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Studying the Frequency of Methicillin-Resistant *Staphylococcus aureus* Through the Molecular Detection of *mec*A

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Abstract

Background Methicillin-resistant *Staphylococcus aureus* is a major cause of serious nosocomial infections and it is very important to have a reliable test to detect these bacteria. *mec*A encodes the penicillin binding protein 2a, which is associated with staphylococcal methicillin resistance.

Objective The study was to determine the frequency of methicillin-resistant *Staphylococcus aureus* in different specimens from Iraqi patients and to genetically characterize and type the samples of methicillin-resistant *Staphylococcus aureus* through the detection of *mec* A gene.

Methods Sixty clinical isolates of *Staphylococcus aureus* were submitted to DNA extraction. Genomic DNA was submitted to conventional polymerase chain reaction assays, employing MR1-MR2 primers (primer set). The results were compared to the cefoxitin disks agar diffusion method.

Results Fifty seven of the sixty isolates showed positive results for *mec*A amplification while three isolates (5%) showed negative results for *mec*A gene.

Conclusion Good correlation between the *mec*A gene detection by PCR and the cefoxitin disk diffusion methods was obtained.

Key words *mec*A gene, *Staphylococcus aureus*, methicillin resistance *Staphylococcus aureus*

List of abbreviation: MRSA = Methicillin-resistant *Staphylococcus aureus*, PBP2a = penicillin-binding protein2a, OSS = oxacillin salt screening, PCR = polymerase chain reaction

Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is a major cause of the increasingly prevalent, difficult and expensive-to-treat nosocomial infections worldwide. Methicillin-resistance in staphylococci constitutes resistance to all of the β-lactam antibiotics and their derivatives. The major mechanism of resistance is the acquisition of the *mec*A gene that encodes for additional penicillin-binding protein2a (PBP2a) [(1)]. The phenotypic methods such as cefoxitin disk agar diffusion and oxacillin salt screening test (OSS) are widely used in routine microbiological laboratory for the detection of MRSA [(2,3)]. The problem with phenotypic methods is that they can be influenced by culture condition such as temperature, medium pH and NaCl content in the medium [(4)]. Several PCR-based methods have been developed to detect the *mec*A gene [(1,5)]; however, one pair of PCR primers are most commonly used [(1,6)]. The purpose of the present work was to determine the frequency of MRSA in different specimens from Iraqi patients and to genetically characterize and type the isolated MRSA through the detection of *mec*A gene.

Methods

Bacterial isolates

One hundred and five samples were collected from different body sites and lesions (UTI, wounds, and ear swabs...etc.) of in-and-out patients from both sexes who attended Al-Imamain Al-Kadhimain Medical City, Baghdad during the period from November-2012 to March-2013. The isolates were identified using standard microbiological procedures [(7)] in Medical Microbiology Laboratories of College of Medicine, Al-Nahrain University. API Staph System, produced by bio-Merieux was also used for the confirmation of the primary identification.

Cefoxitin sensitivity test

According to the method described by Murakami [(1)], olonies from an overnight nutrient agar plate culture were transferred to a tube containing 3 ml of 0.85% normal saline**.** The turbidity was adjusted to 0.5 McFarland equal to 1.5×10^8 CFU/ml.

A sterile cotton swab was dipped into the bacterial suspension; excess fluid was removed by pressing the swab against the tube wall. The bacterial suspension was inoculated into Muller Hinton agar plates and the plates were left to dry for 10 minutes. Each individual petri dish was divided into two sections so as to test two different isolates simultaneously. The antimicrobial discs of cefoxitin (30 µg) were placed on the surface of the medium using sterilized forceps and the plates were incubated at 37 ^{o}C for 24 hour.

When the incubation was complete, the zones of inhibition around the disks were measured and compared with the break points of Clinical Laboratory Standards Institute (CLSI) [(2)] and decided as susceptible (S) and resistant (R).

DNA extraction

DNA was extracted according to Vogelstein [(8)] adopted by the manufacturer of DNA extraction kit **(Geneaid)®.**

Step 1: Cell Harvesting/Pre-lysis

Bacterial cells (1×10^9) were transferred to a 1.5 ml microcentrifuge tube and they were centrifuged for 1 minute at 14-16,000 xg and the supernatant was than discarded. Two hundred µl of lysozyme buffer were added to the tube and the cell pellet was re-suspended by vortexing or pipetting, after that, the lysate was incubated at room temperature for 10 minutes. During incubation, the tube was inverted every 2-3 minutes. Lysis Step of the Cultured Cell Protocol was then preceded.

