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Phenotypic & Genotypic Study of Biofilm and Effectiveness on the Ability of CONS to Oxacillin Resistance Using Muller-Hinton Agar & Mannitol Salt Agar

KEYWORDS: CONS, Biofilm, mecA, ica A, ica D, Oxacillin Resistance

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ABSTRACT *The aim of this study is to analyze the phenotypic and genotypic indicators of biofilm formation of Coagulase negative staphylococci (CONS) and Oxacillin resistance by using many different detection methods. Thirty-six CONS isolated from patients with chronic otitis media diagnosed using API Staph . PCR amplification was used to detect ica A and D gene and also to detect mec A gene, cultured strains were tested for biofilm formation ability with Congo red agar (CRA), phenotypic prediction of mecA gene positive or negative (historically referred to methicillin resistance, MR and methicillin susceptible, MS) was conducted by both Oxacillin and Cefoxitin disk diffusion tests also agar screening test both of tests on Muller Hinton agar (MHA) and mannitol salt agar (MSA) and minimum inhibition concentration (MIC) was determined by agar dilution method with MHA corporation with MIC on MHA containing 2% NaCl both of them Oxacillin concentration (0.25 to 256 µg/mL). The above tests applied to compare their MRCONS and MSCONS detection the sensitivity & specificity were determined to compare between the tests which is used in this study.*

A total of 36 CONS isolates from patients with chronic otitis media studied, the high percentage of this isolates belonging to staph xylosus 20 isolates (55%), of a total 36 isolates 31(86%) were found to be CRA plate test positive, ica A and D genes were found to be present in 30 isolate, the concordance between phenotypic and genotypic of biofilm formation was 90.9% ($p<0.05$). All isolates of study were divided in to MRCONS 29(80.5%) mecA positive and MSCONS 7(19.4%) mecA negative, all phenotypic tests used to detect MSCONS gave false resistance with high percentage (19.4%). Cefoxitin disk diffusion on MHA & MSA yielded the highest sensitivity value (100%), Oxacillin disk diffusion on MHA gave a specificity of 16.6%, Oxacillin screen MSA(2 µg/mL) gave the highest sensitivity value (93%), Oxacillin screening on MHA (6 µg/mL with 4% NaCl) gave the highest specificity value (100%)and MIC on MHA & MSA with 2% NaCl gave the same highest sensitivity value(100%), from 31 isolates positive biofilm CONS isolates 24 (77.4%) were confirmed as MRCONS while 7(23%) confirmed as MSCONS, negative biofilm CONS isolates found only between MRCONS isolates 5(14%) which means an essential role of resistance to Oxacillin referred to biofilm.

Introduction

In last two decades, Coagulase negative staphylococci (CONS) have emerged as significant pathogens in a variety of infection [1] . CONS produces an extracellular matrix called slime layer (Biofilm), the ability of CONS to form biofilm is considered their main virulence determinant [2] , biofilm in CONS have been divided in to three categories: biofilm positive, biofilm weak and biofilm negative [3] .

The biofilm made of carbohydrate and protein molecules, major part of the biofilm is called polysaccharide intercellular adhesion (PIA) encoded by the *ica* ADBC operon [4], the present of the *ica* ADBC did not always correlate with biofilm production, determined under different conditions, but there was evidence to suggest a correlation when at least two gene (*ica* AD) were co- transcribed [5]. The CONS bacterial cell become at least partially tolerant to human innate immune system after growing as biofilm [6,7] , the biofilm made CONS multiresistant to antibiotics and the recent study showed that several antibiotics (Oxacillin, cefotaxime and vancomycin) had reduced penetration throughout *staphylococcus aureus* and *staphylococcus epidermidis* biofilm [8,9] .

