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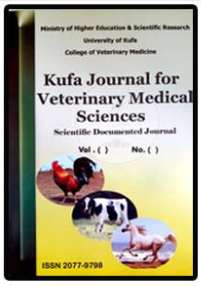

Detection Of Intracellular Adhesion Gene (*Icaa* And *Icad*) And Biofilm Formation *Staphylococcus Aureus* Isolates From Mastitis Milk Of Cow

Hasan I. Idbeis Mohammed H. Khudor

Department of Microbiology, College of Veterinary Medicine ,University of Basrah, Basrah,Iraq

Email(haldriee@uowasit.edu.iq)

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Abstract

In the present study, a total of 75 mastitis milk samples were collected from cow and were analyzed for the presence of *S.aureus*. The obtained results indicate that this bacterium observed in 26.66%. The study of antibiotic susceptibility test to 9 different antibiotics showed that *S. aureus* was 100% resistant to penicillin and 100% sensitive to Vancomycin, Gentamycin , Clarithromycin and Chloramphenicol . Whereas for cefoxitin (alternative to methicillin) resistance was 50%.There were a variable resistance percentage for the rest of antibiotics: Tetracycline (30%), Ciprofloxacin and Clindamycin (22%). The biofilm-forming ability of *S. aureus* was evaluated via microtiter plates and the result revealed that, all the studied isolates were either moderate biofilm producer or weak biofilm producer while the non-biofilm producer and strong biofilm producer were not detected among the tested isolate. PCR analysis was applied to DNA extracted from *S.aureus* isolates from milk samples .The results of PCR assay revealed that all *S.aureus* isolates gave positive results for both *icaA* and *icaD* genes (100%) with Product size 151 and 211 bp, respectively.

Results of this study indicate that biofilm producing *S.aureus* have a major role player on the occurrence of bovine mastitis in addition, there were high prevalence of MRSA isolates (50%) in mastitis milk at the study area.

Keywords: *Staphylococcus aureus*, Mastitis, Biofilm, *icaA* and *icaD* gene, antibiotic resistance

الكشف عن جينات الالتصاق الداخل خلويه وتكوين الغشاء الحيوي في جرثومة المكورات العنقوديه المعزوله من حالات التهاب الضرع في الابقار

حسن اكريم ادبيس محمد حسن خضر

الخلاصه

تم في هذه الدراسة جمع 75 عينة من من الابقار المصابه بالتهاب الضرع من مناطق مختلفه من جامعة البصره . تم تحليل هذه العينات لغرض التحري عن وجود بكتريا المكورات العنقوديه الذهبيه واظهرت النتائج ان هذه البكتريا لوحظت بنسبة 20٪ من مجموع هذه العينا أظهرت دراسة اختبار الحساسية للمضادات الحيوية على 9 مضادات حيوية مختلفة أن بكتيريا المكورات العنقوديه كانت مقاومه 100٪ للبنسلين و 100٪ حساسة للفانكوميسين ، جنتاميسين ، كلاريثروميسين و الكلورامفينيكول في حين كانت المقاومة للسيفوكسيتين (بديل للميثيسيلين) 50٪ .وكانت نسبة المقاومة متغيرة لباقي المضادات الحيوية كمايلي: ، تتراسيكلين و الكليندامايسين (30 ٪) ، سيبروفلوكساسين (25 ٪) تم تقييم مقدرة المكورات العنقودية الذهبية على تكوين الغشاء الحيوي عن طريق (microtiter plates)وكشفت النتيجة أن جميع العزلات المدروسة كانت إما منتجه للغشاء الحيوي

بصوره معتدله او ضعيفه ولا وجود للعزلات الغيرمنتجه او المنتجه بصوره قويه للغشاء الحيوي .تم استخلاص الحمض النووي من جميع عزلات المكوورات العنقوديه الذهبيه واستخدم اختبار تفاعل البلمرة المتسلسل من اجل التحري عن الجينات المنتجه للبايوفيلم icaAو icaD وأظهرت نتائج الاختبار أن جميع العزلات أعطت نتائج إيجابية لكلا الجينين (100 ٪) (مع حجم المنتج 151 و 211 bp، على التوالي .

