Development of SYBR Green Real time PCR for identification methicillin-resistance *Staphylococcus aureus* (MRSA)

الكشف عن البكتريا العنقودية الذهبية المقاومة للمثسلين Methicillin-Resistant *Staphylococcus aureus* باستخدام طريقة التضاعف التسلسلي المزدوج SYBR Green Real time PCR

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Abstract:

The increasing resistance of staphylococci to ß -lactam antibiotics has become a major clinical problem. Development of rapid and sensitive techniques for detection of MRSA is an important aim for public health. A duplex PCR were established for specific identification of methicillin-resistance *Staphylococcus aureus* (MRSA) in clinical samples. In this work a duplex SYBR Green real time PCR was developed for rapid identification of MRSA in local methicillin-resistance S. *aureus* isolates. Twenty methicillin-resistance *S. aureus* isolates, as determined by disc diffusion method, were subjected to DNA extraction and PCR amplification. Two genes were amplified successfully, *mec*A (533bp) and *fem*A (314bp), as targets for methicillin-resistance and specific identification of *S. aureus,* respectively using conventional PCR. Sensitivity of the duplex PCR showed that the minimum concentration of DNA that gave positive results for the two genes was 30ng/µl. In order to develop rapid and sensitive test for identification of MRSA, serial dilutions of purified DNA were amplified gradually according to their concentrations using SYBR Green real time PCR. These results indicated that the SYBR Green real time PCR can be used for identification of methicillin-resistance *S. aureus* (MRSA) in clinical samples.

المستخلص

تعد صفة المقاومة لدى بكتريا الـ *S. aureus* لمضادات البيتا لاكتام من المشاكل الطبية المنتشرة في العالم.لذلك يعد تشخيص بكتريا S.aureus المقاومة للمثسيلين من خلال تطوير طريقة سريعة و حساسة لهذا التشخيص من اهم الاهداف للصحة العامة . استخدمت طريقة التضاعف التسلسلي المزدوج لعينات البكتريا السريرية ، حيث تم تطوير طريقة الـ Real-Time PCR ببادئات مزدوجة للتشخيص السريع لعينات البكتريا المحلية . استخدمت عشرون عزلة بعد تشخيصها بواسطة الطريقة التقليدية (disk diffusion method) وبعد ان تم استخلاص الدنا منها و اجراء عملية التضاعف التسلسلي لها باستخدام بادئان بنجاح احدهما متخصص لصفة المقاومه *mec*A بحجم 533 قاعدة نايتروجينية و اخر متخصص لتعريف هذا النوع *fem*A بحجم 318 قاعدة نايتروجينية . ان حساسية هذا الاختبار قد وصلت الى تركيز 30مايكرو غرام /مل . ولغرض ايجاد طريقة سريعة وحساسة في الوقت نفسه للتعرف على هذه البكتريا ، اجريت عدة تخافيف متسلسلة على الدنا المستخلص وتم اجراء فحص التضاعف التسلسلي بطريقه الـ Real-Time باستخدام صبغة سايبر الخضراء . واثبتت النتائج امكانية استخدام هذه الطريقة لتعريف البكتريا المقاومة للمثيسلين في العينات السريرية .

Key words: methicillin-resistance, *Staphylococcus aureus,* (MRSA), PCR, *mec*A, *fem*A, SYBR green real time PCR.

Introduction:

Staphylococcus aureus is one of the most common human pathogens, especially in hospital patients. This gram positive bacterium is responsible for various infectious diseases, and has a great capacity to develop resistance to antimicrobial agents [1]. Accurate and rapid identification of methicillin-resistant *Staphylococcus aureus* (MRSA) in clinical specimens is essential for timely decisions about isolation procedures and effective antimicrobial chemotherapy. Numerous approaches that improve turnaround time for the identification of MRSA have been described [2].

Multiplex PCR were developed for rapid identification of MRSA through the amplification of *mec*A and *fem*A genes [3,4,5]. The *mec*A gene encoding a PBP(Penicillin Binding Protein) with low affinity with β-lactam antibiotics. The *femA* gene encodes a factor which is essential for methicillin resistance and is universally present in all *S. aureus*). The aim of this work is to develop SYBR Green real time PCR for identification of methicillin-resistance *Staphylococcus aureus* (MRSA) isolated from clinical samples in Iraq.

Materials and methods:

Staphylococcus aureus isolation and identification:

All *S. aureus* isolates were isolated from urine, boil and nasal swab samples and identified primarily by routine laboratory procedures (morphological and biochemical tests). Susceptibility of all *S. aureus* isolates to antibiotics was determined by disc diffusion method [6].

