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Detection and Evaluation of Methicillin-Resistant *Staphylococcus aureus* by Duplex PCR

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Abstract

*Staphylococcusaureus*is avirulence pathogenic bacterium.Detection of methicillin-resistant *S. aureus*(MRSA) using conventional culture and biochemical methods is labor and time consuming. Many MRSA isolates are heterogeneously resistant to ß-lactams, for example only 1 daughter cell out of 10^4 to 10^6 cells appears phenotypically resistant when routine antimicrobial susceptibility tests are performed. In this work two genes were used for detection of MRSA in urine, boil and nasal swabs using conventional PCR, *mecA* gene (533bp) were used for detection of methicillin resistant and *femA* gene (318bp) were used for *S.aureus* identification. It was found that not all resistant *S. aureus* tested with disk diffusion method carry the *mecA* gene that caused the resistant phenomena there were only 55% of all MRSA carrying *mecA* gene while *femA* gene gave 100% positive for *S. aureus* and this will lead us to appoint that penicillin-bindingprotein 2a (PBP2a) produced by *mec*A gene is not the only cause of resistant phenomena.

Introduction

The drug resistant (DR) phenomenon is being worldwide concern especially in the last 20 years. Among the most threatening antibiotic-resistant pathogens known are strains of methicillin-resistant *S. aureus* (MRSA), they are resistant to ß-lactams and other cell-wall-active agents. MRSA strains were first described in England in 1961; most MRSA infections are acquired in hospitals (HA-MRSA) or long-term care facilities (LTCFs). MRSA has been acquired in the community (CA-MRSA) by intravenous drug users in few cities [19,10].

The *mecA* gene confers resistance to methicillin in *S. aureus*. The gene is located on the staphylococcal chromosome cassette *mecA* and encodes penicillin binding protein 2a (PBP2a). PBP2a is located in the bacterial cell wall and has a low binding affinity for β-lactams, which is not present in methicillin-susceptible *S. aureus* (MSSA) [7, 19]. *femA*gene is implicated in cell wall metabolism and is found in large amounts in actively growing cultures [10].

In this study we investigated the application of PCR as a rapid and reliable diagnostictest which detected resistance to methicillin in *S. aureus*isolates and to compare the disc diffusion test with PCR for detection of methicillin resistance from Iraqi patient, which would be an improvement in thediagnosis of staphylococcal infections and help cliniciansto treat them faster and more efficiently.

Materials and Methods

*Staphylococcus aureus*isolation and identification:

All *S. aureus* isolated from urine, boil & nasal swab and identified primarily by routinelaboratory procedures which included the colonial and microscopical morphology and biochemical tests including β-hemolysis on blood agar, catalase 3%, oxidase, manitol, urase, DNase, coagulase tube and slide, latex and for last api staph [6,16].

Antibiotic susceptibility test:

Susceptibility test for 23*S. aureus* isolates to antibiotics was determined by disc diffusion method. Prepared by paper discs impregnated with antibiotic solutions placed on the surface of the plate (Molar Hinton agar) inoculated all over with bacterial culture incubated at 37°C overnight and the inhibition zone around the disc was measured [18].

DNA extraction:

A single colony was taken from a nutrient agar, which had been incubated overnight and emulsifiedinto 1000 µL distilled water. Centrifuged at 14000g for 2 min, supernatant was discarded then extracted as **promega** purification kit protocol instructions.

PCR assay:

The PCR procedure was based on a modification by Unal*et al* [4], and this was used as the 'gold standard'for all isolates. Oligonucleotides used *mecA*forward primer(5'-AAAATCGATGGTAAAGGTTGGC-3') and *mecA* reverse primer (5'-AGTTCTGCAGTACCGGATTTGC-3'),which expected to give 533bp, and *femA* forward primer (5'-CATGATGGCGAGATTACAGGT-3') and *femA* reverse primer (5'-GTCATCACGATCAGCGAAAGC-3'), which expected to give 318bp. All primers were supplied by Alpha DNA.PCR was performed on personal aircooled thermal cycler (eppendorf) using a reaction mixture of 25 µl consisting of 2µl of bacterial DNA, 12.5 µl *GoTaq*® *Green*Master Mix (Promega, CA), 0.5 µl of 25mM $MgCl_2$, 2µl of 10 Pmol\ µl of each primers pair,6 of nuclease free distilled water. The extracted DNA was amplified for30 cycles designed as 60 sec at 94°C for denaturation, 60 sec at 63°C for annealing and 60 sec at 72°C for extension with initial denaturation of 95°C for 5min and final extension of 72°C for 5min, 10 microlitres of the PCR product was then analyzedby agarose gel electrophoresis [1.5% agarose prepared in TBE (0.5x) buffer]. Gels were stainedwith ethidium bromide (0.5µg\ml) and photographed with gel documentation system under UV light (260nm) [13].