Methicillin resistance in CONS is primarily mediated by the over production of penicillin binding protein (PBP) 2a, and altered PBP with extremely low affinities for β- lactam antibiotics. The *mec A* gene encoding PBP2a has very high levels of homology in methicillin resistant MRCONS, so the *mec A* gene is considered a useful molecular marker of methicillin resistance in all staphylococci [10] . Detection of Oxacillin resistance in CONS by phenotypic method is often difficult [11], so the clinical and laboratory standards Institute (CLSI) standardized a disk diffusion method to predict resistance mediated by the *mec A* gene in *staphylococcus spp*. Using a Cefoxitin disk (30 µg), In fact, studies indicate that this is the best phenotypic test to predict resistance to β-lactam agents among CONS [12,13] , Antunes et al [14] showed that important to perform tests with Oxacillin (1µg) and Cefoxitin (30µg) disks to detect resistance to methicillin in CONS . Mannitol salt agar (MSA) was first investigated as a medium for susceptibility testing in 1985 [12] , Kampf *et al*., [13] shows that MSA is a suitable medium for disk diffusion test (1 µg of Oxacillin) and agar screen testing (2 µg of Oxacillin per mL) to detect Oxacillin resistant with high sensitivity and specificity.

Several study have been done for detection CONS biofilm formation and resistance to methicillin and other antibiotics, Cabrera-Contreras *et al*., [14] which showed that higher percentages of resistant to antibiotics and number of resistance markers were found in biofilm forming clinical strains than non biofilm forming *staph. epidermidis* strains while Granslo *et al*., [15] and Quy [16] showed that biofilm positive CONS isolates displayed higher levels of antibiotics resistance than biofilm negative isolate.

The aim of this study was to analyze the phenotypic and genotypic indicators biofilm formation in CONS, phenotypic method by using Congo red agar and genotypic by detection *ica A* and *D* gene also compared the conventional methods (Muller Hinton Agar (MHA) for disk diffusion test 1 µg Oxacillin & 30µg Cefoxitin & agar screen test with MHA 6 µg/mL Oxacillin + 4%NaCl) with MSA in disk diffusion and agar screening tests also detection Minimum inhibitory concentration (MIC) using MHA without NaCl and compared the result with MIC using MHA(2% NaCl), sensitivity and specificity were detected to all these Oxacillin phenotypic methods depending on a *mec A* – based polymerase chine reaction (PCR) for CONS.

Material and methods

Bacterial strains and Identification :

A total 36 Coagulase negative Staphylococcus isolates were

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collected from patients with chronic otitis media, all these strains identified according to morphological features and biochemical test and these identification was confirmed by Api-Staph system (bio Merieux).

Biofilm test :
The biofilm production for CONS isolates was assessed by Congo red agar plates (CRA). This culture medium (CRA) was prepared with brain heart infusion agar (BHI)(Oxoid, England), supplement with Congo red dye (0.8g/L) (BDH, England) and sucrose (36g/L) (oxoid, England) . All Cons strains to be tested for biofilm formation were inculcated on CRA plates. These CRA plates were incubated at 37C° for 24hr then after the plates were maintained at room temperature for 24hr. PIA –positive strains appear as black colonies, and PIA – negative strains were red [17] .

PCR to detect *ica A* and *ica D* genes :
The purpose of using simplex and multiplex PCR was to determine the distribution biofilm genes in 36 genotypically different clones of CONS . Total chromosomal DNA was extracted by boiling method, was prepared according to Ruppé *et al*.(18) Briefly, many isolated colonies of overnight growth bacteria were suspended thoroughly in 1 mL distilled water and boiled in a water bath for 10 min. After centrifugation, supernatant was used as template DNA for the PCR [19] . PCR mixture was composed from 12.5 µl of GoTaq®Green Master Mix(2x)(Kapa, south Afreqa), 1.5 µl(10 pmol) from each forward and reverse primers ,5µl of DNA template(prepared by boiling method) and 4.5 µl of nuclease free water to get final volume of 25 µl. multiplex PCR was carried out with an initial denaturation step of 10 min at 95°C, followed by 33 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C), and extension (1min at 72°C); the reactions were finalized by polymerization for 10min at 72°C.

The amplified PCR product were analyzed by agarose gel electrophoresis according to [19] using 1% agarose supplied with 0.5 µg/mL Ethedium bromide for 1 hour and a half (7 Volts/ cm²) . DNA ladder (100bp and1000bp) were used to assess PCR product size, then PCR products were visualized by UV light at 336 nm, and photographs were taken using digital camera.

Susceptibility test:
The isolated CONS were tested for their on their Oxacillin, OX(1µg)(Bioanalysa, Turkey) and Cefoxitin; Fox(30 µg) (Bioanalysa, Turkey), susceptibility using the disk diffusion technique. A 0.5 McFarland standard suspension of each isolate was on to Muller-Hinton agar (Himedia, India) [20] and mannitol salt agar (Himedia, India) [21] . The diameters of the inhibition zones were interpreted according to the criteria recommended by the CLSI [22] , considering a break point diameter for susceptibility inhibition of 18mm to Oxacillin and 25mm to Cefoxitin.