Step 2: Cell Lysis

Two hundred µl **of GB Buffer** were added to the sample and vortexed for 5 seconds. It was incubated at 70 ^{o}C for 10 minutes or until the sample lysate is clear. During incubation, the tube was inverted every 3 minutes. At this time, the required **Elution Buffer** (200 µl per sample) was incubated at 70 ^{o}C (for Step 5 DNA Elution).

RNA Degradation

After incubation in water bath at 70ºC, 5 µl of RNaseA (10 mg/ml) was added to the sample lysate and mixed by vortex. Then, it was incubated at room temperature for 5 minutes.

Step 3: DNA Binding

Two hundred µl of absolute ethanol was added to the sample lysate and vortexed immediately for 10 seconds. Precipitate was broken by pipetting. GD Column was placed in a 2 ml collection tube; all of the mixture (including any precipitate) was transferred to the GD column. The mixture was centrifuged at 14-16,000 xg for 2 minutes and 2 ml collection tube containing the flow-through was discarded and the GD column was placed in a new 2 ml collection tube.

Step 4: DNA Washing

Four hundred µl of W1 Buffer were added to the GD column. The mixture was centrifuged at 14-16,000 xg for 30 seconds. The flow-through was discarded and the GD column was placed again in the 2 ml collection tube, six hundred µl of Wash Buffer was added to the GD column. The mixture was centrifuged at 14-16,000 x g for 30 seconds. The flow-through was discarded and the GD column was placed again in the 2 ml

collection tube, to dry the column matrix, it was centrifuged for 3 minutes at 14-16,000 x g.

Step 5: DNA Elution

The dried GD column was transferred to a clean 1.5 ml microcentrifuge tube. One hundred (100) µl of preheated elution buffer or TE were added to the center of the column matrix. Let stand for 3-5 minutes or until the elution buffer or TE is absorbed by the matrix. The final step was the centrifugation at 14-16,000 xg for 30 seconds to elute the purified DNA.

Polymerase chain reaction PCR

The sequence of oligonucleotide primers (MR1 and MR2) that were used in conventional PCR to detect the presence of *mec*A was according to [9] and synthesized in Bioneer® (South Korea).

MR1 F GTG GAA TTG GCC AATACA GG
MR2 R TGA GTT CTG CAG TAC CGG AT

The DNA template of *S. aureus* was prepared and the primers (*mec*A gene) were diluted by adding nuclease free water according to the manufacturer instructions. The master mix contents were thawed at room temperature before use. One µl of template DNA was transferred to master mix tubes which contained 5µl of master mix ; 1µl of the diluted primers was added to the tubes. The volume was completed to 20 µl with Deionized Nuclease – Free water (12 µl for conventional PCR) as shown in table 1.

Table 1. Composition of PCR reaction mixture used for amplification of *mecA* gene (Convetional PCR)

Reagents	Volume (µl)	Concentration (pmol) *mecA* gene
Forward Primer	1	0.988
Reverse primer	1	1.042
DNA template	1	
PCR mastermix	5	2x
(DNAse free) water	12	
Total volume	20	

Tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components. The tubes were placed on the PCR machine and the PCR program, with the right cycling conditions pre-installed, was started. Cleaver Scientific Thermal Cycler TC32/80 was used for all PCR amplification reactions. PCR mixture without DNA template (non-templete negative control) was used as negative controls (Table 2).

Table 2. The PCR thermocycler program for *mecA*

Steps	Temp.	Time	Cycles
Initial denaturation	94 °C	5 min	
Denaturation	94 °C	60 sec	30
Annealing	60 °C	60 sec	
Elongation	72 °C	70 sec	
Final extension	72 °C	10 min	
Hold	4 °C		

Electrophoresis

DNA samples were electrophoresed by horizontal agarose gel electrophoresis according to Sambrook and Russell [10] Agarose at concentrations of 2% was prepared, the agarose was left to cool at 60 °C before adding ethidium bromide in a concentration of 0.5 µg/ml and poured into the taped plate. A comb was placed near one edge of the gel. The gel was left to harden until it became opaque; gently the comb and tape were removed. TBE buffer (0.5X) was prepared, poured into the gel tank and the slab was placed horizontally in electrophoresis tank. About 5 µl of prepared loading buffer, was applied to each 10 µl of DNA sample. The wells were filled with the mixture by a micropipette and adding 5 µl of 100bp DNA ladder to one well. The power supply was set at (5 V/cm (70) for 1 hour) for genomic DNA and PCR products electrophoresis.