Introduction:

Mastitis means inflammation of the udder and is a common disease among dairy animals worldwide. It is often associated with bacterial intramammary infections (IMI) and influence milk quality and yield negatively, therefore, mastitis is of major economic concern for the farmer (1,2).

Staphylococcus aureus is generally regarded as one of the major etiologic agents of mastitis in dairy animals (3–5). This pathogen has the potential to develop resistance to almost all the antimicrobial agents used for the management of the disease (3,5,6). *S. aureus* is also well known for its tolerance to a wide range of adverse circumstances. This tolerance is related to diverse genetic capabilities including the ability to form biofilms in the host, which contributes to the resistance of this microorganism against antibiotics (7,8).

S. aureus biofilms are considered major facilitators of different animal and human infections contributing 80% of all infections (9) .The major component of *S. aureus* biofilms is an exopolysaccharide, Polyβ-1, 6-linked N-acetylglucosamine (PNAG)(10). Four proteins including *IcaA, IcaD, IcaB* and *IcaC* encoded by the *icaADBC* operon are associated with the production of PNAG. IcaA and IcaD are the most important proteins for the production of PNAG(11). Carriage of the *ica* operon is a characteristic of most clinical *S. aureus* strains (12) and Production of the extracellular polysaccharide in *S. aureus* is currently the best understood mechanism of biofilm development, this ica operon can be further differentiated to the *icaA, icaD, icaB* and *icaC* loci each responsible for relevant pathogenic and virulent factors involved in polysaccharide intercellular adhesin synthesis (13).

This study aimed to determine the isolation rate of *S. aureus* from cow mastitis cases, potential of these isolates to carriage ica operon and it is phenotypic evaluation of antibiotic susceptibility and biofilm formation.

Materials And Methods:

Samples collection

A total 75 milk sample were collected from clinical and subclinical mastitis of cow The samples were collected from different area in AL- Basra provenance after cleaning the udder by a piece of cloth then using cotton moistened by alcohol 70% and removing the first flowage of milk and collecting 10 ml in sterile tube, transported with ice box. The subclinical mastitis was confirmed with California mastitis test according to (14). From each sample, 1 ml of milk was pipetted into sterile microcentrifuge tubes and centrifuged at 5000 rpm for 5 min at room temperature. The supernatant was then discarded and the pellet was directly inoculated onto plated of mannitol salt agar(14).

***Staphylococcus aureus* isolation and identification.**

Milk samples were inoculated on mannitol salt agar and incubated for 24hrs at 37°C. All colonies from primary cultures were purified by subculture on brain-heart infusion (BHI) agar and then inoculated onto MSA and incubated at 37°C for 24 hr. (15).

Suspected colonies on mannitol salt agar were identified by coagulase test(15) , chromogenic agar (CHROMagar™ Staph

aureus) (16,17) and VITEK 2 compact system according to its manufactures instructions.

Antibiotics susceptibility test

The antimicrobial susceptibility patterns of isolates to different antimicrobial agents was determined and interpreted according to (18). Nine antibiotics were chosen for the study. The antibiotic tested were from (Bioanalyse/ Turkey), as it was shown in table (1).

Table (1): Zone diameter interpretation standards according to (18)

N0.	Antimicrobial disc	Disc concentration µg or U/dis	Zone Diameter		
			R	I	S
1	Penicillin 10 units	10 units	≤ 28	-	≥ 29
2	Cefoxitin	30 µg	≤24	-	≥25
3	Vancomycin	30 µg	-	-	≥15
4	Gentamicin	10 µg	≤12	13-14	≥15
5	Clarithromycin	15 µg	≤13	14-18	≥18
6	Tetracycline	30 µg	≤14	15-18	≥19
7	Ciprofloxacin	5 µg	≤15	16-20	≥21
8	Clindamycin	2 µg	≤14	15-20	≥21
9	Chloramphenicol	30 µg	≤12	13-17	≥18

Biofilm formation assay

Biofilm formation was assayed phenotypically by the ability of cells to adhere to the wells of 96-well microtiter plate as described by (19).