Agar Screen test :
An aliquot of 10 µl of a 1:100 dilution of a bacterial suspension (McFarland standard 0.5) was placed on Muller-Hinton agar with 6 µg\mL Oxacillin (str. Valea lupuluinr., Romania) with 4%NaCl as described in [22] and also on mannitol salt agar with 2 µg\mL Oxacillin as described in [21] . Plates were incubated for 48hr at 37C° and growth was observed after14, 24 and 48hr.

MIC determinations:
The MICs (of Oxacillin) was done by using agar dilution methods recommended by the CLSI/NCCLs [23] . Two µL of bacterial suspension, diluted to a final concentration of 10^7 CFU/mL (inoculums size of 2 10^4 CFU/spot), was inculcated on Muller Hinton agar with 2%NaCl containing Oxacillin concentration from 0.25-256 µg/mL . Also the same method above was done by using Muller Hinton agar without NaCl. The inoculated plates were then incubated at 37C° for 24hr. The MIC was defined as the lowest concentration of antibiotics that prevented visible growth. According to the current Oxacillin break point for methicillin susceptibility, an Oxacillin MIC break point of 0.25 µg/mL was considered as methicillin susceptible in CNS.

Detection *mecA* gene by PCR :
All isolates were submitted to genotypic study using PCR. The oligonucleotide primers specific in this study showe in (table1). PCR mixture was composed from 12.5 µl of GoTaq®Green Master Mix(2x) (kappa, south afreqa), 1.5 µl(10pmol) from each forward and reverse primers ,5µl of DNA template(prepared by boiling method) and 4.5 µl of nuclease free water to get final volume of 25 µl.

PCR mixture without DNA template was used as a negative control. PCR was carried out with an initial denaturation step of 10 min at 95°C, followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 57°C), and extension (1min at 72°C); the reactions were finalized by polymerization for 10min at 72°C [24] .

Table (1) :The primers used in the current study for PCR amplification.

Primers name	primers sequence 5′————→3′	Product size (bp)	Anneling temperature	Reference from
icaA	F-5-ACACTT-GCTG-GCGCAGT-CAA-3 R-5-TCTG-GAAC-CAACATC-CAACA-3	188bp	51	Atshan, *et al*., 19
icaD	F-5-ATGGT-CAAGCCCA-GACAGAG-3 R-5-AG-TATTTTCAAT-GTTTAAA-GCAA-3	198bp	51	Atshan, *et al*., 19
mecA	F- GTA-GAAATGACT-GAACGTC-CGATAA R- CCAATTC-CACATT-GTTTCG-GTCTAA	310 bp,	54	Cabrera *et al*., 24

Statistic:
Sensitivity and specificity rates were calculated for all method was assessed in this paper to discriminate between Muller Hinton agar and Mannitol salt agar to choose the best for disk diffusion test and agar screen test, also to found the corrodance rate between phenotype and genotype test. The specificity is based on the number of correct negative results, i. e., the number of MSCONS strains that were correctly identified. The sensitivity is based on the number of MRCONS that were correctly identified.

Result and Discussion
A total of 36 CONS isolate were recovered from patients with chronic otitis media. The CONS were assigned by the Api-Staph system in to five species : *staph xylosus* (20 isolate),

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staph epidemidis (6 isolate), *staph capitis* (5 isolate), *staph lentus* (4 isolate) and *staph hominis* (1 isolate). Which show in figure (1).

Prevalence of both phenotypic and genotypic Biofilm formation and methicillin resistance of CONS were study. Detection of slime producing phenotype of CONS was performed with congo red agar (CRA) plate test, 31(86%) were CRA plate test positive and this agreement with Jain & Agarwal [25] that found CRA excellent method that could be used to determine whether an isolate has the potential for biofilm production or not , figure (2). Relationship between presence of the *icaA/ icaD* gene (figure 2)and phenotype is summarized in table (2), of the 36 CONS, 30 isolate of (*icaA/ icaD*)$^{+}$/ Biofilm^{+}, 3 isolate (*staph. capitis* and two *staph xylosus*) false negative (*icaA/ icaD*)$^{+}$ /Biofilm^{-} , one isolate *staph. capitis* false positive (*icaA/ icaD*)$^{-}$ / Biofilm^{+} and 2 isolate staph. xylosus and staph. epidermidis with (*icaA/ icaD*)$^{-}$ / Biofilm^{-} .