DNA extraction:

A single colony of cultivated bacteria, which had been incubated overnight, suspended into 1ml of distilled water, centrifuged at 14000xg for 2 min., then the supernatant discarded, after that 120µL of lysostaphin (10 mg/L; Sigma) was added. DNA extracted using mini DNA extraction kit (Promega) according to manufacture instructions.

Duplex PCR:

Specific primers were designed for amplification of *mecA* [7] and *femA* genes, table (1). These primers synthesized by Alpha DNA Company, Canada.

PCR reaction was conducted in 25µl of a reaction mixture containing 2µl of DNA, 12.5 µl *GoTaq*® *Green* Master (Promega, CA), (0.5 µl) 25mM $MgCl_2$, 2µl of (10 Pmol\ µl) of each primer, 2µl of distilled water. Amplification program was 1 cycle at 94°C for 1 min; 35 cycles of 94°C for 1min, 63°C for 1min, 72°C for 1min; 72°C for 10min, using the Mastercycler (Eppendorf). The amplified product was subjected to 1.5% agarose gel electrophoresis, and visualized under UV (Imagemaster VDS, Pharmacia Biotech, USA) after ethidium bromide staining.

Table (1): Primers used for the amplification of *mec*A and *fem*A genes.

Gene	Primers	Primer sequence (5'–'3)	Genebank Accession number	Amplicon size (bp)
*mec*A	Forward	AAAATCGATGGTAAAGGTTGGC		
	Reverse	AGTTCTGCAGTACCGGATTTGC	AB033763	533bp
*fem*A	Forward	CATGATGGCGAGATTACAGGT		
	Reverse	GTCATCACGACCAGCGAAAGC	GQ284644	314bp

SYBR Green Real-Time PCR

Serial dilutions of extracted DNA (4.9, 2.4, 1.2, 0.6, 0.30, 0.15, 0.07, 0.03, 0.01) µg/µl were used. Real-time PCR was carried out on Exicycler^{TM}96 (Bioneer-korea) using the *AccuPower*® 2X Greenstar qPCR Master Mix (Bioneer-korea) according to manufacture instructions. The amplification program consisted of 1 cycle of 95°C with a 5 min, followed by 26 cycles of 94°C with a 1min annealing temperature at 63°C with a 1min, and 72°C with a 1min. This is followed by dissociation curve analysis, consisting of 1min at 95°C and a gradual increase from 65°C to 90°C at a rate of 0.3°C/sec with fluorescence acquisition at each temperature transition. A negative control was included in each run to access specificity of primers and possible contamination. The threshold cycle (Ct) was determined as the PCR cycle at which increased reporter fluorescence above the baseline could be detected.

Results:

Two gene, *mec*A and *fem*A, successfully amplified identification of MRSA. DNA extracted from isolated *S. aureus* figure (1-A) and both genes were amplified and detected according to their size, *mec*A (533bp) and *fem*A (314bp) using agarose gel electrophoresis figure (1-B).

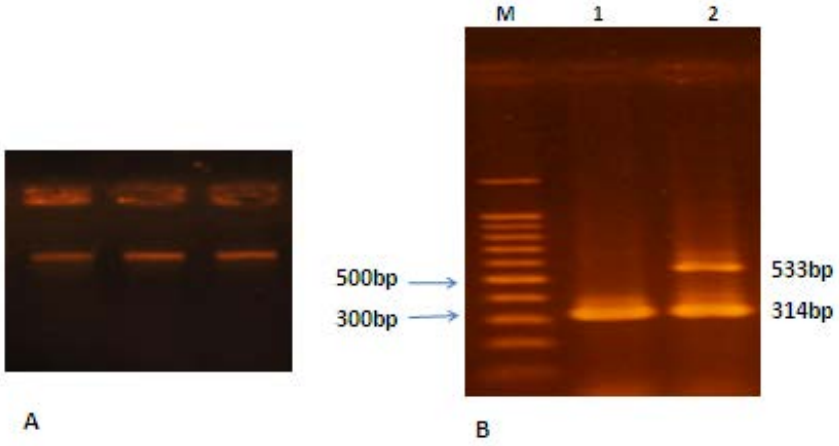


Fig (1): Ethidium bromide-stained agarose gel (1.5%) electrophoresis of: A) genomic DNA isolated from methicillin-resistance *Staphylococcus aureus* (MRSA) clinical isolates. B) DNA fragments generated by duplex PCR amplification of methicillin-resistance *Staphylococcus aureus* (MRSA). Lane (M): 100bp DNA ladder (promega), lanes (1-2): PCR product of *femA* gene (314bp) and *mecA* gene (533bp).