Briefly, the inoculum was prepared from bacteria grown in TSP broth, the culture was diluted 1:100 in TSB supplemented with 1% glucose, and 200 µl was poured into the wells. The negative control wells contained 200 µl of TSB supplemented with 1% glucose. The tissue culture plates were incubated for 24hours at 37°C. After incubation, the content of each well was gently removed by tapping the plates. The wells were washed 3 times with 0.2 ml of phosphate buffer saline (PBS), fixed by methanol (0.2 ml) for 20 min, dried at room temperature and finally stained with 0.1% crystal violet. The crystal violet dye bound to the adherent cells was dissolved with 200 µl 95% ethanol per well, and the plates were read at 490nm (A490) using ELISA reader. Optical density cut-off (ODc) was determined. It is defined as average OD of negative control + 3× standard deviation (SD) of negative control. Biofilm production is considered;

(Non-biofilm producer (OD < ODc), 0**)** **(**Weak biofilm producer (ODc < OD < 2×ODc), +**)** **(** Moderate biofilm producer (2×ODc < OD < 4×ODc), ++**)** **(** Strong biofilm producer (4×ODc < OD). +++**)**

Bacterial "DNA extraction" and PCR Method:

P C R technique was performed for detection ,icaA gene and icaD gene in "Staphylococcus aureus" isolated from mastitis milk samples by following steps:-

1-D N A extraction: Genomic DNA of *S.aureus* isolates were extracted by using Genomic DNA Kit (Geneaid . U S A) and according to manufacturing instructions

2-Nano drop: The extracted DNA was estimated by "nanodrop device" at 260 /280 n m, and then kept at deep freezer until used in P C R method.

3-Primers: The PCR primers that used in this study for detection *icaA* and *icaD* genes were design by (20).These primers were provided by (Bioneer company, Korea) as in the Table (2).

Table (2): Primers for amplification *icaA* and *icaD* genes .

Primer	Sequence		Product size (bp)
***icaA* gene**	**F**	**5-GAGGTAAAGCCAACGCACTC-3**	**151**
	R	**5-CCTGTAACCGCACCAAGTTT-3**	
***icaD* gene**	**F**	**5-ACCCAACGCTAAAATCATCG-3**	**211**
	R	**5-GCGAAAATGCCCATAGTTTC-3**	

4- The "PCR master mix preparation"The reaction mixture was prepared by adding 1µl of both forward and reverse of the primers specific for the each gene, 3µl of DNA template to AccuPower ® PCR PreMix(20 µl reaction volume) and the volume was completed to 20 µl by adding nuclease free water. After that, all the P C R tubes transferred into "vortex centrifuge" for 3 minutes. Then transferred into thermo cycler (Bioneer. Korea).

5- PCR thermo cycler conditions:-

Table (3): Table (3) PCR thermo cycler conditions

Step	Temperature, °C	Time	Cycle
Initial denaturation	95	5 min	1
Denaturation	95	20 s	40
Annealing	60	20 s	
Extension	72	20 s	
Final extension	72	3min	1

6- P C R product analysis: The P C R products (151 b p and 2011 b p) were examined by electrophoresis in a 1.5% "agarose gel" using "1X TBE buffer", stained with "ethidium bromide", and conceive under "gel documentary".

Results:

Bacterial isolation and identification

According to the results of isolation and identification there were out of 75 tested samples analyzed 20 (26.66%) were *S.aureus* positive .All *S. aureus* isolates were identified by culture of samples on mannitol salt agar , Chromogenic agar, and tested for its ability to produce the coagulase.

All *S. aureus* isolates convert the medium of mannitol salt agar from red to yellow color

(fig.1.A), form pink to mauve colonies on chromogenic agar (fig.1.B).and give positive result for coagulase test.