As result, the concordance between phenotype and genotype of Biofilm formation was 90.9%(P 0.05), PPV of 96.8% and NPV of 40% for the *icaA/ icaD* gene assay. Our result presented an increased incidence that *icaA/ icaD* gene essential for Biofilm formation, which are involved in the production of the polysaccharide intercellular adhesion (PIA) that is functionally necessary for cell to cell adhesion, biofilm accumulation and virulence on CONS and this discrepancy with Kogn *et al.*, [26] and Rohde *et al.*, [27] which suggested that PIA and its encoding *icaA* gene might not be of universal important CONS Biofilm, the result of false negative may be due to mutations that produced Biofilm negative phenotype, while the result of false positive may be due to PIA- independent Biofilm formation.

Table (2): relationship between presence of the icaA/ icaD gene and phenotype on Congo red agar (CRA).

Presence genes	Biofilm Formation on CRA	Not Biofilm Formation on CRA	Total
Presence *icaA/ icaD*	30	3	33
Absence *icaA/ icaD*	1	2	3
Total	31	5	36

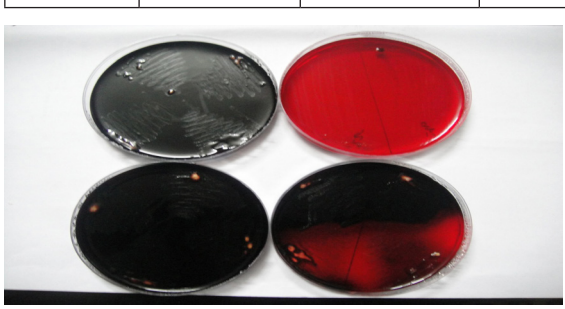

Figure 2: Slime layer production on the Congo red agar (CRA). A and B: Strong slime layer production by Staph. Spp. (ORS) on the CRA, C: Negative production of slime layer by Staph. Spp (ORS) on CRA, D: Strong slime layer production by two isolates of Staph. Spp (ORS) on the CRA and other two isolates give negative production slime layer.

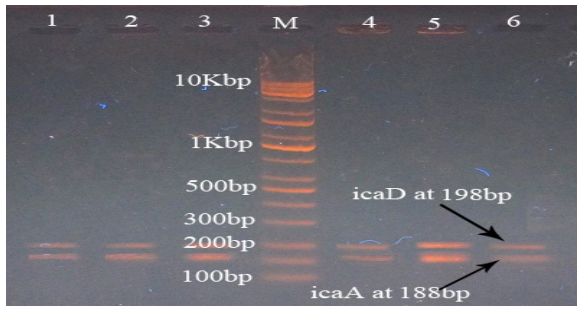

Figure 3: electrophoresis (1% agarose, 7 V/cm for 90 min) to Multiplex PCR amplification of biofilm genes icaA, icaD for some staph. Spp. isolates using boiling method, line M 1000bp DNA ladder, lines (1,2,3,4, 5,6) positive results with 1100bp and 900bp for icaA, icaD genes respectively , while line 7 give negative result.

The resistance to methicillin among the isolated CONS was declared using disk diffusion test, agar screen test, MIC of Oxacillin and PCR , the gold standard method for the detection of resistance mediated by *mecA* . Table (3) shows the result of both phenotypic and genotypic method which used for detection *mecA* gene (methicillin resistance) according to PCR (figure 4) results 29 isolates (80.5%) were *mecA* positive, while 7 isolates (19.4%) *mecA* negative .This finding was very much similar to other study done by Ferreira *et al.*, [28] , Hussain *et al.*, [29] from Canada, from Brazil by Secchi *et al.*, [30] and by Kilic *et al.*, [31] from Turkey in which they found 67%, 54%, 71(169/238) and 59%(19/32) respectively of total strains as *mecA* positive. The relatively increased rate of MRCONS isolates in our study might be due to the fact that our specimens were taken from patients with chronic infection. Both Oxacillin and Cefoxitin disk diffusion tests were used for detection methicillin resistance as we show in table (4) and the sensitivity and specificities for the different methods used in this study are shown in table (3) testing of Oxacillin (1 µg) on MHA correctly detected (26) isolate giving only (6)isolate false positive and (3) isolate false negative at a sensitivity of 89.6% and specificity of 16.6%. The poor level of specificity of this method is largely due to the high number of false positive, 8 from 26 isolate *mec A* gene positive shows zone of inhibition (7 to 16) mm while testing of Oxacillin (1 µg) on MSA correctly detected (23) giving only (7) false positive and (6) false negative at a sensitivity of (79%) and specificity of (0%) , (8) from (23) isolate shows zone of inhibition (9 to 15)mm.

