NUCLEOTIDE SEQUENCE ANALYSIS OF METHICILLIN RESISTANCE 
STAPHYLOCOCCUS AUREUS IN KURDISTAN REGION-IRAQ

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Abstract

In this study a total of 241 clinically important specimens were collected from three hospitals in Kurdistan region; from which 64 isolates of S. aureus were isolated representing 26.5% of the total samples. The molecular confirmation of S. aureus isolates were done by PCR using nuc primer as a species-specific primer producing 280bp DNA fragment for nuc gene. 31 of these isolates were resistance to methicillin representing 48.4% of total isolates. Resistance to Methicillin was confirmed by using mecA primer producing 310bp DNA fragment for mecA gene. Three isolates (SaDu5, SaEr17 and SaSul24) were subjected to partial DNA sequencing of the mecA gene to assess the relation of Kurdistan isolates with other global sequences from GenBank. Sequence analysis showed that the sequence identity of all three mecA genes among the Kurdistan isolates was ranged between 97.5-98.5%, whereas the sequence identity among the Kurdistan isolates and other global isolates selected from GenBank was ranged between 92.6-96.1%. The sequenced product of mecA genes from Kurdistan found similarities and showed partial homology with S. aureus strains (UK/NTCC124, Turkey/HMS, South Korea/ YSSA11 and India/TN/CN/1/12) penicillin binding protein 2a mecA gene, partial cds, which is available in public databases. The phylogenetic tree of the aligned sequences was produced by using MEGAS Beta version 6.1 online software, and showed a typical relatedness among Kurdistan isolates and GenBank reference strains. This study will gives insights in to the molecular diagnosis of S. aureus and to find better synthetic drugs or organic to control expression of antibiotic genes in different pathogenic organism in general and in particular MRSA strains.

Key words: MRSA, mecA gene, DNA Sequencing.

Introduction:

Staphylococcus aureus is one of the most significant and serious human pathogens, which is known as a threat to human health for more than a century. This pathogen is responsible for a wide range of diseases, causing both nosocomial and community-acquired infections (Askari et al., 2012). Acquisition of resistance to several antimicrobial agents by this pathogen has been a major problem for the treatment of infections in the hospital and the community settings. Resistance to β-lactam antibiotics such as methicillin and oxacillin, is named "methicillin resistance" and is considered of great importance since the multidrug resistant phenotype of MRSA strains and their intrinsic β-lactam resistance make them difficult and costly to treat (Paradisi et al., 2001). Most strains of Methicillin Resistance S. aureus (MRSA) are also resistant to other β-lactam antibiotics, with the exception of glycopeptide antibiotics (Tiwari et al., 2006). Methicillin resistance is mediated by the presence of PBP-2a which is highly conserved among staphylococci and is encoded by the chromosomal gene mecA (Malik et al., 2006). mecA is located on a genetic element called the staphylococcal cassette chromosome (SCC) in Staphylococcus aureus. SCCmec is a group of mobile DNA fragments of about 21 to 67 kb that is integrated into the chromosome of methicillin resistant S. aureus at a unique site (attBscC) located near the S. aureus origin of replication (Hanssen et. al., 2004). The incidence of MRSA has become increasingly prevalent in the world since it first reported. Subsequently, the occurrence of MRSA particularly in hospitalized patients has increased steadily and nosocomial infections caused by such strains have become a serious problem worldwide. Therefore, rapid and accurate identification of MRSA is essential (Terry et. al., 2011). Improved strain characterization would improve our understanding of the epidemiology of this pathogen and will allow the development of a rapid assay for monitoring and controlling of MRSA. In view of this, the detection of mecA gene has been used as an alternative way of detecting or confirming MRSA either by use of DNA probe, commercially available fluorescence test, latex agglutination test, and PCR (Ieven et. al., 1995, Cavassini et. al., 1999 and Rohrer et. al., 2001). Molecular typing techniques are widely applied in studies of S. aureus epidemiology, especially MRSA, with the aim of distinguishing isolates that are epidemiologically related from those unrelated. S. aureus outbreaks in hospitals are frequently
considered as short duration events of localized epidemiology and, in these situations, most of the molecular typing methods are able to distinguish the isolates that belong to the outbreak (Shopsin and Kreiswirth, 2001).

