

Comparison of Oxacillin Disc Diffusion Test with *mecA* Polymerase Chain Reaction and Cefoxitin Disc Diffusion Test for the Detection of Oxacillin-resistant *Staphylococcus aureus* Collected from Baghdad Hospitals

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Abstract

This study was carried out to evaluate the accuracy of oxacillin and cefoxitin disc diffusion test compared with *mecA* gene-positive PCR isolates. Two hundred and six clinical swabs from nose, hand and ear were collected from four different hospitals in Baghdad. The diagnostic results showed that (154) out of (206) samples gave positive bacterial culture, (61) of them were confirmed as *Staphylococcus aureus*; of which 47 (77%) were oxacillin-resistant *Staphylococcus aureus* and exhibited multiple resistances to other tested antibiotics. Depending on the MICs values, 23 isolates were chosen. Plasmid profiles revealed that (11) out of (23) had a plasmid with different molecular weight. Using polymerase chain reaction assay, 19 isolates were found to have *mecA* gene and results of agarose gel revealed the presence of DNA band at (200 bp.); the results detect a correlation between the positivity of the isolates for cefoxitin resistant test and the molecular study for the presence of (*mecA*).

Keywords: *S.aureus* MRSA/ORSA; cefoxitin disc test; *mecA/nuc* genes.

Introduction

One of the major ecological problems in a society is the problem of health-care facilities (hospitals, inpatient and outpatient departments). Hospital represents a special environment, serving health care to patients and as a work environment for medical and other staff [1]. Nosocomial or hospital-acquired infections; are those infections acquired in hospital by a patient who was admitted for a reason other than that infection [2]. The microbiological factor is the most risky one for immuno-compromised patients because it has the potential to start nosocomial infections [1]. Any microbial group, bacteria, viruses, fungi, or parasites can cause a nosocomial infection, but bacteria are the most prevalent organisms. The natural bacterial world has always coexisted with humans and their extended environments [3].

Staphylococcus aureus is a unique microorganism as compared with other clinically relevant bacteria in three respects that the organism expresses a variety of virulence factors; continues to demonstrate the ability to develop resistance to include a broad array of antimicrobial classes, and *S.aureus* is a prominent pathogen in both hospital and the community settings [4]. Thus; it has been recognized as one of the major human pathogens and is by far one of the most common nosocomial organisms [5]. It

permanently colonizes the epithelium of 20% of the population, transiently occurs in more than 60% and 20% never carry the organism [6].

Despite the availability of novel drugs as an approach to staphylococcal therapy, the bacteria seem to be able to rapidly develop resistance to these drugs [7]. In recent years, *S. aureus* became resistant to both new and traditional antibiotics and thus, treatment of antibiotic resistant bacteria represented a therapeutic problem. For this reason, the determination of the susceptibility pattern is useful to determine the future challenges of effective therapy. Methicillin-resistant *S.aureus* (MRSA) (which also may be called multi drug resistant *S.aureus* and oxacillin resistant *S.aureus* "ORSA") represents a serious problem in hospitals worldwide, increasing infected patients mortality and morbidity and raising treatment costs [8]. Hospital-acquired MRSA/ ORSA are usually associated with increased expression of multiple antibiotic resistance genes including those genes coding for aminoglycoside resistance [9]. *S.aureus* developed resistance to β -lactam antibiotics through the acquisition of the *mecA* gene that encodes penicillin-binding protein 2a (PBP2a), which has a significantly reduced affinity for β -lactam antibiotics, thereby conferring β -lactam resistance [10]. Thus; resistant strains usually expressed

PBP2a that was absent from susceptible ones, making it a useful molecular marker of β -lactam resistance. Therefore; the detection of *mecA*, by the Polymerase Chain Reaction (PCR), is considered a gold-standard technique for oxacillin resistance detection [11].