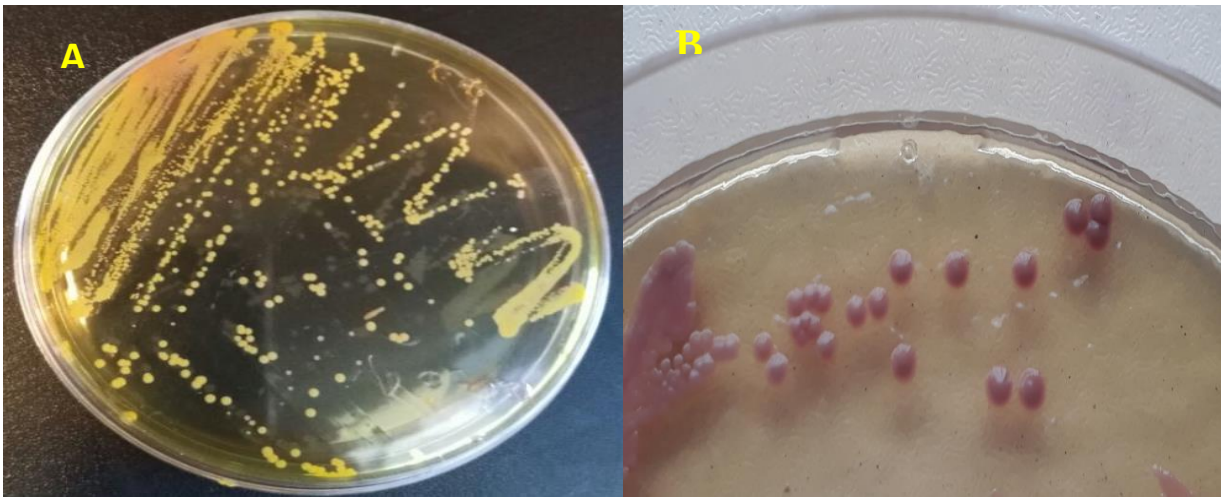

Fig .3-1: A-*S. aureus* colonies on MSA, B-*S. aureus* colonies on chromogenic agar

The identification was confirmed with automated VITEK-2 copact system using GP cards with ID massage confidence level as excellent (probability percentage from 95-99).

Antibiotics susceptibility test

After the identification of *S. aureus*, susceptibility test was performed for all *S. aureus* (20isolates) by disk diffusion method to examine 9 different antibiotics as clarified in table (4).

The results showed that, the highest resistant rate was against pencillin (100%) fallowed by cfoxitine (55%), tetracycline (30%), clindamycin (30%) and ciprofloxacin (25%), .On the other hand, all the tested isolates showed 100% sensitivity toward vancomycin, gentamycin and Chloramphenicol high sensitivity to clarithromycin (95%) . There was a significant difference among the antibiotics resistancy (P< 0.01).

Table (4).Antimicrobial susceptibility of *S.aureus* isolates from mastitic milk of cow, goat and sheep to ward nine antimicrobials.

Penicillin 10 units	30 µg cefoxitin	Vancomycin 30 µg	Gentamicin 10 µg	clarithromycin 15 µg	Tetracycline 30 µg	Ciprofloxacin 5 µg	Clindamycin 2 µg	Chloramphenicol 30 µg
R (20)100 %	R (11)55%	R (0) 0%	R (0) 0%	R (0) 0%	R (3) 15%	R (1) 5%	R(2) 10%	R (0) 0%
I (0) 0%	–	I (0) 0%	I (0) 0%	I (1) 5%	I (3) 15%	I (4) 20%	I (4) 20%	I (0) 0%
S (0) 0%	S (9) 45%	S (20) 100%	S (20) 100%	S (19) 95%	S (14) 70%	S (15) 25%	S (15) 70%	S (0) 0%

P< 0.01

Biofilm formation assay by micro titer plat.

The ability of *S. aureus* isolates to produce biofilm were evaluated by using pre-sterilized 96-well polystyrene microtiter plates and then absorbance was determined at 580 nm in an ELISA reader for the determination of the degree of biofilm formation for studied isolates that adhered on the surface of the microtiter well.

Absorbance values represented the degree of the biofilm thickness that formed by the studied isolates on the surface of the microtiter well. All *S. aureus* isolates assayed for the production of biofilm, and the results obtained are categorized into four groups based on Statistical analysis of biofilm forming capacity: weak or non-producers (OD580nm < 0.064), modrate producers (OD580nm 0.064– 0.128), strong producers (OD580nm ≥ 0.128)

The results of the present study revealed that, all the tested isolates were found to be biofilm producer at different level (fig 2).

As shown in table (5), out of a total 20 tested isolate, 8(40%) isolates were moderate biofilm producer and the remaining isolates 12 (60%) were weak producer,

Table (5): biofilm producing ability of *S.aureus* on microtiter plate.

