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Comparison Study of *mecA* Gene-Based PCR With Phenotypic Methods For Detecting Biofilm Forming Methicillin Resistant *taphylococcus aureus* Isolates and Comparison of *mecA* With *femA*, *femB*, and *mecC* Genes.

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ABSTRACT

Staphylococcus aureus is opportunistic human pathogen that causes a variety of diseases. Out of 580 clinical specimens, 100 *Staphylococcus aureus* isolates were isolated and identified depending on cultural, morphological and different biochemical tests , in addition to molecular identification by using PCR with specific primer *16S rRNA*. For biofilm detection, method of polystyrene microtitre plate was used and the results showed that 61% were biofilm producer and 39% were non-biofilm producer isolates. The different methods were applied for detection of methicillin resistant *Staphylococcus aureus* isolates and the identification of *mecA* gene by PCR was considered as the gold standard method comparing with other four phenotypic tests in sensitivity, specificity, positive predictive value, negative predictive value and accuracy of method. The results of cefoxitin showed that 50 (81.97%) were similar to *mecA* gene PCR 50(81.97%) and sensitivity, specificity, positive predictive value, negative predictive value and accuracy of these test were 100%, while, the method of oxacillin disk diffusion , oxacillin agar screening and MIC for oxacillin showed less specificity ,positive predictive value and accuracy. Whereas, sensitivity and negative predictive value for all methods equal to 100%. The results of cefoxitin disk diffusion test showed the highest specificity, accuracy compared to other phenotypic tests that were low precision in the determination of methicillin resistant *Staphylococcus aureus* isolates. On the other hand, It was found that there was a relation between *femA* and *mecA* genes, while no relation was observed between *femB* , *mecC* and with

1-Introduction:

Staphylococcus aureus has the ability to colonize and form biofilms which defined as groups of microorganisms in which cells affix to each other on a surface a polymeric, mixture of biofilm composed of extracellular DNA, proteins, and polysaccharides(1).

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Biofilms are of a great relevance to the medical community , they have been associated with a variety of persistent infections which include: urinary tract infections, gastrointestinal tract, cystic fibrosis infections, heart valves, infections of permanent indwelling devices such as joint prostheses, and for chronic administration (2).Indiscriminate uses of antibiotics without suitable susceptibility tests for quick therapy are some key agents for emergence of resistant organisms versus antimicrobial drugs particularly in developing nations(3). The emergence of antibiotic resistance among pathogens is a rising problem of universal worry (4). In 1959, methicillin (the first β-lactamase-resistant penicillin) was licensed in England. In 1960 the first MRSA isolates were identified in England (5). In 1961, British scientists identified the first MRSA isolates while the first stated

human case of MRSA in the United States arrive in 1968 (6). Resistance to methicillin is due to *mecA* gene that is portion of the staphylococcal cassette chromosome this gene encodes the protein PBP2A (protein binding to penicillin) which inhibits the activity of β-lactam antibiotics (7). The *mec* operon which consists of *mecA* and its regulatory genes *mecI* , *mecR1* and the cassette chromosome recombines complex ccr (8). In the divergence in genetic material, it is fully known that *mecA* gene is described as a proper molecular marker in defining MRSA(9), however a new *mecA* variant *mecC* has lately been described in a novel staphylococcal cassette chromosome *mec* termed type XI (10). The *femA* and *femB* operon act as regulator genes which are essential for the expression of methicillin resistance in *S. aureus* (11). *femA* protein is concerned in the synthesis of the Staphylococcal cell wall (12). *femA* and *femB* are encoded by the *femAB* operon. *femA* directs the incorporation of the glycines Gly2-Gly3 while *femB* that of the glycines Gly4-Gly5 (13). Accurate detection of MRSA is the most importance to secure effective treatment for the patient and control of methicillin-resistant staphylococci in the hospital environment and subsequently block further transmission (14).