Material and Methods

Two hundred and six clinical samples were collected during the period from February to October 2010; these samples were obtained from Al-Kaddimia Teaching Hospital, Al-Yarmuk General Teaching Hospital, Al-Kindi General Teaching Hospital and Central Pediatric Teaching Hospital. Samples from nose (both anterior nares), hand-swab (especially hands of the hospital personnel) and ear-swab were collected from different patients attending the four hospitals, health care workers such as staff-nurses, ward boys and indoor environment of these hospitals. Preliminary identification of the isolates was performed as described by [12] on the basis of colonial morphology, cultural characteristics on agar media, grams staining, catalase activity, coagulase test, and growth on mannitol salt agar and thermonuclease activity of clinical isolates. In addition; a confirmatory examination was carried out using the (VITEK 2 System) at Al-Kaddimia Teaching Hospital.

Antibiotic susceptibility profile

The antibiotic susceptibility profile for these isolated was tested against fifteen antibiotics included (β -lactam antibiotics, fluoroquinolones, aminoglycosides, macrolides, glycopeptide, phenicols and tetracycline). The disc diffusion (DD) assay was performed using Kirby-Bauer method according to the [13] guideline. All the *S.aureus* isolates that were previously identified by morphological and biochemical tests, plated by streaking on Muller-Hinton agar medium (with a turbidity equivalent to 0.5 McFarland tube; containing approximately 1 to 2×10^8 cfu/ml). The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye [13]. This gave a profile of drug susceptibility vis-à-vis antibiotic resistance [14].

Identification of bacteria with VITEK 2 System

A specific number of *S. aureus* isolates was selected according to the antibiotic susceptibility profile were chosen to confirm their identification and antibiotic susceptibility using the VITEK 2 System provided by Al-Kaddimia Teaching Hospital.

Detection of MIC by Broth microdilution method for oxacillin

MICs were determined using the broth microdilution method. Oxacillin stock solution was prepared at a concentration of 1280 μ g/ml in deionized water. The antibiotic was serially diluted with 2.0% NaCl as recommended by the NCCLS to give working concentrations of 64-0.125 μ g/ml and bacterial suspension of 0.5 McFarland. Turbidity standard was added to all the wells and were incubated for 24 hours at $35 \pm 2^\circ\text{C}$. the MIC breakpoint of oxacillin (1 μ g/ml) for *S.aureus* was ≥ 4.0 - < 2 (μ g/ml).

Extraction of the bacterial chromosomal and plasmid DNA

DNA extraction mini kit (INtRON biotechnology) and Plasmid mini preparation kit (Fermentas) were used according to the manufactures' instructions to isolate the chromosomal and plasmid DNA from *S.aureus* clinical isolates.

Spectrophotometer determination of DNA

Ten micro liters of each DNA sample were added to 490 μ l of D.W. Using spectrophotometer, the optical density was measured at wave length of 260 nm and 280 nm. An optical density of 1 corresponds to approximately 50 mg/ml for double stranded DNA; the concentration of DNA was calculated according to formula described in [15]. DNA concentration (μ g/ml) = optical density at 260 nm X 50 X dilution factor.

Detection of *S. aureus* specific gene

PCR amplification was performed according to [16]. A partial *nuc* gene was amplified using *nuc* gene primers (*nuc*F5'GCGATTGATGGTGATACGGTT3' and *nuc*R5'AGCCAAGCCTTGACGAACTAAGC3'). These primers were synthesized by Cinna Gen, Iran. Using temperature gradient program starting from 50.7°C to 55°C with

0.5°C increment each time; annealing temp. was recorded as 55°C.

Detection of *mecA* gene by PCR

PCR-based identification used in this study for accurate detection of *mecA* gene which mediates oxacillin resistance in staphylococci using the primers (*mecA* F 5'AACAGGTGAATTATTAGCACTTGTAAG3'and*mecA*R 5'ATTGCTGTTAATATTTTTTGAGTTGAA3') according to [17] which were provided by Cinna Gen, Iran. All the *nuc* positive strains (n=23) that were characterized as ORSA by the (oxacillin DD and MIC test) were subjected to PCR to detect the presence of *mecA* gene using thermal cycler; starting with 94°C for 5 minute, 30 cycles at 94°C for 1 minute, at 55°C for 1 minute, then at 72°C for 1 minute followed by final extension for 5 minute at 72°C.