NO.of isolates	Biofilm producer			
	None NO. (%)	Weak NO. (%)	Moderate NO. (%)	Strong NO.(%)
20	0	12(60)	8 (40)	0

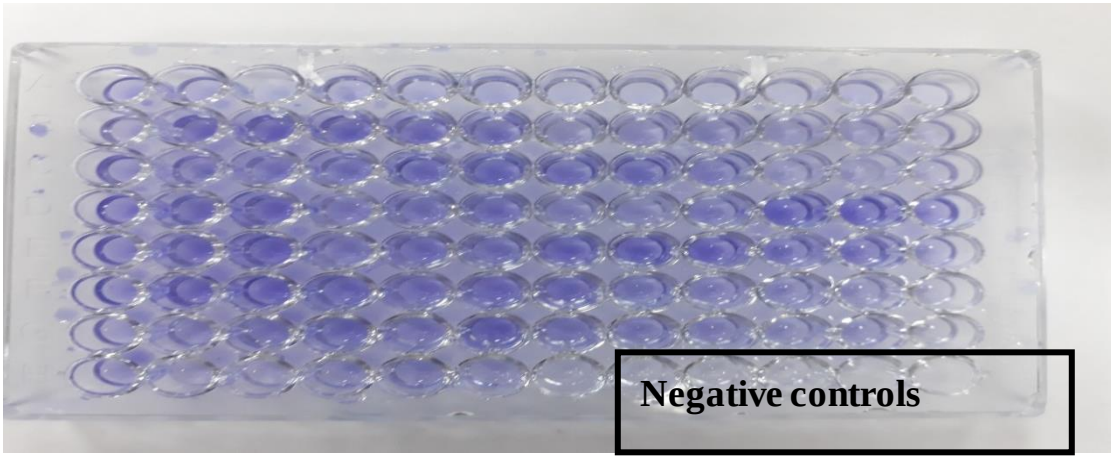

Fig 2: Biofilm formation of *S.aureus* on microtiter plate after staining with 1% crysal violet

3.7.1. Detection of *icaA* and *icaD* gene.

The PCR analysis was applied to DNA extracted from *S.aureus* isolates from milk samples and the results of PCR assay revealed that all *S.aureus* isolates gave positive results for both icaA and icaD genes (100%) with Product size 151 and 211 bp, respectively (fig 3 and 4).

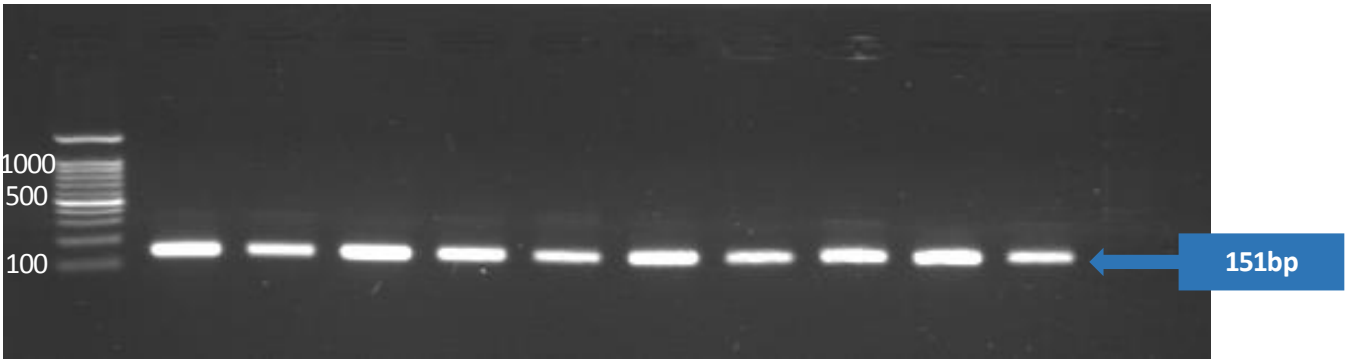

Fig 3 : Agarose gel electrophoresis of *icaA* gene amplification, where M:ladder, 11:negative control , 1-10:positive results.