There are several phenotypic methods like MIC determination, oxacillin screen agar (OSA) method and disk diffusion (DD) testing, for recognizing of methicillin resistant staphylococci. Phenotypic expression of resistance can alter depending on the growth conditions (e.g. osmolality, temperature and culture medium supplements such NaCl or sucrose) (15). The discovery of *mecA* by the Polymerase Chain Reaction (PCR) is supposed a gold-standard technique for oxacillin resistance detection (16). The *femA* gene encodes a protein *femA* which essential for the expression of resistance to methicillin, despite this sharing in resistance, the gene *femA* is also found in methicillin-sensitive strains (17). The aim of present study was to identify *S. aureus* isolates that form biofilm by phenotypic and molecular assays, this study also applied PCR for *mecA* gene as a ‘‘gold standard’’ assay, we estimated the accuracy of phenotypic methods for detection of methicillin resistance in compression with *mecA* based PCR . However, the contribution of *mecC, femA* and *femB* genes to methicillin resistance are not visibly known.Thus, we also scanned the association of *mecA* gene and *femA, femB* and *mecC.*

2. Materials and Methods

2.1. Bacterial isolates

In this study, 580 clinical specimens were collected during December 2015 to April 2016 from different patients at different hospitals in Erbil city/ Iraq. The specimens included; 120 specimens from urinary tract infections , 65 from nose infection, 127 from wound infections , 78 from burns, 85 from tonsillitis, 45 from vaginitis and 60 specimens from ear infections and all specimens were taken by disposable cotton swabs or sterile containers. The specimens were plated on mannitol salt agar (MSA) media, (Oxoid, England), and incubated overnight at 37C◦ for 24 hours. Single, well-isolated colonies with the typical appearance of *S. aureus* were sub cultured and confirmed by Phenotypic and *molecular* identification.

2.2.The conventional identification of *Staphylococcus aureus.*

Conventional identification was performed through microscopic morphology, coagulase, DNase, motility, oxidase, catalase, urease ,voges-proskauer, hemolysis and mannitol fermentation test (18).

2.3. Isolation of DNA from bacterial cell

DNA extraction was isolated from bacterial cells by using Presto Mini™ gDNA bacterial kit (Geneaid).

2.4. Molecular identification by PCR amplification of *16S rRNA gene*

To confirm the isolated *S.aureus* bacteria by phenotypic method, the monoplex PCR was used by using specific primer for *16S rRNA* gene. The primers of this study was designed by using primers program on the NBCI website and supplied by Bioneer (Korea) as showed in Table (1). These primers amplify 1487bp region of *16S rRNA* gene fragment of

S.aureus. PCR reaction kit (AccuPower PCR PreMix) was purchased from Bioneer Company. The PCR reaction was carried out in 20 µl as shown in Table (2), after that lyophilized blue was dissolved by pipette and the PCR programed as illustrated in Table(3) by using thermal cycler (Eppendorf, Germany) ,10 µL of the PCR product was resolved into amplified fragments by electrophoresis in 1.5% agarose gel (containing ethidium bromide dye with final concentration 0.1 µg/ml) with 45 v for 15 minutes and Completed with 100 V for 1 hour . The ladder 100bp was used to evaluate the molecular weight of fragments and The amplicons observed under the UV radiation.

Table (1): Primers sequences and their product size.

Primer name	Primer sequences	Product size bp	Annealing Temperature
16s rRNA F	CCTGGCTCAGGATGAACG	1478	55
16s rRNA R	AATCATTTGTCCCACCTTCG		
femA F	TTCACGCAAACTGTTGGCCACT	104	59
femA R	AAGCAAGCTGCAATGACCTCGT		
femB F	ACCGAGAGACTGAAGCTAGAACTGG	295	
femB R	TCGCCATCTGTTTGACGTAATTCACT		
mecA F	AGCTGATTCAGGTTACGGACAAGGT	499	
mecA R	GCAACCATCGTTACGGATTGCTTCA		
mecC F	TCACTACATCACCAGGTTCAACCCA	381	
mecC R	TCTCGCCTTGGCCATATCCTGA		

16s rRNA: 16s ribosomal RNA; R: Reverse; F: Forward, *mec:* methicillin resistance , *fem:* Auxiliary genes

Table (2): Monoplex PCR reaction mixture for *16 S rRNA* gene.