Results and Discussion

The bacterial isolates were dignosed using morphologicaland biochemical tests [catalase 3% +ve, urase +ve, heamagglutination test +ve, oxidase –ve, coagulase +ve, manitol fermentation +ve, DNase +ve, β-hemolysis on blood agar +ve and apisystem staph. (*S. aureus*)], then the antibiotic profiles of all 23 clinical *S. aureus* isolatesexamined for methicillin (ME), ciprofloxacin (CIP),oxacillin (OX), tetracycline (TET), gentamycin (GN), vancomycin (VAN), the results shown in Table (1). There were 20 MRSA and 3 methicillin-sensetive *S. aurius* (MSSA). *S. aureus* had variable antibioticprofiles. There was 20 isolates resistant to methicillin, whereas all 20 staphylococcal isolates were susceptibleto vancomycin, also showed variant susceptibility against oxacillin, ciprofloxacine, tetracyclin, and gentamycin.

Table (1)

Antibiotic sensitivity test for S. aureus isolates determined by conventional disk diffusion susceptibility test.

Sample no.	*Sample source*	*ME 5mg*	*CIP 5mg*	*OX 1mg*	*TET 30mg*	*GN 10mg*	*VAN 30mg*
1	B	R.	R.	S.	R.	R.	S.
2	N.S	R.	S.	R.	S.	I.	S.
3	N.S	R.	S.	R.	S.	I.	S.
4	B	R.	I.	S.	R.	R.	S.
5	B	R.	R.	S.	R.	S.	S.
6	U	R.	S.	S.	R.	R.	S.
7	B	R.	R.	R.	R.	S.	S.
8	U	R.	R.	R.	R.	R.	S.
9	U	R.	I.	R.	I.	S.	S.
10	B	R.	R.	R.	R.	R.	S.
11	B	R.	I.	R.	S.	R.	S.
12	U	R.	R.	S.	I.	R.	S.
13	U	R.	S.	R.	R.	I.	S.
14	U	R.	S.	R.	R.	I.	S.
15	U	R.	S.	R.	S.	I.	S.
16	N.S	R.	S.	I.	S.	S.	S.
17	U	R.	S.	R.	R.	R.	S.
18	U	R.	S.	R.	R.	I.	S.
19	U	R.	S.	R.	R.	I.	S.
20	U	R.	S.	R.	R.	I.	S.
21	B	S.	S.	S.	S.	S.	S.
22	U	S.	S.	I.	S.	I.	S.
23	N.S	S.	S.	S.	S.	S.	S.

R: resistant; I: intermediate resistant; S: sensitive; B: Boil; U: Urine; N.W: Nasal Swab.

DNA extracted and characterized by electrophoresis as shown in (Fig.(1)). Results of PCR for presence of *mecA*gene responsible for the resistant phenomena and *femA*gene specific for *S. aurius* were 55% of the MRSA samples carry *mecA* gene and 100% of all *S. aurius* isolates carry *femA*gene .

Results showed in Table (2), the 23 clinical samples; 20 samples were methicillin-resistant as examined with disk diffusion susceptibility test and 3 samples were methicillin-sensitive, and all MRSA samples was *femA* positive with 318 bp product but not all of them gave positive results for the *mecA* gene presence which is 533bp product as shown in Fig.(2), while the 3 MSSA samples showed positive results for *femA* but negative results for *mecA* gene.