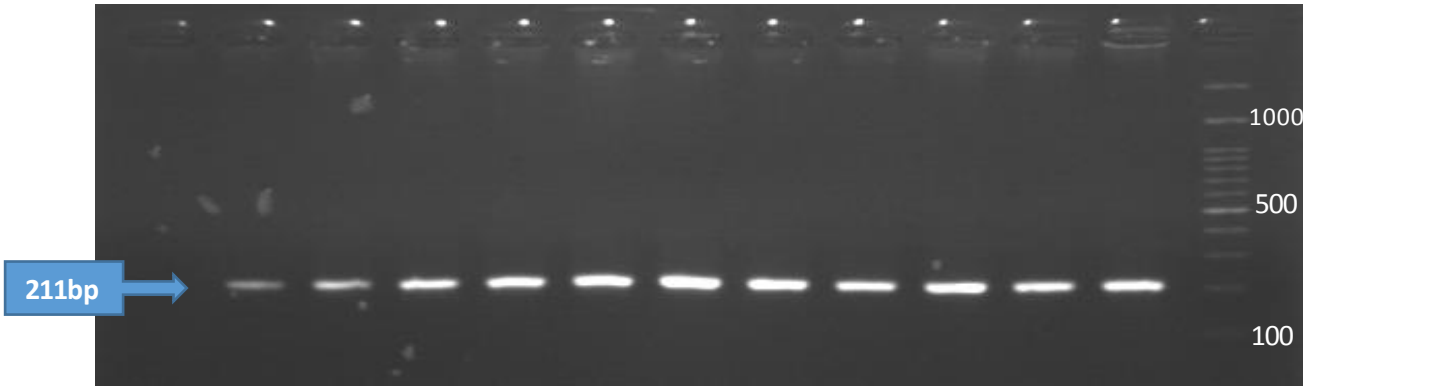

Fig 4: Agarose gel electrophoresis of *icaD* gene amplification, where M:ladder, 12:negative control , 1-11:positive results.

Discussion

Distribution of *S. aureus*

S.aureus is one of the main etiological agents of mastitis in different mammalian species (2).

Different works from different parts of the world give varying frequency of *S.aureus* isolation from mastitis milk of dairy animals , some of which agree while others disagree with the findings of the present study.

In the present study, 26.66% of mastitis bovine milk samples were positive for *S.aureus* .These results are in line with many studies such as (21) (22) (4) (23) and (24) Who recorded the isolation rate of *S.aureus* from mastitis bovine milk in 30% , 29.7%, 25.53% and 28% .On the other hand, lower ratios of *S. aureus* isolation was recorded by (25) (26) (27) (28) Who recorded the isolation rate of *S.aureus* from mastitis bovine milk in 10.16 % , 20.60% , 20.6% , 21% . respictivily. And higher rate of isolation was recorded by (29–31) Who recorded the isolation rate in ,37.5% , 44.44% and 55%, respectively.

Staphylococcal mastitis prevalence in dairy animals varies widely between different countries and may reflect the fact that different policies for infection control.

A comparison of the results of the present study and those reported by other authors is difficult because the occurrence of *S.aureus* as a causative agent of mastitis varies according to the area, handling practices of the animals and hygienic conditions during milking(32)

Antibiotic susceptibility test

All the *S.aureus* isolates were resistance to penicillin and sensitive to vancomycin ,

Gentamicin , clarithromycin and chloramphenicol , this results compatible to many studies dealing with *S.aureus* isolated from mastitis milk of dairy animals(29,33–35) who report all *S.aureus* isolated from mastitis were resistance to penicillin 100% and sensitive to Gentamicin , clarithromycin chloramphenicol and vancomycin 100% . On the other hand ,VRSA have been reported by (6,8,36,37) in a percentage 8.6% ,21% ,50%,76% respectively and chloramphenicol resistance were detected in a percentage of 17% by(37) , 12 % by (8) and 42% by (6).The high sensitivity rate toward theses antibiotic in the current result may belong to low rate of usage in the animals host.

In the present study, cefoxitin was used for detection MRSA strains. According to (18) , oxacillin or cefoxitin replace methicillin as this antibiotics is stable under storage conditions, and methicillin actually is an excellent inducer of the *mecA*gene. However, methicillin is not the agent of choice for MRSA recognition and its not preferred to evaluate methicillin resistance, so it should be replaced by oxacillin or cefoxitin for detection of MRSA isolates , moreover the cefoxitin disk test is easier to read and thus is the preferred method in comparison with oxacillin and methicillin (18).