Components	Size for single sample (µl)	concentration
Primer Forward	1	10 Pmol/µl
Primer Reverse	1	10 Pmol/µl
DNA template	2	50- 100 ng
D.W	16	—
The total volume	20	—

Table (3): PCR program for amplification of *16S rRNA* gene.

Step	Temperature °C	Time	Number of cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	1min	35
Annealing	55°C	1min	
Extension	72°C	1:30min	
Final extension	72°C	7min	1

2.5. Quantitative biofilm formation assay:

All isolates were tested for biofilm formation by using the quantitative method of Microtitre plate method (MtP) (19).The test strains were inoculated in brain heart infusion broth and incubated for 24 hours at 37 °C. After incubation each culture was diluted 1:100 with sterile fresh medium, 200 µL of the samples were added to each well of a 96-well microplate and sterile broth was used as blank. The microplate were incubated at 37 °C for 24 hours. After incubation, content of each well was gently removed. The wells were washed three times with sterile distilled water to take off unbound bacterial cells subsequently the plates were exposed to air-dry and 200 µL of 0.1% w/v crystal violet solution was added to each well and incubated at room temperature for 30 minutes . The plates were washed off with distilled water and kept for air-dry. The bound bacteria were quantified by addition of 200 µL of absolute ethanol to each well and the absorbance of dissolved dye was measured at a wavelength of 570 nm by using 96-flat wells of ELISA (BioTek ,USA). The isolates were classified according to biofilm production relying on the criteria place down by (20).

2.6. Oxacillin resistance testing

2.6.1. Phenotypic detection of biofilm forming methicillin resistant *S. aureus*

This was achieved by using oxacillin screen agar (OSA) method.The isolates were spread on Mueller Hinton(Rashmi, India) supplemented with 6µg/mL oxacillin (Himedia, India) and 4% NaCl **;** Also the disk diffusion (DD) method with antibiotic discs oxacillin 1µg and cefoxitin 30 µg (BBL, England) was performed in Mueller Hinton agar. Additionally, the Minimal Inhibitory Concentration (MIC) for oxacillin for each isolate was specified by the broth macrodilution method on MH broth (Rashmi,

India) which supplemented with 2% NaCl and oxacillin, in serial dilutions from 0.5 µg/mL to 256 µg/mL (21) *S. aureus* ATCC 25923 was used as control in this experiment**.**

2.6.2. Molecular Detection of *mecA, mecC, femA* and *femB* Genes.

S. aureus isolates were undergone to detect *mecA*, *mecC*, *femA* and *femB* genes by multiplex PCR and by using specific primer pairs which designed for this studyTable (1). AccuPower multiplex PCR PreMix was purchased from Bioneer Company and the PCR reaction was carried out in 20 µl as shown in Table (4). After that the amplification was performed for methicillin resistant genes and negative control (water in state of DNA) in a thermal cycler as illustrated in Table (5). In addition, 10 µL of the PCR product was resolved into amplified fragments by electrophoresis as mentioned above.

Table (4) :Multiplex PCR reaction mixture for methicillin resistant genes .

Components	Size for single sample(µl)	Final concentration
Primer Forward	4	20 Pmol/µl
Primer Reverse	4	20 Pmol/µl
DNA template	2	50-100 ng
D.W	10	—
The total volume	20	—

Table (5) :PCR program for amplification of the *mecA, mecC, femA* and *femB* genes.

The main steps	Temperature	Time	Number .of cycle
Initial Denaturation	94	5 min	1
Denaturation	94	1min	35
Annealing	59	1:30 min	
Extension	72	1min	
Final Extension	72	7min	1

3. Results and Discussion:

In this study, 100 (17.24%) of *S. aureus* strains were isolated from wound infection (31%), burns (12%), UTI (15%), tonsillitis (8%), vaginitis (3%), ear infection (13%) and nose infection (18%). All isolates of *S. aureus* were identified by conventional phenotypic tests and there were 100% agreement between the conventional identification results and the amplification of the 1478 bp fragment of the species-specific *16S rRNA* gene Figure (1).

