Phenotypic and Genotypic Detection of Methicillin Resistance in Locally Isolated Staphylococcus aureus

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Total (150) isolates of Staphylococci species were isolated from different clinical samples. They were distributed as (41) isolates from urine, (59) isolates from wound infections and (50) isolates from ear swabs. These isolates were diagnosed using different morphological and biochemical test followed by the complementary api 20E test and mast staph kit. Out of 150 isolates, (58%) were Staphylococcus aureus. Results of primary screening test for methicillin resistance using disk diffusion method revealed that 84 isolates (96.5%) were proved to be methicillin resistant Staphyllococcus aureus (MRSA). Twenty five isolates were selected for the genotypic study to detect methicillin resistance gene (mec A) using the polymerase chain reaction (PCR). Two target sequences related to this gene were chosen in the current study, the first represent the total gene segment (1339 bp) since the forward and the reverse primers were picked up from the upstream and the downstream region from the original gene while the other amplified segment represent part of the gene (314 bp). Total genomic DNA was used as a Template and it was prepared either by direct colony or by boiling method using distilled water or TE buffer. Results showed that all the selected 25 isolates (100%) were harboring mec A gene based on the presence of 314 bp or 1339bp clear bands in 1% agarose gel as compared with DNA ladder. Direct colony was better in preparing the template DNA compared with the boiling method which gave a negative results with the two amplified segments.
INTRODUCTION
Methicillin resistant *Staphylococcus aureus* (MRSA) is a significant pathogen causing both nosocomial, community acquired infections, and its high prevalence in hospitals has been reported from many Countries[1]. Since most of these bacteria carry multiple resistance genes against commonly used antibiotics, they show multiple antibiotic resistance patterns thus causing important treatment problems[2]. MRSA has the ability to resistance β-lactam antibiotics either by production β-lactamase enzyme which binds specifically to β-lactam ring in the β-lactam antibiotics rendering them inactivate[3] beside that, resistance could be due to altering or modification target site represent by penicillin binding proteins((PBP)), once the organism alters the PBP, the β-lactam antibiotics significantly decreases its affinity for their substrate that is to say to PBP [4] The low-affinity to (PBP2a) is encoded by mec A gene which represent the main factor responsible for methicillin resistance in *Staphylococcus*[5] The mec A gene have never been found in methicillin-susceptible *Staphylococcus aureus*(MSSA) , while they have been detected in almost all MRSA isolates[6]. The mecA gene becomes a useful molecular tool for identification of MRSA since molecular techniques( mostly based on polymerase chain reaction ) had been used for the rapid detection of MRSA[7]. The aims of this study are to isolate *S. aureus* from different infections then to detect MRSA using primary screening test followed by genotypic detection for mecA gene using PCR technique using DNA template prepared by different methods.

MATERIALS AND METHODS
Specimen's collections and Diagnosis:
Total of (150) clinical isolates primary identify as *Staphylococcus species* were obtained from hospitalized and non hospitalized patients from two hospitals: Al-habibia and Noaman hospital in Baghdad city during the period from January to April 2011. Types and the numbers of these clinical samples were distributed as (41) isolates from urine, wounds include (59) isolates from wound infections and (50) isolates from ear swabs. Bacterial diagnosis including morphological and biochemical tests were done according to [8] which involve coagulase test and the culturing on specific media like mannitol salt agar, staph 110 and Baird parker agar. The diagnosis was followed by the complementary api 20E test and mast staph kit for *S. aureus* and the latter was done according to (BioMérieux /France)and (MastGroup Ltd., Bootle, Merseyside, / U.K)
Phenotypic detection of Methicillin resistant
All isolates were tested for primary screening test of antimicrobial susceptibility depending on the [9]. Disk diffusion tests was done by placing 5µg/disc of methicillin on Mueller-Hinton agar cultured with the bacterial isolate. The zone of inhibition was measured after 24 hours of incubation at 37°C and the results were compared with the negative control represent by the standard strain ATCC 25923.

PCR amplification:
The selected resistance isolates with positive phenotypic tests were subjected to molecular screening study using PCR amplification technique according to following steps:

DNA template preparation
The preparation of DNA template was conducted by two ways:
1-direct colony: It was performed according to [10] in which a sterile wooden stick was simply attached with a single isolated bacterial colony then it was carefully suspended in the wall of Eppendorf tube containing the PCR mixture, then it was mixed by vortex to get fully dissolved mixture.
2-Boiling method
TE buffer method: Template DNA was prepared by dissolving 1µl of bacteria in 1ml of TE buffer, centrifuged at 5000 rpm for 5 min, and the pellet resuspended in 100µl of TE buffer. The suspension was boiled at 100°C for 10 min before centrifugation at 5000 rpm for 5 min. The supernatant served as PCR template [11]
Distilled water method: It was prepared according to Ruppé et al.[12] Briefly, 5 isolated colonies of overnight growth bacteria were suspended thoroughly in 1 mL distilled water and boiled in a water bath for 10 min. After centrifugation, supernatant was used as template DNA for the PCR.