Results

Cefoxitine sensitivity testing

By the disc-diffusion method, Staphylococcal isolates were tested for their sensitivity to

cefoxitine (30 mg). A zone of inhibition with a diameter of ≤ 21 mm was considered as an indication for resistance to methicillin (Fig. 1).

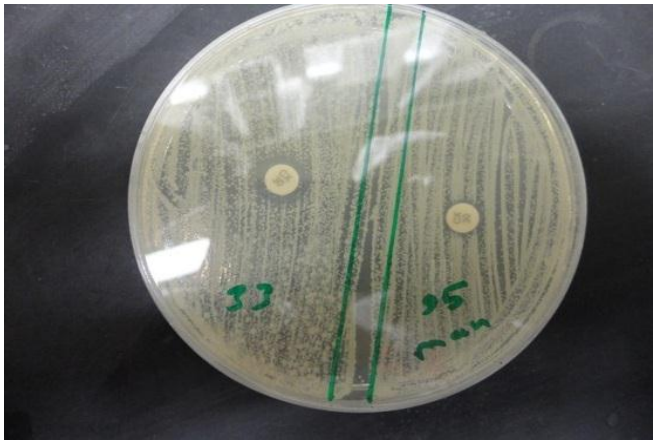

Fig. 1. Antibiotic sensitivity profile of the isolates enrolled in the current study. A zone of inhibition with a diameter of ≤ 21 mm was considered resistant to methicillin

Out of the one hundred and five samples enrolled in this study there were only sixty samples (57.1%) showed resistance to cefoxitin (30 mg) and were considered as methicillin resistant *Staphylococcus aureus*.

DNA extraction

The final concentration of extracted DNA ranged from 4.9 to 167.8 ng/µl and purity ranged from 1.17 to 1.9.

Polymerase Chain Reaction (PCR)

Conventional PCR screening for *mecA* gene

The results of the present study indicated that *mec*A was successfully amplified in fifty seven out of the sixty MRSA isolates, with a product size of 1339 bp, (95 %) of the isolates while only three isolates (5%) lacked this gene and gave negative amplification results (Fig. 2).

Discussion

The frequency of MRSA in many countries is increasing in hospitals in some areas, more than half of all *S. aureus* disease isolates are MRSA. MRSA strains are becoming increasingly multi-resistant, and have developed resistance to vancomycin, used successfully to treat MRSA for more than 30 years. Nosocomial methicillin-resistant *Staphylococcus aureus* infections represent a major challenge to hospital microbiologists because of the emergence and spread of clones with decreased susceptibility to many antibiotic classes. Since the mid to late 1990s, hospital MRSA isolates have increased in prevalence in Europe, the USA and elsewhere (European Antimicrobial Resistance Surveillance System (EARSS) annual report 2001) [(11)].

In one European study of 25 university hospitals, one-quarter of 3051 *S.aureus* isolates collected were MRSA, with a geographical bias towards higher rates in southern countries such as Italy (50.5%) and Portugal (54%), and lower rates in northern European countries, including Netherlands (2%), Austria (9%) and Switzerland (2%) [(11)]. Epidemiological data on MRSA in Africa are scarce. The prevalence of MRSA was determined in eight African countries between 1996 and 1997 and was relatively high in Nigeria, Kenya, and Cameroon (21 to 30) and below 10% in Tunisia and Algeria [(12)]. In Algeria, the rate of MRSA increased to 14% in 2001. MRSA infections are associated with increased morbidity; mortality and length of hospital stay, and represent a major financial burden on healthcare services [(13)].

