیار بوون بۆ ڤانکۆمایسین.هەموو جیاکراوەکان تێست کران بۆ دەردانی ئەنزیمی بێتا لاکتمێز وە ئەنجامەکان دەریانخست کەوا هەموو جیاکراوەکان توانای دەردانی ئەنزیمیان هەیە. تەکنیکی PCR بەکارهات بۆ دۆزینەوەی جینی mecA لە جیاکراوەکانی S. aureus ئەنجامەکان دەریانخست کەوا 62٪ لەم جیاکراوانە ئەم جینەیان تێدایە بەلاّم 38٪ لە جیاکراوەکان نێگەتیڤ بوون بۆ ئەم جینە. هەموو جیاکراوەکان تێست کران بۆ دروست کردنی زیندە توێژال لە ناو تێست تیوب بەبەکارهێنانی دوو ڕێگا ئەویش ڕێگای کوتگۆرید و قاپی مایکرۆتایتر. ڕێژەی دروست بوونی زیندە توێژالەکان 94٪ و 100٪ بوو یەک لە دوای یەک. تەکنیکی PCR بەکارهات بۆ دۆزینەوەی جینی icaAD لە S. aureus وە ئەنجامەکان دەریانخست کەوا 94٪ جیاکراوەکان هەڵگری ئەم جینەن وە 6٪ هەڵگر نەبوون بۆ ئەم جینە.

الخلاصة

نمط مقاومة المضادات الحيوية وتكوين الغشاء الحيوى فى بكتريا العنقوديات الذهبية المرضية

في هذه الدراسة تم جمع مئة و عشرين عينة من المرضى اللذين دخلوا مستشفى طوارئ غرب أربيل، مستشفى الطوارئ، و مستشفى رزكَاري التعليمي خلال المدة ما بين 1\3\2015 الى 20\5\2015. العينات المجموعة كانت من المصادر التالية: 50٪ من الجروح، 32٪ من جروح العمليات، 10٪ من تسوسات الأسنان، و 8٪ من عينات البول. تم تشخيص العينات عن طريق الزرع و الفحص المجهري للبكتريا و الفحوصات الكيموحيوية، و تم التأكد من التشخيص باستخدام جهاز الVITEK. خمسون عينة تم تشخيصها على إنها من المكورات العنقودية الذهبية. تم إجراء اختبار حساسية العزلات البكتيرية للمضادات الحياتية بطريقة الأقراص حيث استُخدم عشرون نوع من المضادات الحيوية شائعة الاستخدام في هذا الاختبار وكانت النسب المئوية للمقاومة كما يلي : 100٪ لكل من AMC، AP، AX و PG. 92٪ ل ME، 68٪ ل CAZ، 64٪ ل TM، 62٪ ل T، 60٪ ل E، 50٪ ل CRO، 48٪ ل CTX، KF و S، 46٪ ل L، 42٪ ل RA، 40٪ ل DA، 36٪ ل CIP، 34٪ ل C و 4٪ ل GM، وقد كانت جميع العزلات كانت حساسة لمضاد الفانكومايسين VA . تم اختبار قابلية العزلات على انتاج إنزيم البيتا لاكتاميز و كانت جميعها منتجة لهذا الإنزيم. استُخدمت تقنية تفاعل البلمرة المتسلسل (PCR) للكشف عن وجود جين الـ*mecA* في عزلات بكتريا المكورات العنقودية وقد أظهرت النتائج أن 62٪ من العزلات كانت تمتلك هذا الجين، في حين ان 38٪ كانت غير حاوية على هذا الجين. كذلك تم اختبار جميع العزلات من حيث قدرتها على انتاج الغشاء الحيوي خارج الجسم الحي (*in-vitro*) باستخدام طريقتين، الأولى كانت باستخدام اكَار الكونكَو الاحمر أما الثانية فكانت باستخدام اطباق العد الدقيق (microtiter plates) وكانت النسبة المئوية لإنتاج الغشاء الحيوي 94٪ بالطريقة الاولى و 100٪ بالطريقة الثانية. تم الكشف عن وجود جين الـ*icaAD* في جميع العزلات و اظهرت النتائج أن 94٪ من العزلات كانت حاملة للجين في حين أن 6٪ فقط كانت لا تمتلك هذا الجين.