The current result revealed that, the resistance to methicillin was 55%. These results are in line with many local studies dealing with mastitis milk of dairy animals (8,29) who report the percentage of methcilline resistance in *S.aureus* isolated from mastits milk of dairy animals was 61% , 60%,respictivily. On the other hand, higher results were obtained by (38)who recorded the occurrence of MRSA was 88% and lower results were detected by (33) who found only 10 % of *S.aureus* was MRSA.

Methicillin resistance is clinically the most important, since single genetic element can convers resistance to most commonly prescribed class of antimicrobials-the beta lactam antibiotics, which include penicillins, cephalosporin and carbapenems(39,40).

The reason behind continuous increasing in resistant to β-lactam antibiotics is caused by the overuse or misuse of these antibiotics and by the use of poor quality antibiotics. It also results from natural genetic changes, or mutations, within the organisms that cause diseases. Different classes of antibiotics such as vancomycin, linezolid, quinupristin/dalfopristin (streptogramin) and newer fluoroquinilones were used for treatment of severe MRSA infection caused by multidrug resistant strain (39). However, since 1990, MRSA strains with intermediate resistance to vancomycin (MIC, 8-16 µg/ml) and strains fully resistant to vancomycin (MIC ≥ 32 µg/ml) have been reported (41)

The results of the present study showed that, , the resistance against tetracycline and clindamycin were 30%.These result are in line with the local study of (38) who found the clindamycin resistance of *S.aureus* isolated from dairy animals was 25% .similar findng also reported by (29) (42)who found the resistance rate of *S.aureus* against tetracycline and clindamycin from 10 -30%.

Biofilm Formation .

The isolated *S. aureus* were evaluated for biofilm formation capability using phenotypic screening as well as molecular detection of *icaA* and *ica D* genes. Microtiter plate (MTP) showed that, 20/20 (100%) isolates were able to form biofilm .In addition, all *S.aureus* isolates were investigated for biofilm associated genes, *icaA* and *icaD*. Molecular investigation revealed that both *icaA* and *icaD* genes were present in the 100% of isolates.

These data are in accordance with those reported by (43)who detected *icaA* and *icaD* in all *S. aureus* isolates by PCR techniques. Similar results were obtained by (44) who

found that all the isolates were biofilm producing and contain ica locus .

The current results also were compatible with the studies of (45), (46).Whom found all clinical isolates of *S. aureus* were biofilm producer and positive for both *icaA and icaD* genes .In addition ,the present study are in line with local study of (8) who found 94.117% of biofilm production in strains of *S. aureus* isolates from Bovine Mastitis. On the other hand , slightly lower percentage of biofilm production were reported by the study of (29) reported that, 80.6% of *S.aureus* isolates were biofilm positive when tested by MTP method.

However, our results are in contrast with the data reported by (47), who detected *icaA* and *icaD* genes in only (12.5%) of 23 *S. aureus* isolates and (48) who detected *icaA* and *icaD* genes in 70% of *S. aureus* isolates. The variations in the presence of *icaAD* genes from different studies might be due to the heterogeneity in the genetic origins of *S.aureus* (48)

In this study, a high percentage of agreement (100%) was observed between the genotypes and phenotypes of isolates, determined by PCR and MTP, respectively. Broad applicability, reliability and high reproducibility of the MTP were previously verified for bacterial biofilms (49). On the other hand , failure of *S. aureus* strains that possess the ica locus to form biofilm has been reported *in vitro* (50) and biofilm producing *S.aureus* that lack ica operon also reported by many studies such as (51,52)

These results suggest that biofilm production is regulated by the interaction of different regulatory mechanisms and the expression of *ica* genes is strongly influenced by environmental factors such as glucose, temperature, osmolarity, and growth in anaerobic conditions (53). Indeed, transcriptional regulation of the *ica* operon is complex, involving the interdependent and independent activity of several activators and repressors. Differential transcriptional regulation of the locus and/or putative *ica*-independent biofilm mechanisms can influence biofilm production phenotype (54). Insertional inactivation and point mutations in the *ica* locus were reported as other plausible mechanisms to give rise to biofilm-negative variants in *S.aureus* (55). Thus, the difference between phenotypic and genotypic characterization may be due to the heterogeneity in the genetic origins, and not because of the presence or absence of genes required for the biofilm formation.Therefore, a combination of phenotypic and genotypic assays should be employed for improved confidence in identifying biofilm-producing *S. aureus* isolates.

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