Primers used for PCR: Oligonucleotide primers specific for the methicillin resistant genes(mec A) were chosen in this study to get two amplified segment, one of them represent the total gene sequence since it was picked from the upstream and the downstream region and the amplified size was 1339bp, the other represent part of the gene with amplified size 314bp[14]. Table 1 shows the details of these two primers.
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Table-1 : The primers used in the current study for PCR amplification.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer name</th>
<th>Nucleotide sequences and direction 5<code> −−−−−−→3</code></th>
<th>MW μg/µmol</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA</td>
<td>mecA1 (F)</td>
<td>CCTAGTAAAGCTCCGGAA</td>
<td>1024</td>
<td>314</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>mecA1 (R)</td>
<td>CTAGTCCATTTCGCTGTCGA</td>
<td>732</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mecA</td>
<td>mecA2 (F)</td>
<td>GTGGAATTGGCCAAATACAG</td>
<td>6296</td>
<td>1339</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>mecA2 (R)</td>
<td>TGAGTTCTGAGTACCGGAT</td>
<td>6148</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR amplification procedure: Twenty five selected isolates were submitted to genotypic study using PCR. The oligonucleotide primers specific for the mecA genes (table1) were diluted using nuclease free water according to the manufacture company information (alpha Canada) to get primary concentration equal to 100 pmol. The amplification was performed in a TECHNE (TC-3000) thermal cycler and the reaction mixture was prepared according to the procedure that suggested by the manufacture company (Promega, USA). PCR mixture was composed from 12.5 µl of GoTaq® Green Master Mix(2x), 1.5 µl (10 pmol) from each forward and reverse primers, 5 µl of DNA template (prepared by boiling method) and 4.5 µl of nuclease free water to get final volume of 25 µl. In case of using direct colony method, the volume of the DNA template was considered zero and the volume of the PCR mixture was completed with nuclease free water (9.5 µl instead of 4.5 µl) to get final volume of 25 µl. PCR mixture without DNA template was used as a negative control. Table 2 represent Conditions for PCR amplification which were set according to this study for the two primers and was started with predenaturation step at 95°C for 5 min followed by 30 repeated cycles of denaturation at 95°C for 45 sec, annealing step at 56°C for 45 sec (annealing temperature was increased to 60°C for 1 min for mecA2) and an extension step at 72°C for 45 sec for mecA1 (72°C for 1 min for mecA2), finally one extension step at 72°C for 7 min. The amplified PCR product were analyzed by agarose gel electrophoresis according to [13] using 1% agarose supplied with 0.5 μg/mL ethidium bromide for 1 hour and a half (7 Volts/cm²). DNA ladder (100bp and 1000bp) were used to assess PCR product size, then PCR products were visualized by UV light at 336 nm, and photographs were taken using digital camera.

Table-2: PCR programs applied in this study.

<table>
<thead>
<tr>
<th>PCR</th>
<th>Initial Denaturation</th>
<th>Cycles No.</th>
<th>Denaturation</th>
<th>Primer annealing</th>
<th>Elongation</th>
<th>Final elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA</td>
<td>95°C/5 min</td>
<td>30</td>
<td>94°C/45 sec</td>
<td>58°C/45 sec</td>
<td>72°C/45 sec</td>
<td>72°C/7 min</td>
</tr>
<tr>
<td>mecA</td>
<td>95°C/5 min</td>
<td>30</td>
<td>94°C/45 sec</td>
<td>60°C/1 min</td>
<td>72°C/1 min</td>
<td>72°C/7 min</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Spread of methicillin-resistant Staphylococcus aureus (MRSA) and coagulase-negative Staphylococci (CNS) is an important concern in hospitals and other health care settings[15]. In the current study, a total of 150 specimens showed significant growth as Staphylococcus species. To distinguish S.aureus from others, the isolates were submitted to different biochemical test including culturing on mannitol salt agar and here S.aureus isolates were able to grow on this media converting the
red color to yellow due to mannitol fermentation [16] while growing on Baird-Parker agar, the colonies of this species appeared black, shiny, circular and convex, surrounded by a clear zone[17] Coagulase test also was performed since it considered a remarkable diagnostic test and only *S. aureus* has the ability to give positive result [18] Another important diagnostic test was performed using specific kit known as Mast staph which is an agglutination test specific for detecting protein A in this species. Positive results appeared as a clear agglutination after a few seconds from the addition of one drop mast staph solution, while the negative result appears transparent (clear) without any agglutination according to (Mastgroup ltd., Bootle, Merseyside, /U.K) catalog. figure 1 represent a positive and negative results.