Results

Depending on the conventional cultural procedures, results showed that; 154 samples gave positive culture and many of them showed beta haemolytic activity on blood agar plates. Primary test for species identification was done by Gram staining and standard biochemical tests and results revealed that 61 isolates were detected as *S.aureus* as they appear Gram positive. The clinical isolates ferment mannitol and produced yellow color due to the acid production; they were catalase

positive due to the production of catalase enzyme which distinguishes them from *Streptococcus* spp. Isolates were positive for coagulase which distinguished them from other *Staphylococcus* spp. characterizing them as *S.aureus*. In addition; a confirmatory examination was carried out using the (VITEK 2 System) at Al-Kaddimia Teaching Hospital; this documented system enable rapid identification for the suspected specimens to the species level.

The tested pathogenic isolates were found to exhibit obvious level of resistance against the antibiotics used and the susceptibility pattern for these clinical *S. aureus* isolates are shown in Fig. (1) and Table (1).

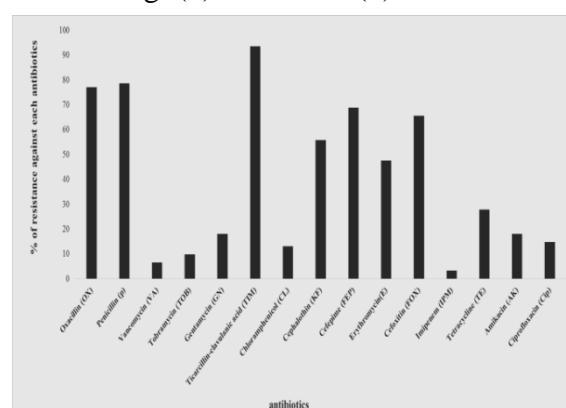


Fig. (1) Antibiotic susceptibility profile of the tested *S. aureus* isolates to different groups of antibiotics.

Table (1)
Susceptibility of *S. aureus* isolates to 15 different antibiotics groups.

Antibiotic	Resistans	%	Intermediate	%	Sensitive	%
Oxacillin	47	77.1	1	1.6	11	18
Penicillin	48	78.7	1	1.6	12	19.7
Vancomycin	4	6.6	9	14.8	48	78.7
Tobramycin	6	9.8	0	0.00	55	90.2
Gentamycin	11	18	7	11.5	43	70.5
Ticarcillin-clavulanic acid	57	93.4	0	0.00	4	6.6
Chloramphenicol	8	13.1	8	14.8	45	73.8
Cephalothin	34	55.7	9	14.8	18	29.5
Cefepime	42	68.9	3	6.6	16	26.2
Erythromycin	29	47.5	4	6.6	28	45.9
Cefoxitin	40	65.6	3	4.9	18	29.5
Imipenem	2	3.3	0	0.00	59	96.7
Tetracycline	17	27.9	4	6.6	40	65.6
Amikacin	11	18.0	2	3.3	48	78.7
Ciprofloxacin	9	14.8	1	1.6	51	83.6

The percentages of resistance for *S. aureus* isolates to ticarcillin-clavulanic acid, penicillin, oxacillin, cefepime, cefoxitin, cephalothin were (93.4, 78.7, 77.1, 68.9, 65.6 and 55.7 %), respectively, followed by a moderate resistance against erythromycin (47.5%) and tetracycline (27.9%) while, the percentage of resistance of gentamicin and amikacin was (18%), ciprofloxacin (14.8%) and (13%) for chloramphenicol. On the other hand, the tested isolates exhibited a high degree of susceptibility towards vancomycin (6.6%), tobramycin (9.8%) and imipenem with only (3.3%). Results showed that (77%) of the total number of isolates exhibited multiple resistances as determined by their ability to resist more than three types of antibiotics as described in Table (2). These isolates were oxacillin resistant and thus they were designated as multidrug-resistant oxacillin-resistant *S. aureus* (MDR-ORSA).

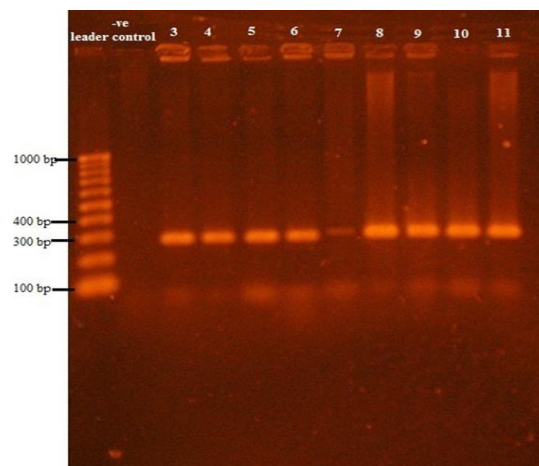
Table (2)
Multiple antibiotic resistance of ORS Isolates.