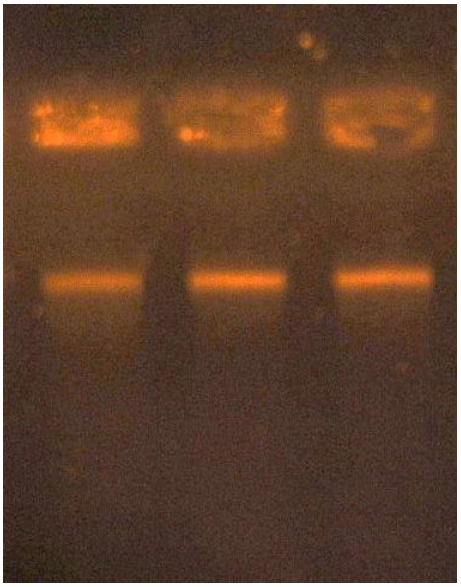

Fig. (1) Gel electrophoresis (0.8%) for total DNA after extracted from different samples under UV light (260nm).

Table (2)
PCR results for mecA&femA gene presence and samples sours.

Sample no.	*mecA presence*	*femA presence*
1	+	+
2	-	+
3	+	+
4	-	+
5	+	+
6	+	+
7	+	+
8	+	+
9	-	+
10	+	+
11	+	+
12	+	+
13	-	+
14	-	+
15	+	+
16	+	+
17	-	+
18	-	+
19	-	+
20	-	+
21	-	+
22	-	+
23	-	+

Several studies were used PCR for identification of *S. aureus*strains according to *femA* or for methicillin resistance phynomina on the basis of *macA* gene amplification [17,12]. Optimization for the optimum annealing temperature done according to the melting temperature, the results showed that 63°C optimum for amplification of the target sequences but with a faint bands for *mecA* gene whereas sharp bands for *femA* gene. In order to improve efficiency of PCR and better bands, 0.5 µl of 25mM $MgCl_2$ concentrations were added, in addition to the initial concentration of $MgCl_2$, which was 0.75mM/ 25 µl to the reaction to get sharper bands for *mecA* gene. Increasing the concentration of $MgCl_2$ in PCR reactions tends to promote binding of primers and bring the dNTPs, adding $MgCl_2$ can also be useful in cases

where no amplification products are observed. If the concentrations are too high, nonspecific primer binding can occur resulting in amplifications that are multi-banded or smeared bands on agarose gel [11].

In order to get bands without smears and without nonspecific products, number of cycles should also be optimized, for this aim the optimal number of cycles were 30 cycle.

Previous studies have shown mostly high sensitivity of PCR up to 97.3% compared to the disk diffusion method[12], Some other studies shows 70.3% [23], While other study gave 59.2% for oxacillin resistant MRSA and 55.2% for cefoxitin resistant MRSA [22,24]. In this study the sensitivity was 55% indicating that the methicillin resistant in *S. aureus* may not come from *mecA* gene, it may come from over production of β-lactamase by other gene which have mutation in its regulatory gene, including various length sequences deletion, or conjugation and transformation occur between bacterial cells causing constitutive expression for the β-lactamase [15].

Some strains of *S. aureus* that over produce ß-lactamase may have "borderline-resistant" minimum inhibitory concentrations (MICs) to methicillin but are susceptible to few other ß-lactam and many non-ß-lactam antibiotics. A few *S. aureus* strains with decreased susceptibility to ß-lactam antibiotics have been shown to possess modified penicillin binding proteins PBPs (but not low-affinity penicillin-binding protein PBP-2) [9].

Heterogenicity of methicillin resistance has complicated the detection and identification of these strains at the clinical microbiology laboratories. The usage of ß-lactam antibiotics stored under improper circumstances, the short incubation period (18 instead of 24 hours), and incubation at 37°C or low inoculum quantity may cause false results [8]. As it is well known, the detection of *mecA* gene by PCR is “a gold standard” to determine the resistance to methicillin of *S. aureus*isolates. However it should not be forgotten that sensitive strains which have *mecA* gene might not express it [16]. On the basis of these results, the multiplex PCR strategy could give rapid and reliable information to clinicians not only for the identification of pathogenic bacteria but also for therapeutic management. Especially MRSA has become a major nosocomial pathogen not only in tertiary care hospitals but also in chronic care facilities [14].The frequent resistance of MRSA to several antibacterial agents has prompted the overuse of vancomycin in first-line therapy and even in prophylaxis therapy [3,20]. However, both the selection of vancomycin resistance [2] and the potential transmission of such resistance between species [5], encourage restricted use of glycopeptides. An early and specific diagnosis might help clinicians face this problem [1].