The disk diffusion results on both MHA and MSA for Cefoxitin (30 µg) correctly identified (29) MRCONS giving (7) false positive, a sensitivity and specificity for this tests were 100% and 0% respectively. The present study show that MSA and Cefoxitin failed in detection MSCONS referrer that both of them MSA and Cefoxitin induced ability of CONS to biofilm formation.

These differences between result of both Oxacillin and Cefoxitin disk can be attributed to inoculums size , growth conditions (e.g. the temperature or osmolarity of the medium) and biofilm formation , making susceptibility testing of methicillin resistant staphylococci very difficult, the disk diffusion tests were reported to lead to false positive and false negative results, and this good agreement with murakami *et al.*, [32]. According to Skoy *et al* [33], false resistance among CONS is expected to be present in (1 to 9%) of the cases, Although found no technique alone display 100% of sensitivity and specificity to detect Oxacillin resistance among CONS while the current data showed that percentage of false resistance 19.4% and this high percentage refer to ability of CONS to biofilm formation. This phenomenon make disk diffusion tests disadvantages to call *mec A* negative strains.

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Table (3): Comparison of results between presence of mecA gene and various phenotypic methods for detection of MRS-CONS & MSCONS.

No. of isolates Disk diffusion				Total No. of isolates	Agar screen test			Total No. of isolates	MHA(2% NaCl)	
Presence of mec A gene					MHA (4% NaCl +6µg\mL)				≤0.25µg\mL	≥0.5µg\mL
mec A positive	Oxacillin (1 µg))	MHA	≤ 17 R	26	MHA (4% NaCl +6µg\mL)	Gr.	3	29(80.5%)	0	29
			≥ 18 S	3		No Gr.	26		0	29
		MSA	≤ 17 R	23	MSA 2µg\mL	Gr.	27		0	29
			≥ 18 S	6		No Gr.	2		0	29
	Cefoxitin	MHA	≤ 24 R	29	MHA	≥0.5µg\mL	29		≤0.25µg\mL	≥0.5µg\mL
			≥ 25 S	0					0	29
		MSA	≤ 24 R	29					≥0.5µg\mL	29
			≥ 25 S	0					0	29
mec A negative	Oxacillin (1 µg))	MHA	≤ 17 R	6	MHA (4% NaCl +6µg\mL)	Gr.	0	7(19.4%)	0	7
			≥ 18 S	1		No Gr.	7		0	7
		MSA	≤ 17 R	7	MSA 2µg\mL	Gr.	7		0	7
			≥ 18 S	0		No Gr.	0		0	7
	Cefoxitin	MHA	≤ 24 R	7	MHA	≥0.5µg\mL	7		≤0.25µg\mL	≥0.5µg\mL
			≥ 25 S	0					0	7
		MSA	≤ 24 R	7					≥0.5µg\mL	7
			≥ 25 S	0					0	7
Total	Oxacillin (1 µg))	MHA	≤ 17 R	32	MHA (4% NaCl +6µg\mL)	Gr.	3	36	0	36
			≥ 18 S	4		No Gr.	26		0	36
		MSA	≤ 17 R	30	MSA 2µg\mL	Gr.	27		0	36
			≥ 18 S	6		No Gr.	2		0	36
	Cefoxitin	MHA	≤ 24 R	36	MHA	≥0.5µg\mL	36		≤0.25µg\mL	≥0.5µg\mL
			≥ 25 S	0					0	36
		MSA	≤ 24 R	36					≥0.5µg\mL	36
			≥ 25 S	0					0	36

MHR: Muller Hinton Agar, MSA: Mannitol Salt Agar, R: Resistance, S:Sensitive, Gr: Growth, No Gr: No Growth , MIC: Minimum Inhibitory Concentration.