No. of antibiotic	No. of the isolates	Percentage (%)
3	2	(3.3)
4	3	(4.9)
5	7	(11.5)
6	7	(11.5)
7	8	(13.1)
8	6	(9.8)
9	6	(9.8)
10	4	(6.6)
11	2	(3.3)
13	1	(1.6)
14	1	(1.6)

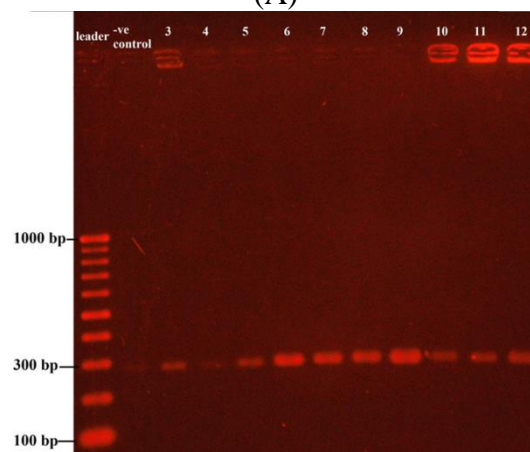
All staphylococci that characterized previously as *S. aureus* by conventional methods were further identified using a set of *nuc* primer. All the selected isolates (n=23) expressed *S. aureus* specific sequence gene in their PCR products which confirmed the obtained results that all the isolates were *S. aureus* as shown in Figs. (2 A,B and C). The amplified PCR product appeared as a single DNA band with a molecular size of about (300bp) when compared with the size marker

(100 bp.) ladder, this result is slightly close to the previously published results by [18] who detected a (276 bp) DNA fragment in all tested *S. aureus* isolates. Also it was in accordance with the findings of [16] who indicated that; the quantity of the 270-bp that he got as PCR product varied with the number of *S. aureus* CFU isolated from the specimen in such a way that the *nuc* PCR detected 20 viable *S. aureus* cells.

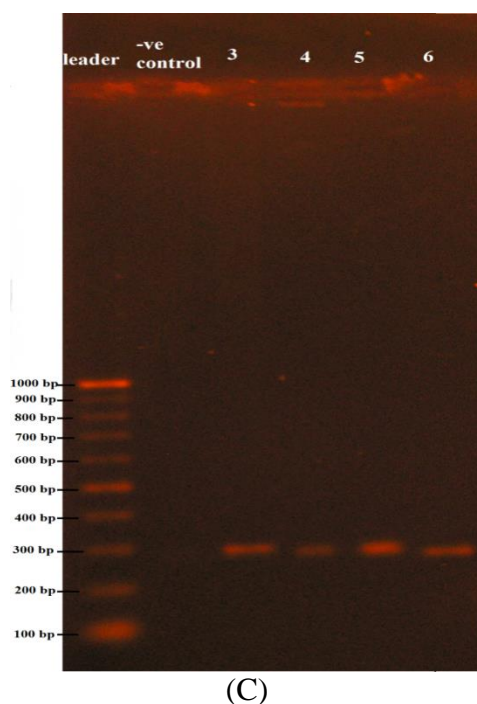
The results of plasmid profiles revealed that (11) out of (23) contain a plasmid and six of them appear to have two plasmids with a molecular size of (5200 bp) and (10,000 bp) as shown in Fig.(3A); while the plasmid profile of the rest five isolates Fig.(3B) revealed a plasmid with large molecular size (out of the borderline of the ladder) except one isolate; (S67) which showed three types of plasmids with closely determined molecular sizes of (4000 bp, 10,000 bp) and the third band was out of the borderline of the ladder.