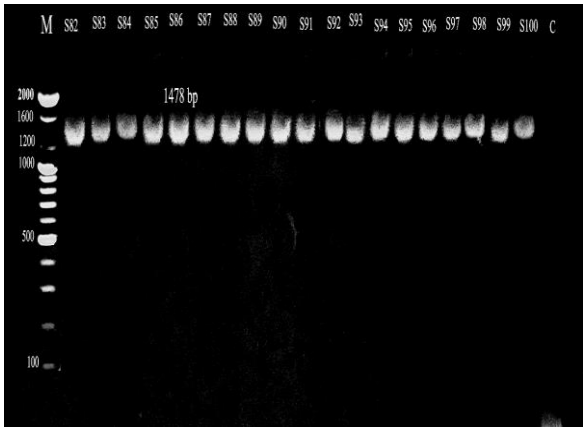

Figure (1): Polymerase chain reaction products on gel electrophoresis (1.5%) for *16S rRNA* gene. M: DNA ladder (100 bp). Lane S82 to S100: Amplified PCR product of 16S rRNA gene (1478 bp) for *S.aureus* isolates. C: negative control.

These results in agreement with (22) who identified 100 clinical isolates of *S. aureus* depending on phenotypical and biochemical tests, in addition to molecular identification by using species specific *16S rRNA* pairs for PCR. This result also consistent with the study conducted in Baghdad and Al- Anbar governorate which were 43 (20.7%) (23), 15 (14%) (24) and less than in Turkey and other study in Baghdad,49(46.7%) (25) and 64 (57.14) (26) respectively. On the other hand, the results of polystyrene microtitre plate method showed that 61% were biofilm producer and 39% were non-biofilm producer isolates. These results were in agreement with (27) which indicated that 64.89% of *S. aureus* isolates were biofilm producers and 35.11% strains were non biofilm producer. Whereas (28) found that 69% of the isolates were biofilm producers and 31% of the isolates were non-biofilm producers .while (29) stated that (54.19%) of the isolates were biofilm producers and (45.81%) of the isolates were non-biofilm producers by using Mtp method Figure(2).

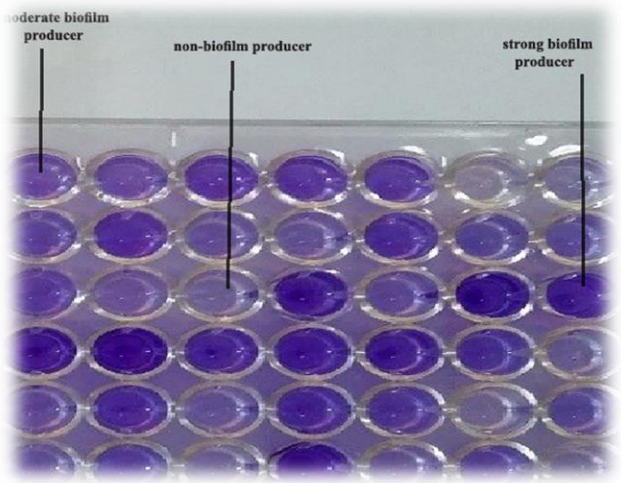

Figure (2): Detection of biofilm production in *S. aureus* by Microtitre plate method

The different methods were applied for detection of MRSA through the world. The identification of *mecA* gene by PCR was counted as the gold standard, but it was not routinely available in generality clinical laboratories. In this study, the results of the tested isolates with multiplex PCR methicillin resistant genes have been shown in Figure (3).The results obtained by the PCR *mecA* gene test and phenotypic have been listed in Table (6), and Sensitivity, specificity, Positive predictive value (PPV), Negative predictive value (NPV) and accuracy of the four phenotypic tests in comparison with *mecA*-based PCR method are given in Table (7). Out of 61 biofilm forming isolates of *S. aureus* 49 (80.33%) were MRSA and 12(19.67%) were MSSA with MICs of oxacillin method. While for disk diffusion method of cefoxitin and oxacillin it were 50(81.97%) and 47(77.05%) classified as MRSA and 11(18.33%), 14(22.95%) were classified as MSSA respectively. Whereas for oxacillin screening agar it were 46(75.41%) and 15(24.59%) for MRSA and MSSA respectively; the amplification of *mecA* gene target by polymerase chain reaction showed that 50(81.97%) were positive for *mecA* gene, while 11(18.33%) were negative for *mecA* gene.