The detection of MRSA isolates is important for appropriate patienttreatment and to help for recognition and management of MRSA outbreaks and cross-infection [15].

In conclusion, the multiplex PCR approach can be a beneficial way to standard microbiological methods for rapid and specific identifications of pathogens and resistance patterns. It could also be used as a tool to guide and reduce the use of glycopeptides in the clinical setting to avoid further resistance probability.

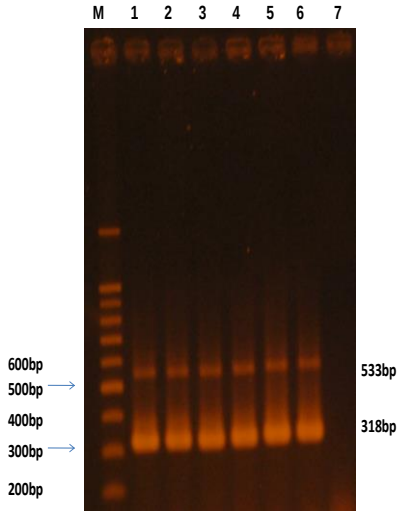

Fig.(2) Gel electrophoresis (1.2% agarose) for PCR product of femA and mecA gene in MRSA strains isolated from urine, boil and nasal swab [M:100bp ladder DNA marker, lanes 1,2,3,4,5&6 represents samples with femA (318bp) and mecA (533bp) positive and lane 7 represents negative control].

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الخلاصة

ان بكتريا الـ *S. aureus* هي بكتريا أمراضية ضارية بسبب قدرة سلالات معينه منها مقاومة المضادات الحيوية . ان تشخيص البكتريا المقاومة بالطرق التقليدية هي تحتاج الى وقت و جهد. وهناك عزلات مقاومتها متغايرة للبيتا لاكتم كمثل خليه واحده تظهر من 10^4 الى 10^6 خلية تظهر مقاومة مظهرية عندما تم فحصها بالطرق التقليدية لفحص المقاومة. لهذا يمكننا القول انه خاصية مقاومة المشلين لايتم الكشف عنها بصورة جيدة بالطرق فحص الحساسية التقليدية. في هذا البحث تم أستخدم زوجين من البادئات لعينات أخذت من الادرار و البثورو المسحات الانفية والتي هي بادء *mecA* (٥٣٣ قاعدة نايتروجينية) الخاص للبحث عن خاصية المقومة للمشلين وبادء *femA* (٣١٨ قاعدة نايتروجينية) الخاص لتشخيص الـ *S.aureus*. وقد تم تشخيصها بالطرق التقليدية و فحص صفة المقاومة بطريقة Disk Diffusion Method وتم استخلاص الدنا منها وتم استخدام بادء *mecA* و الحصول على قطعة بحجم ٥٣٣قاعدة نايتروجينية للجين المشفر لمقاومة المشلين بطريقة التفاعل التسلسلي المتضاعف و كانت النسبة ٥٥% من العينات التي اضهرت مقاومتها بطريقة الـDisk Diffusion Method وبادء *femA* للحصول على قطعة اخرى بحجم ٣١٨ قاعدة نايتروجينية للجين المتخصص لهذه السلالة من هذا النوع. في هذا العمل نجد ان ليس جميع البكتريا المقاومة هي حاملة لجين الـ *mec A* فقط ٥٥% منها اظهرت انها حاملة لجين الـ*mecA* المسؤول عن ظاهرة المقاومة وجميعها تحمل الجين المتخصص للـ *S.aureus* و نستنتج من ذلك ان بروتين الارتباط بالبنسلين المحمول على جين الـ*mecA* هو ليس العامل الوحيد الذي يسبب ظاهرة المقاومة.

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