(A)



(B)



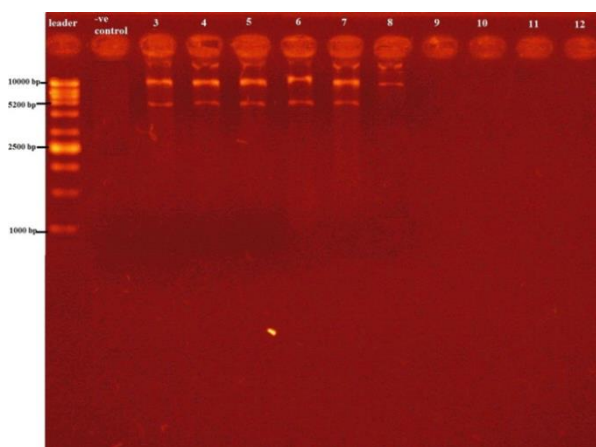
(C)

Fig. (2) Ethidium bromide-stained agarose gel (1.5%) electrophoresis shows the results of PCR amplification for nuc gene in hospital samples. Lane (1): 100bp DNA ladder (promega) Lane (2): PCR product of negative control (without DNA template).

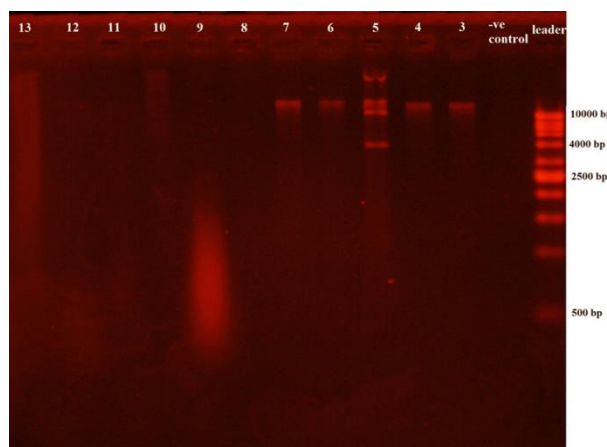
A) Lane (3-11) PCR product for Staph aureus specific nuc gene of nine isolates arranged as (S5, S10, S12, S14, S21, S34, S35, S41 and S45).

B) Lane (3-12) PCR product for the isolates (S76, S79, S81, S82, S86, S87, S88, S90, S100, and S102).

C) Lane (3-6) PCR product for the isolates (S50, S51, S56 and S67).



(A)



(B)

Fig. (3) Ethidium bromide-stained agarose gel (1.5%) electrophoresis shows plasmid profile for
A) The isolates (S5, S10, S14, S21, S35 and S45) and
B) The isolates (S51, S56, S67, S76 and S79).

The results of PCR for *mecA* gene (which mediates oxacillin resistance in staphylococci) exhibited that *mecA* gene was detected in 19 out of 23 oxacillin-resistant *S. aureus* and as shown in Fig.(4 A and B).