The rapidly expanding number of sequenced microbial genomes has served as a catalyst for the development of a variety of molecular typing approaches that focus on either single or multiple chromosomal loci. Single loci sequence typing (SLST) Sequence data for specific loci (genes for virulence, pathogenicity, drug resistance …etc.) from different strains of the same species have revealed variability in a specific gene, such as single-nucleotide polymorphisms and areas with repetitive sequence that demonstrate potential for epidemiologic application. SLST shows potential for a typing and clearly represents an important and promising sequenced-based approach to epidemiologic analysis (Singh et al., 2006). Thus the objectives of this study were molecular identification of S. aureus by using nuc primer as a species-specific primer and to determine the nucleotide sequence of mecA gene. Furthermore, analysis and determine the phylogenetic diversity and nucleotide sequence comparison of S. aureus isolates depending on the sequence of mecA gene in Kurdistan Region-Iraq.

Materials and Methods:

Bacterial isolation
Isolates of S. aureus used in this study were collected from Azadi, Dr. Khalid and Rizgary General Hospitals in Duhok, Koya and Erbil cities respectively in Kurdistan Region-Iraq. These samples were taken from patients, healthcare staffs and hospital environment. Basing on the source of infections, the isolates were obtained from: urine, nose, throat, wounds, burns, skin infections and hospital environments. These isolates were confirmed at molecular level as S. aureus by using nuc primer.

Antimicrobial Susceptibility Testing
Disk diffusion testing was performed as recommended for staphylococci by Clinical and Laboratory Standards Institute (CLSI, 2012). Strains were considered susceptible to oxacillin if the zone of inhibition around the 1μg disks was >13 mm, intermediate if 11-12 mm, and resistant if the zone was < 10 mm.

Oligonucleotides sequences
Oligonucleotides of nuc gene as species-specific primer and mecA for Methicillin Resistant gene detection were used in this study provided by MWG Company, Germany (Table: 2) (Merza 2009).

PCR amplification:
The PCR amplification was carried out in 50-μl reaction mixture volumes containing 4μl of genomic DNA, 2 μl of each primer (50 pmol/μl), 5 μl of 10× PCR buffer (100mM Tris-base pH8.3, 500mM KCl, 20mM MgCl₂ and 0.001 gelatin), 5 μl of 100 mM deoxynucleoside triphosphates, and 2 units of Taq DNA polymerase (Gold Taq). DNA amplification was carried out in Applied Biosystem (USA) thermocycler, with the following thermal cycling profile for nuc gene: initial denaturation at 94°C for 4 min was followed by 30 cycles as follows: denaturation at 94°C for 45sec, annealing at 50°C for 45sec, and DNA extension at 72°C for 1 min. The reaction was achieved with a final extension at 72°C for 2 min. (Tiwari et al., 2006).

The reaction condition for detection mecA gene was programmed with the initial denaturation, 4 min at 94°C; 30 cycles with a 45-s denaturation step at 94°C, a 45-s annealing step at 56°C and a 30-s extension step at 72°C and 2 min extension step at 72°C (Geha et al., 1994). Five microliters of the amplified PCR products were used for detection of amplified products by electrophoresis in a 2.0% agarose gel and visualization with UV illumination after staining with ethidium bromide for 15 min. Standard DNA ladder 1500-100bp promega Company (USA) were included in each run.

DNA sequencing:
The amplification products were purified by using Illustra™ GFX™ PCR DNA and GeL Band Purification Kit (GE Healthcare Company, UK) following the manufacturers protocol before being used in a sequencing reaction. Sequencing was carried out on each DNA strand with BigDye Terminator Ready Reaction Mix v3.1 (AB Biosystems, HITACHI) by using the same primer set for mecA gene under the following conditions: initial denaturation at 96°C for 1 min, 30 cycles of 10 sec at 96°C, 5 sec at 51°C and 2 min at 60°C. Unincorporated dye terminators were removed by precipitation with 95% alcohol. The reaction products were separated and detected on an Applied Biosystem
genetic analyser 3130 (AB Biosystems) using a standard sequencing module with a Performance Optimised Polymer 7 (POP7) and 36 cm capillary array.

**Nucleotide sequence analysis:**

The sequences were compared to the nucleotide sequence database from the National Center for Biotechnology Information (NCBI) using the BLAST tool (http://www.ncbi.nlm.nih.gov/sites/entrez). Restriction map was performed by using ChromasPro (version 1.5) online software. Multiple sequence alignments were performed by using ClustalW (http://align.genome.jp) software (www.ebi.ac.uk/clustalw). Phylogenetic tree was performed by using MEGA5 Beta version 6.1 online software (www.megasoftware.net).