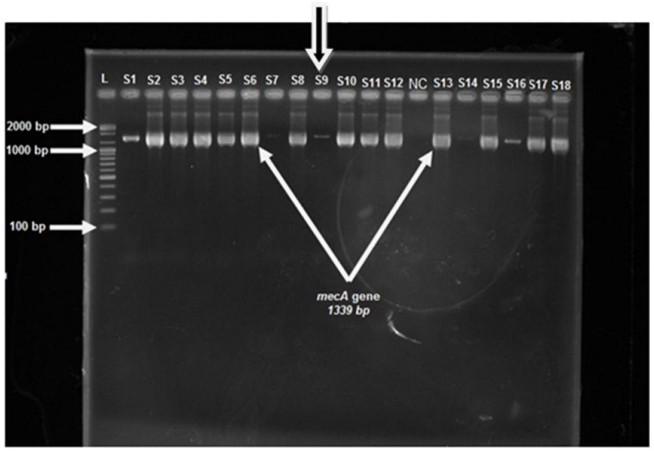

Fig. 2. Gel electrophoresis of conventional PCR products of *mecA*gene (1339bp); negative control; MW,2000 bp ladder; (2% agarose, 5v/cm (70)1hr), the (N.C) between S12 and S13.

The rapid development of resistance is due to mutational events and/or gene transfer and

acquisition of resistance determinants, allowing strains to survive antibiotic treatment. Methicillin-resistant staphylococci depend on efficient penicillin binding protein (PBP2′) production and are modulated by chromosomal factors. Depending on the genetic background of the strain that acquired *mecA*, resistance levels range from phenotypically susceptible to highly resistant. A common characteristic of most methicillin-resistant staphylococci is the heterogenous expression of resistance, which is due to the segregation of a more highly resistant subpopulation upon challenge with methicillin. Maximal expression of resistance by PBP2′ requires the efficient and correct synthesis of the peptidoglycan precursor. Genes involved in cell wall precursor formation and turnover, regulation, transport, and signal transduction may determine the level of resistance that is expressed [(14)].

Detection of the *mecA* gene or its product, penicillin binding protein (PBP2a), by PCR is considered the gold standard for MRSA detection [(15)]. In this study, however, three PCR negative isolates out of the total sixty isolates enrolled in the present study were recorded and this might be explained by some other mechanism rather than the absence of the *mec*A gene. These mechanisms are: 1) *mec*-encoded resistance, 2) overproduction of penicillinase and 3) modifications of normal penicillin-binding proteins [(16)].

Oxacillin may fail to detect them while cefoxitin is strong inducer for production of PBP2a, and do not appear to be affected by hyper-production of penicillinase which may show methicillin resistant [(17)]. Further, cefoxitin has higher affinity for staphylococcal PBP4 than that for PBP2 and overproduction may also contribute in methicillin resistant [(18)]. The present study emphasized the use of a cefoxitin disc diffusion (DD) test for the detection of meticillin resistance in staphylococci. A total of 60 clinical isolates of *Staphylococcus aureus* showed resistant cefoxitin (30 mg disc) as an indication for methicillin resistance. The sensitivity and specificity of the cefoxitin DD test were (95%). The accuracy of the cefoxitin DD test was better than that of the oxacillin DD test for the detection of MR staphylococci. It also does not require special testing conditions such as a lower incubation temperature (35 ^{o}C) and NaCl supplementation in the testing media, as required by the oxacillin DD test [(19)]. Cefoxitin is considered to be a better predictor than oxacillin for the detection of heteroresistance because it is a stronger inducer than oxacillin of penicillin-binding protein 2a (PBP2a) [(18)].

In the current study, sixty samples of 105 showed resistance to cefoxitin (30 mg) (57.1%) and can be considered as methicillin resistant *Staphylococcus aureus*. The widespread emergence of MRSA, especially in various types of nosocomial infections, is a serious clinical problem worldwide. The incidence of methicillin resistance among nosocomial isolates of *S. aureus* is higher than 70% in some Asian countries such as Taiwan, China, and Korea (20).

It is concluded from this study that there is good correlation between the *mec*A gene detection by PCR and the cefoxitin disk diffusion methods.

After this research it is recommended that a large scale multi-center studies are being done both in human patients and normal healthy population to determine more precisely MRSA prevalence depending on mec *A* gene and depending on this study purpose of determination the frequency of methicillin-resistant *Staphylococcus aureus* (MRSA) in different specimens from Iraqi patients and to genetically Characterize and type the samples of methicillin-resistant *Staphylococcus aureus* (MRSA) through the detection of *mec* A gene.

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Author Contribution

Rafeef conducted the sampling, isolation, and molecular work. Ahmed and Amir guided and finished writing and editing the study.

Conflict of Interest

The authors declare no conflict of interest.

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