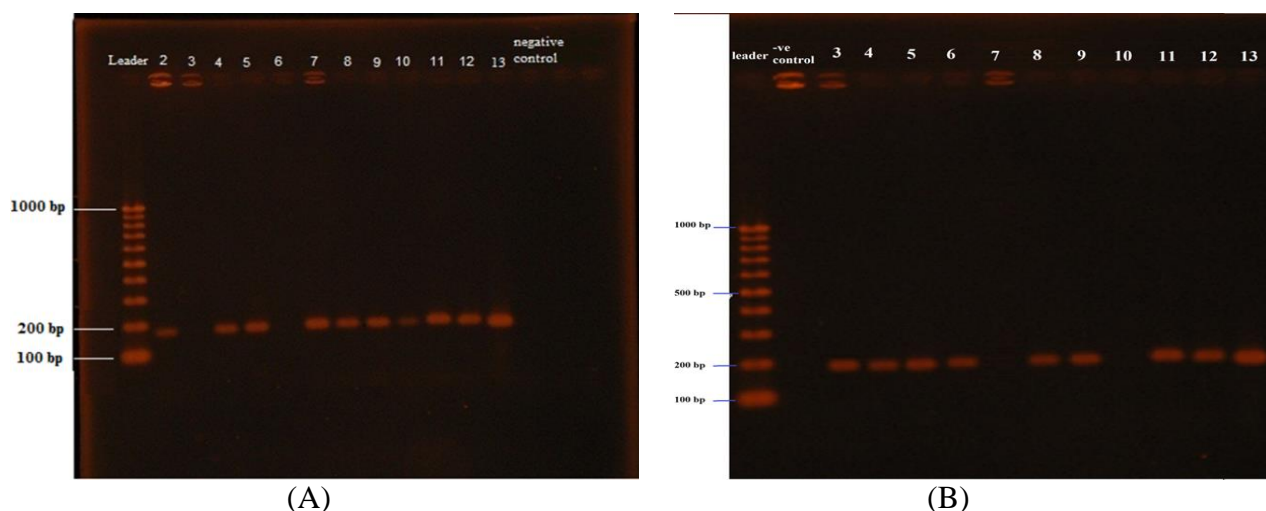


Fig. (4) Ethidium bromide-stained agarose gel (1.5%) electrophoresis showd the results of PCR amplification for *mecA* gene in *S. aureus*.

Lane (1): 100bp DNA ladder (promega) Lane (2): PCR product of negative control (without DNA),

A) PCR product forsuspectedMRSA(S5, S10-ve, S12, S14, S21-ve, S34, S35, S41, S45, S50, S51 and S56).

B) PCR product for the isolates (S67, S76, S79, S81, S82-ve, S86, S87, S88-ve, S90, S100 and S102).

Discussion

Oxacillin is the agent recommended by the CLSI for phenotypic tests to predict resistance to penicillinase-stable penicillins (PSPs) because of its stability and superior sensitivity over other PSPs used for susceptibility tests. The results of our work demonstrated that out of 61 tested *S. aureus* isolates; 47 isolates showed a high level of resistance (77%) to oxacillin (ORSA); the target antibiotic. The majority of ORSA were resistant to all β -lactam antibiotics in accordance with that obtained in Saudi Arabia by [19], [20]. *S. aureus* isolates recorded (78.6%) resistance against penicillin; which represent the same class of antibiotic as oxacillin that kills the bacteria by the inhibition of the cell wall synthesis. Our results are in agreement with the earlier study of [18] who reported 100% resistance to penicillin and oxacillin against *S. aureus* isolates from clinical hospitals in Cairo. Cefoxitin which is a type of the cephamycin a subclass of β lactams antibiotic has been recommended by the CLSI in (2011) as a surrogate for the accurate detection of *mecA*-mediated resistance to oxacillin and other (PSPs), i.e., methicillin and cloxacillin, thus; the recorded result of (65.6%) resistance among our tested isolates represent methicillin resistant among these isolates and in

agreement with the published studies of [21] [11] in which they recorded the effectiveness of cefoxitin disc diffusion method to detect *mecA* mediated oxacillin-resistance. Vancomycin represent one of the most potent antibiotics against our hospital origin *S. aureus* isolates as all the tested isolates were appeared to be susceptible to vancomycin and only (6.6%) of them showed resistant. This may be due to the rare use of vancomycin as a terminal drug of choice against MRSA/ORSA in our clinical institutes. These findings are in accordance with that obtained by [22]. Maximum number of resistance was observed for ticarcillin-clavulanic acid represented by (93.4%); this may be due to the effect of the clavulanic acid (β -lactamase inhibitors) that has no antibacterial activity. Instead, they bind and inactivate β -lactamases, this protecting the antibiotics that are normally substrates for these enzymes.

According to the findings of [23]; despite aminoglycosides resistance among clinical MRSA isolates being widespread; gentamycin remains active against most MRSA strains; as we recorded (18%) resistance among tested isolates and the same value was obtained for amikacin that was reported by [24] to have no effect on *S. aureus* isolates from hospital personnel. High resistance against the first and

fourth generation of cephalosporins represented by (55.7%) for cephalothin and (68.9%) for cefepime so this indicates that they are not being abused or commonly prescribed.

Within the tested aminoglycoside antibiotics tested, erythromycin that act by inhibition of protein synthesis showed relative activity against our tested isolates; [22] reported the development of moderate susceptibility to erythromycin among tested *S.aureus* isolates.