Table (4) : Sensitivity and Specificity value for the various phenotypic methods for detect MRCONS &MSCONS.

Methods	Sensitivity%	Specificity%	PPV%	NPV%
Oxacillin Disk diffusion (MHA)	89.6	16.6	81	25
Oxacillin Disk diffusion (MSA)	79	0	76.6	0
Cefoxitin Disk diffusion (MHA)	100	0	80.5	0
Cefoxitin Disk diffusion (MSA)	100	0	80.5	0
Oxacillin screen of MHA + 6 µg \mL Oxacillin+4% NaCl	10.3	100	100	21
Oxacillin screen MSA+ 2 µg \mL Oxacillin	93	0	79	0
MIC MHA only	100	0	80.5	0
MIC MHA2% NaCl	100	0	80.5	0

PPV: Positive Predictive Value, NPV: Negative Predictive Value.

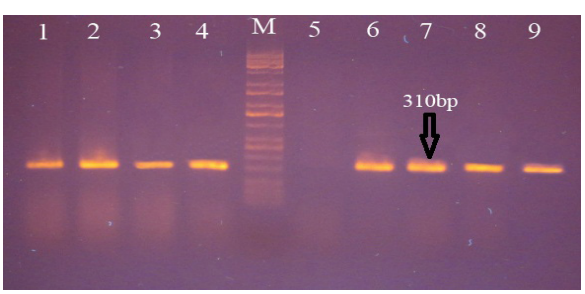

Figure (4): Agarose gel electrophoresis (1% agarose, 7 V/cm, for 90 min) for mecA gene (amplified size 310bp as compared with 1000bp DNA ladder) using template DNA prepared by boiling method for (1,2,3,4,6,7,8,9)lines give positive result while (5)lines give negative result.

The false negative (group of strains carried the *mec A* gene) but was sensitive by disk diffusion test, this can be explained by the possible involvement of other genes in the process of β- lactam resistance which can affect the expression of *mec A* gene [34,35,36,37] . Another cause of such problem was reported to be the heteroresistance phenomenon which is difficult to be classically detected and was reported mainly to occur in CONS, in this case strains produce low levels of PBPs and so escape classic detection and phenotypically misidentified as methicillin sensitive despite having the *mec A* gene [38].

The agar screening tests were also evaluated in this study , CLSI agar screening test to Oxacillin detected only (3) isolate

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(colonies were visible only after (48hr) of incubation) of (29) MRCONS and had a sensitivity of (10.3%) and a specificity of (100%), while agar screen test (2 µg\mL of Oxacillin) on MSA detect (27) isolate (3 isolate were visible after 14hr, 17 isolate after 24hr and 6 isolate after 48hr) of 29 MRCONS giving 7 isolate false positive, the result read one isolate *staph. xylosus* at 14hr, five isolate ((*staph epidermidis* (2), *staph. xylosus* (2) and staph lentus (1))), the results to these isolates read at 24hr and only one isolate *staph. xylosus* read 48hr, a sensitivity and specificity to this test (93% and 0%) respectively, surprising two isolate of *staph. xylosus* (*mec A* positive) did not show grow on both agar screening method. The micro colonies associated with heterogeneously resistant populations were clearer and easier to read when incubation for 48hr, and this agreement with Swenson & Tenover, [39], while the result read at 14hr refer to the population in that at cases with homogeneous expression (methicillin resistance).

This study MIC determined by agar dilution method with MHA containing Oxacillin concentration (0.125 to 256 µg\mL) corporation with MIC on MHA containing 2% NaCl and Oxacillin concentration (0.125 to 256 µg\mL) both method failed to detect the negative result with high sensitivity (100%), all isolates with *mec A* positive MIC 256 µg\mL while isolates with *mec A* negative (5 isolate) MIC 256 µg\mL ,2 isolate of staph xylosus MIC 128 µg\mL and 64 µg\mL).