In order to determine the sensitivity of the assay serial dilutions of DNA were used. The lowest concentration of DNA detected by conventional PCR was (30ng/µl) fig. (2).

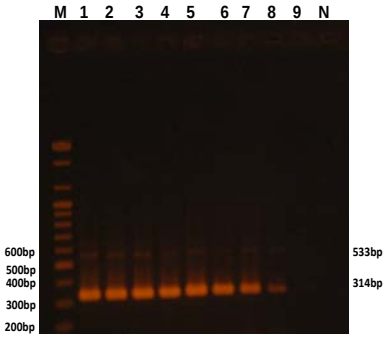
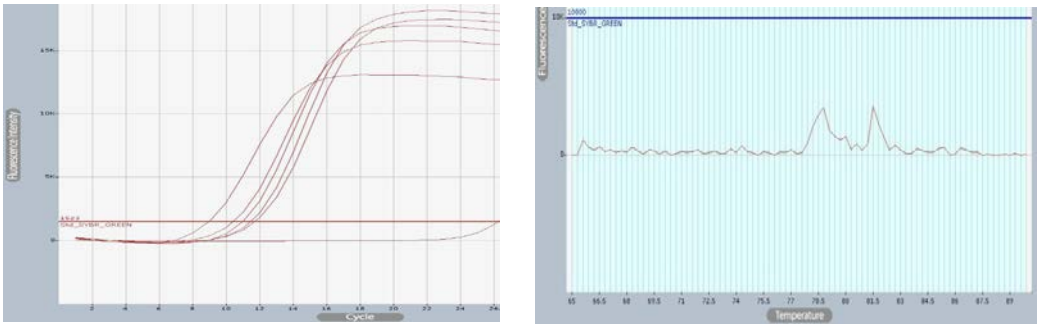


Fig (2): Ethidium bromide-stained agarose gel (1.5%) electrophoresis of DNA fragments generated by duplex PCR amplification of methicillin-resistance *Staphylococcus aureus* (MRSA) DNA dilutions. Lane (M): 100bp DNA ladder (promega), lanes (1-9): PCR product of *femA* gene (314bp) and *mecA* gene (533bp) using serial dilutions of DNA, Lane (N): PCR product of negative control (without DNA template).

SYBR Green real time PCR was used for amplification of both genes using serial dilutions of genomic DNA. Ct values were increased gradually with decreasing the concentrations of DNA. The melting curve analysis showed that the T_m of *mec*A and *fem*A were (82°C) and (79°C) respectively fig (3).



A B

Fig (3): A) Fluorescent graph and B) melting curve analysis of *mec*A and *fem*A genes amplified using SYBR Green real time PCR.

Discussion:

The use of PCR has increased the speed and accuracy for identification of *S aureus* and confirmation of MRSA. For the routine diagnostic laboratory, a multiplex PCR assay using detection of the *mecA* and *femA* genes has been suggested as one approach to rapidly identify MRSA from cultures. This was found to be more accurate and sensitive than routine, culture-based analysis, with results available from the initial use of this technology in 24 hours [8, 9]. SYBR Green real time PCR were used for rapid detection of pathogens in order to avoid the disadvantages of ethidium bromide-agarose gel electrophoresis and facilitates the monitoring of the reaction as it progresses [10]. In this work we detected the MRSA by targeting two genes recognized the methicillin resistance and species specific genes, *mec*A and *fem*A genes respectively. Pair of primers was designed according to general rules for primer design [11] for *fem*A gene to be co-amplified with *mec*A gene in duplex PCR.

The main aim in this work is the gradual detection of serial concentrations of template DNA in order to determine the sensitivity of the assay. In this work the lower concentrations were detected at cycle number 12, and it can be more if we use

lowest concentrations. So the sensitivity of this assay will be superior to conventional PCR. Melting curve analysis showed the presence of both genes as indicated by two peaks. Also we can improve this assay to be used for quantification by providing a suitable standard gene. Another studies were amplified both genes using Taqman chemistry for rapid identification of MRSA [12]. Taqman more specific than SYBR Green but more expensive.

In conclusion this assay can be used for rapid detection of *S aureus* or MRSA isolated from clinical samples. Therefore, the detection of additional targets was not needed. With the benefit of obtaining reliable results in a few hours as opposed to days, real-time PCR can be used for rapid, accurate detection of MRSA that can lead to optimal therapeutic treatment at the earliest time.

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