The amplified PCR product *fnrA* gene appeared as a single DNA band with a molecular size of about (300bp); our results confirmed the classical biochemical and morphological methods for identification of the studied strains at the species level as reported by [14], [25].

Plasmids profiles for our clinical *S.aureus* isolates revealed that 11 isolates produce one or two bands representing their plasmid content that determine the location of genetic determinants for antibiotic as reported by [26]. Plasmids were not detected in 12 of the resistant strains and this may indicate that their antibiotic resistance was located on the chromosome.

Comparing the performance of these PCR assays for *mecA*⁺ strains with those of classical methods of susceptibility testing; we observed discrepant results between oxacillin and cefoxitin when tested by disc diffusion method.

We detected a correlation between phenotypic and genotypic method of 100% for cefoxitin resistance, and these results confirmed the previous findings of [27],[28] who reported that; the results of cefoxitin (DD) tests correlate better with the presence of *mecA* than do the results of (DD) tests using oxacillin. The four *mecA*-negative tested isolates were not detected by the cefoxitin disc, but were detected by oxacillin disc. Thus, we found that the sensitivity in cefoxitin disc diffusion test was slightly better than that for oxacillin disc and this was reported by Ekrami and his colleagues [29]. Our results are in agreement with [30] in that; differentiation between MRSA and BORSA strains is very difficult using phenotypic tests. False positivity of oxacillin (DD) method during our

work may be due to hyper production of beta-lactamase which may lead to phenotypic expression of oxacillin-resistance (borderline oxacillin resistance); this was corroborated by the fact that all the isolates that were resistance to oxacillin but sensitive to cefoxitin were negative for *mecA* gene because they do not possess the usual genetic mechanism for such resistance.

In conclusion; for those who use disk diffusion as their routine susceptibility testing method for staphylococci, they should replace their (1µg) oxacillin discs with (30µg) cefoxitin that result in an easier-to-read test and provide equivalent detection (sensitivity and specificity) of oxacillin resistance in *S.aureus* discs. In addition; using accurate methods for the diagnosis of ORSA in our hospitals and that should be depended on PCR technique and not only by the conventional methods to differentiate between sensitive and resistant strains; that are independent of physical and chemical conditions of bacterial culture.

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أجريت الدراسة الحالية لتقييم دقة الطرق المظهرية المتبعة والمتمثلة بفحص الحساسية بطريقة الانتشار بواسطة الأقراص وبأستخدام مضادات الأوكساسيلين والسيفوكسينين ومقارنتها بالنتائج المعتمدة على استخدام تقنية تفاعل البلمرة المتسلسل للكشف عن العزلات الحاملة لجين *mecA*⁺. تم جمع ٢٠٦ من المسحات المرضية والتي شملت الأنف، اليد والأذن ومن اربع مستشفيات في بغداد وقد أظهرت الفحوص الأولية ان ١٥٤ عينة كانت موجبة للزرع البكتيري ومن ضمنها، ٦١ عذلة تم تشخيصها على انها بكتريا المكورات العنقودية الذهبية وقد ظهر ان من بين هذه العزلات، ٤٧ عذلة والتي تمثل نسبة ٧٧% هي من نوع بكتريا المكورات العنقودية المقاومة لمضاد الأوكساسيلين بالإضافة الى مقاومتها الى عدد من المضادات الحيوية المستخدمة اثناء البحث وبالأعتماد على نتائج تقدير التركيز المثبط الأدنى لعقار الأوكساسيلين ضد هذه العزلات فلقد تم انتخاب ٢٣ عذلة. أظهر تحليل المحتوى البلازميدي للعزلات ان (١١) من أصل (٢٣) عذلة تحتوي على بلازميد وبأحجام جزيئية مختلفة. وبأستخدام تفاعل تسلسل البلمرة فلقد ظهر أن (١٩) عذلة كانت حاملة لجين *mecA*⁺ حيث اظهرت نتيجة التضخيم وجود حزمة من الدنا وبحجم ٢٠٠ زوج قاعدي، لقد أظهرت النتائج وجود تطابق بين ايجابية فحص المقاومة لهذه العزلات تجاه عقار السيفوكسينين وطرق التشخيص الجزيئية المستخدمة للتحري عن وجود جين *mecA*.