In comparison with results of MIC on MHA containing Oxacillin only, 20 isolate *mec A* positive MIC 256 µg\mL, 9 isolate (*staph. xylosus* (No.4) MIC 128 µg\mL, *staphe. Xylosus* (No. 2) MIC 64 µg\mL, one isolate *staph. lentus* MIC 32 µg\mL and tow isolate *staph xylosus* MIC 16 µg\mL) while the result of isolate with *mec A* negative (5 isolate MIC 256 µg\mL, two isolate *staph. xylosus* with MIC 128 µg\mL and 64 µg\mL) we observed Oxacillin resistant to some strains (*mec A* gene positive of CONS increased two- to eight fold with addition NaCl (2%) to the medium except one isolate *staph. xylosus* (*mec A* gene) MIC decreased from 256 to 128 µg\mL, while the MIC for *mec A* gene negative strains did not change in the presence of added salt and this result agreement with Huang *et al*.,[40]. Also this sure that most of CONS strains are heterogeneous group and the addition of salt to medium affect members differentially.

Table (5): correlation between MRCONS & MSCONS and Biofilm phenotype (positive & negative).

Methicillin susceptible	Biofilm Formation	Not Biofilm Formation	Total
MRCONS	24	5	29
MSCONS	7	0	7
Total	31(86.%)	5(14%)	36(100%)

MRCONS: Methicillin Resistance Coagulase Negative Staphylococci, MSCONS: Methicillin Susceptible Coagulase Negative Staphylococci

Out of a total 36, 5(14%) negative Biofilm CONS isolate, all these isolate were confirmed as MRCONS by PCR to *mec A* gene . From 31 positive Biofilm CONS isolate (24)77.4% were confirmed as MRCONS while 7(23%) confirmed as MSCONS, table (5), this study show that an essential role of resistance in MRCONS in the first stage referred to biofilm and not to *mecA* gene and this sure by many study [41,42] that the antibiotics resistant sub population exist in the deeper layer of mature biofilm with biofilm- specific phenotypes, like slow growth owing nutrient limitation the cell viability decreased as the concentration of Oxacillin increased until the highest concentration of Oxacillin killed all young colony biofilm so the subpopulation in the deeper layer which *mecA* positive grow and resistant to Oxacillin and this sure that must result in MIC Oxacillin test heterogeneously repression isolates in this study only small single colony and these colony grew again and formed thick biofilm after Oxacillin treatment was discontinued which one of the mechanisms of antibiotics resistance is the formation by subpopulation show that of microorganism in biofilm of cell with a unique and highly protective phenotype similar to spore formation . Although most bacteria in biofilm rapidly killed by highest concentration of antibiotics subpopulation which might consist of 1% or less of the original population neither grow nor die in the presence of antibiotics and persist despite continued exposure to antibiotics, this agreement with Cabrera-Contreras *et al*.,[14], which show the presence of *mec A* gene seems to be enhanced in Biofilm producers.

In spite of small value of MSCONS isolate in this study but all these have Biofilm with high resistance to Oxacillin and this explained why these infection be chronic with difficult to treatment and this agreement with [42,43,44] these studies showed that reduced susceptibility of biofilm bacteria to antimicrobial agents is a crucial problem for treatment of chronic infection, and biofilm bacteria are 100 to 1000 times more resistance to antibiotics.

In conclusion, current study shows that MHA using (1 µg) Oxacillin, MHA(6 µg\mL+4%NaCl) , agar dilution method (2%NaCl) were a suitable medium for disk diffusion, agar screen testing and MIC test to detect Oxacillin resistance in CONS forming biofilm in spite of small percentage of NPV because biofilm making detection or resistance to Oxacillin very difficult so we need many other study to detection specific media and method to detection MR or MS without false –positive resistance result to avoid un necessary use of antibiotic such as vancomycin and we finding that both MRCONS and MSCONS biofilm formation show high resistant to Oxacillin even sensitive bacteria that do not have a known genetic basis for resistance can exhibit profoundly reduced susceptibility when they form biofilm. So this phenomenon need special attention in determining these relation. The population of CONS in one isolate heterogeneous in ability to methicillin resistance (mecA gene positive) and in ability to biofilm formation this dependent on the incidence that one isolate content two population the first population (*mec A* gene) negative with strong ability to produces biofilm in comparison with other population in the same species (*mec A* gene) which did not have ability to produce biofilm and this incidence sure by that the result of MIC on medium show

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