Out of 150 isolates, 87 (58%) diagnosed as *S. aureus* and 63 (42%) isolates include other *Staphylococcus* spp. This result is comparable to Rallapalli [19] who reported that out of 150 Staphylococcal clinical isolates only 95 isolates were *S. aureus* (63.33%), while results of Farzana and Hameed [20] illustrated that out of 2580 Gram-positive cocci, 1688 isolates were *S. aureus* (65.5%). This percentage may be differ from time to time according to source of the isolates, nature and size of the samples, the type of study and the geographic location that samples were taken from. By using disk diffusion method to detect methicillin resistance, 84 MRSA were identified out of 150 isolates (96.5%) while the remaining 3 isolates (3.5%) showed their susceptibility for methicillin (MSSA). Kader et al. [21] reported that (88.24%) isolates were resistant to methicillin and Oxacillin, while Odonkora and Addob[22] reported that methicillin disc diffusion test detected 54 (21.6%) representing MRSA while 196 (78.4%)
representing MSSA and this result is less than the result of the current study and other studies. Disc diffusion test or the phenotypic method in some cases has low specificity and sensitivity for the detection of MRSA and other type of resistance since the phenotypic expression is influenced by many factors such as inoculums size, incubation time, temperature, pH and salt concentration of the medium[23]. Twenty five isolates of MRSA which gave positive results in phenotypic detection were selected for the genotypic study by PCR technique using two different gene sequence, the first one represent the total genomic segment since the forward and reverse primers were picked up from the upstream and downstream region of the original gene sequence with amplified size equal to 1339bp [13]. The second represent part of the gene and the amplified size was 314 bp [14]. To perform the test, two process was used for template DNA preparation. The results revealed that using the direct colony was more efficient in expressing the results as compared with the boiling method whether distilled water or TE buffer was employed in spite of repeating the experiments for several time, hence that the first method was followed. The results of PCR experiment revealed that all selected isolates demonstrating resistance to methicillin and expressing of mecA gene. Figur 2 illustrates number of isolates harboring mec A based on the presence of 1339 bp bands (as compared with the ladder DNA ladder) on 1% agarose gel.
Figure-2: Agarose gel electrophoresis (1% agarose, 75 v/cm) for mecA-2 gene (amplified size 1339bp as compared with 1000bp DNA ladder) using template DNA prepared by direct colony. Line 1-12 represents positive results. This result is close to the result of Rallapalli (19) who showed that all the 40 isolates determined as MRSA by phenotypic methods, gave positive results using PCR. Also Kader et al. [21] reported that all the 34 strains were harboring mecA gene. The same result was obtained in the current study when another amplified sequence was used and all the selected isolates gave positive results for mecA gene. Figure (3) shows number of isolates harboring mec A based on the presence of 314 bp bands (as compared with 100bp DNA ladder) on 1% agarose gel
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Figure-3: Agarose gel electrophoresis (1% agarose, 75 v/cm) for *mecA* gene (amplified size 314bp as compared with 100bp DNA ladder) using template DNA prepared by direct colony. Positive results in lines 1-10.

Usually those isolates possessing an altered PBP2 are resistant to methicillin, oxacillin, and probably to all other currently available β-lactam antibiotics and such isolates which responsible for serious nosocomial infections will require administration of non β-lactam antimicrobial therapy[23]. Rapid and accurate detection of methicillin resistance in *S. aureus* is important for using other appropriate antimicrobial therapy beside controlling nosocomial spread of MRSA strains[24]. Identification of the *mecA* gene remains the most reliable method of detecting MRSA isolates since there is no optimal phenotypic method for detecting methicillin resistance in *S. aureus*, however not all laboratories can include molecular biology techniques in their routine clinical practice. In conclusion, molecular techniques remain the most sensitive method in detecting *S. aureus* at both genus and species level and with 100% accuracy in detecting MRSA, as compared with the classical identification method.

REFERENCES

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