In this study, the *mecA* gene was observed in 50 (81.97%) of the *S. aureus* isolates by PCR method. The cefoxitin disk diffusion results were like to *mecA* gene PCR 50 (81.97%) and sensitivity ,specificity ,PPV,NPV and accuracy of this test were 100%, however, oxacillin disk diffusion , oxacillin agar screening and MIC for oxacillin showed less specificity ,PPV and accuracy.

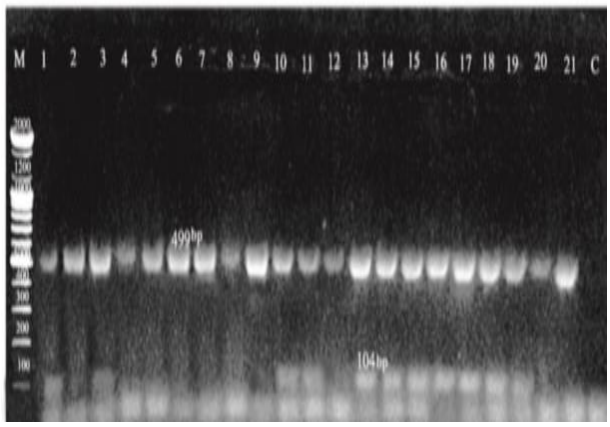

Figure (3): Polymerase chain reaction products on gel electrophoresis (1.5%) for mecA, mecC, femA, femB genes. M: DNA ladder (100 bp). Lane1 to 21 : Amplified PCR product of mecA, mecC, femA, femB genes for S.aureus isolates. C: negative control

Table(6): Comparison between phenotypic methods and PCR for 61 isolates biofilm forming methicillin resistance S. aureus .

PCR mecA detection	Number of isolates	Oxacillin screening agar		Oxacillin disk diffusion		Cefoxitin disk diffusion		MIC for Oxacillin	
		Growth	No Growth	Growth	No Growth	Growth	No Growth	Growth	No Growth
Positive	50	46	4	47	3	50	0	49	1
Negative	11	0	11	0	11	0	11	0	11
Total	61	61		61		61		61	

Table (7): Sensitivity, specificity, PPV, NPV and Accuracy of phenotypic and genotypic methods used for biofilm forming methicillin resistance *S. aureus*.

Testing method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Oxacillin screening agar	100%	73.33%	92%	100%	93.44%
Oxacillin disk diffusion	100%	78.57%	94%	100%	95%
Cefoxitin disk diffusion	100%	100%	100%	100%	100%
MIC for Oxacillin	100%	91.67%	98%	100%	98.36%
PCR for *mecA* gene	100%	100%	100%	100%	100%

PPV: Positive predictive value, NPV: Negative predictive value

These results corresponding with (16) who showed 28 from 50 of *S. aureus* were MRSA by oxacillin disk diffusion test. They also showed 30 isolates were MRSA in oxacillin agar screening and 32 isolates were resistant with cefoxitin disk diffusion test and 32 of them harbor *mecA* gene and they also

showed that sensitivity and specificity of cefoxitin disk diffusion test was 100% but another tests had less sensitivity and specificity while (30) reported that the conventional tests for identification of *S. aureus* and detection resistance to oxacillin showed 100% agreement with the Multiplex PCR results. Whereas (31) showed through Compared phenotypic detection with the molecular detection of methicillin resistance the sensitivities and specificities of the phenotypic tests for oxacillin agar screening were 95% and 95.5%, for oxacillin disc diffusion were 91.7 and 92.8% and for cefoxitin disc diffusion were 100%, respectively. Moreover the result of this study partial agreement with (32) a total of 138 isolates, 118 MRSA isolates and 20 MSSA isolates were estimated by phenotypic methods: cefoxitin and oxacillin disk diffusion(DD) ,agar dilution (AD), oxacillin agar screening (OAS) all methods showed 100% specificity, but only the DD tests were given 100% sensitivity. The sensitivity of the other tests ranged from 82.2% (OAS)-98.3% (AD). The DD test showed high accuracy in the determination of MRSA isolates.

Another studies have shown different sensitivity and specificity for these phenotypic tests for determination of MRSA such as (33) which found that the oxacillin agar screening was 92.3% sensitive and 45.8% specific and they stated that the sensitivity and specificity of the cefoxitin and oxacillin disk diffusion test were 84.6, 84.6, 87.5 and 79.2% respectively. In other report (34) found that the oxacillin agar screening and cefoxitin, oxacillin disk diffusion test was 100% specific but only the oxacillin and cefoxitin disk diffusion test had 100% sensitivity. Furthermore, they showed that the oxacillin agar screening had the lowest sensitivity (82.2%). In general, cefoxitin disk diffusion test had shown the highest specificity, accuracy compairing to phenotypic tests and it was mentioned that the genotypic characteristics of the isolates should be investigated.

On the other hand, the correlation between the presence of *femA, femB* and *mecC* genes with *mecA* gene was investigated, while *femA* gene has been found to associate with *mecA* gene, whereas *femB* and *mecC* not related with *mecA.* Indeed, (35) showed a total of 60 MRSA isolates were detected by cefoxitin disk diffusion and they found that all isolates were positive for *mecA* and *femA* genes and fifteen isolates were positive for *femB.* The association between the existence of *femA, femB* and *femX* genes with *mecA* gene was scanned and *femA* gene has been found to associate with *mecA* gene.

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دراسة مقارنة تفاعل البلمرة المتسلسل المعتمد على الجين *mecA* مع الطرق المظهرية للكشف عن مقاومة الميثيسيلين في عزلات المكورات العنقودية المكونة للغشاء الحيوي ومقارنة جين *mecA* مع جينات *femA*، *femB* و*mecC*

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الخلاصة

المكورات العنقودية الذهبية من ممرضات الانسان الإنتهازية التي تسبب مجموعة متنوعة من الأمراض. من مجموع 580 عينة سريرية ، عزل100عزلة من *Staphylococcus aureus* وتم تشخيصها بالإعتماد على الصفات الزرعية والمظهرية و الاختبارات الكيميائية الحيوية المختلفة فضلاً عن التشخيص الجزيئي بواسطة تفاعل البلمرة المتسلسل باستخدام بادئات متخصصة ل*16S rRNA* ، ولتحديد الغشاء الحيوي أُستخدمت طريقة اطباق المعايرة الدقيقة فأظهرت النتائج 61٪ من العزلات مكونة للغشاء الحيوي ، و 39٪ من العزلات غيرمنتجة للغشاء الحيوي. وطبقت طرق مختلفة لتحديد عزلات المكورات العنقودية الذهبية المقاومة للمثيسيلين وعد تحديد الجين *mecA* بواسطة تفاعل البلمرة المتسلسل كمعيار ذهبي مقارنة مع الطرق المظهرية الاربعة مثل الحساسية، النوعية، القيمة التنبؤية الإيجابية، القيمة التنبؤية السلبية ودقة االطرق ، فاظهرت نتائج السيفوكسيتين 50 (81.97٪) مشابهة لنتاتج تفاعل البلمرة المتسلسل للجين *mecA* 50 (81.97٪) وكانت الحساسية والنوعية والقيمة التنبؤية الإيجابية والقيمة التنبؤية السلبية والدقة لهذين الاختبارين 100٪، بينما أظهرت طرق إنتشار اقراص الأوكساسيلين ، مسح اطباق الاوكساسيلين والتركيز المثبط الادنى للأوكساسيلين أقل خصوصية و دقة. في حين الحساسية والقيمة التنبؤية السلبية لجميع الطرق تساوي 100٪ ، أظهر إختبار إنتشار اقراص سيفوكسيتين خصوصية ودقة عالية مقارنة مع غيره من الإختبارات المظهرية إذ وجد هناك دقة منخفضة في التحديد بوساطة الاختبارات الاخرى، من ناحية أخرى لقد وجد هناك إرتباط مابين *femA* والجين *mecA* بينما لم يلاحظ وجود إرتباط بين *femB* ، *mecC* ، و *mecA* .