**GenBank accession numbers:**

GenBank accession numbers of *S. aureus* sequences used in the analysis are: Turkey/HM5 (EU790490.1), Iran/M795 (GU301104.1), Egypt/1 (EF600988.1), India/TN/CN/1/12 (KC243783.1), South Korea/ YSSA11 (EFS96937.1) and UK/NTCC124 (HQ686324.1).

**Results and Discussions**

In this study a total of 241 clinically important specimens were collected; from which 64 isolates of *S. aureus* were isolated representing 26.5% of the total samples. These samples obtained from three hospitals (Dr. Khalid General Hospital in Koya city, Rizgary General Hospital in Erbil city and Azadi General Hospital in Duhok city) in three districts in Kurdistan-Iraq. These samples collected from patients, healthcare staffs and hospital environment in hospitals mentioned above (Table: 1). Isolates were obtained from urine, nose, throat, wounds, burns, skin infections and Hospital environments. Healthcare staffs included physicians, nurses and workers. Hospital environment samples were obtained from different sites in surgical, intensive care, burn, emergency, laboratory units and delivery ward. The molecular confirmation of *S. aureus* isolates was done by using nuc primer as a species-specific primer for *S. aureus*. The results showed that all isolates were amplified and producing 280bp DNA fragment for nuc gene as illustrated in (Figure: 1.a). All 64 isolates of *S. aureus* were searched for methicillin resistant by using Oxacillin (1μg) disc diffusion method according to the Clinical and Laboratory Standards Institute guidelines (Antimicrobial Susceptibility Testing Standards) (CLSI, 2012). The results revealed that the prevalence of methicillin resistant *S. aureus* in Kurdistan was very high, which found that 31 of these isolates were resistance to methicillin representing 48.4% of total isolates as illustrated in (Table:2). The high prevalence of MRSA immevered in our hospitals may reflect failures in strategies to control infection and/or differences in antibiotic use, the poor hygienic conditions of the patients. Resistance to methicillin is mediated by the acquisition of penicillin binding protein PBP-2a encoded by the meca gene, which exhibits a low affinity for β-lactam antibiotics (Davoodi et al., 2012). Resistance to Methicillin was confirmed by using meca primer producing 310bp DNA fragment for meca gene as shown in (Figure: 1.b).

Three methicillin-resistant staphylococcal isolates (SaDu5, SaEr17 and SaSul24) were subjected to partial DNA sequencing of the meca gene to assess the relation of Kurdistan isolates with other global sequences from GenBank and to find similarity between them for cataloging mutation patterns and to provide useful strain resolution. Sequence analysis shows that the sequence identity of all three meca genes among Kurdistan isolates was ranged between 97.5-98.5%, whereas the sequence identity among Kurdistan isolates and other global isolates selected from GenBank was ranged between 92.6-96.1% (Table: 3). Identical sequences among Kurdistan isolates and other global isolates obtained from the National Center for Biotechnology Information (NCBI) database, supporting the hypothesis that horizontal transfer plays an important role in the dissemination of the meca gene in the *S. aureus* population. Only a few restriction enzymes can be used to differentiate between these closely related isolates (Fig. 2), these enzymes can be used for restriction fragment length polymorphism (RFLP) to identify and differentiate between them. Multiple sequence alignment of three Kurdistan isolates with other 6 global isolates from GenBank has been performed using ClustalW 2.0.12 multiple sequence alignment software (Fig. 3). The sequenced product of *S. aureus* meca genes from Kurdistan when compared with meca gene of *S. aureus* strain from GenBank found
similarities and showed partial homology with S. aureus strains (UK/NTCC124, Turkey/HM5, South Korea/ YSSA11 and India/TN/CN/1/12) penicillin binding protein 2a meca gene, partial cds, which is available in public databases. The phylogenetic tree of the aligned sequences was also produced using MEGA5 Beta version 6.1 online software (www.megasoftware.net), and showed a typical relatedness among Kurdistan isolates and GenBank reference strains (Fig. 4). As a result, the structure of the same gene reported by different labs can be different! Multiple sequence alignment and phylogenetic tree analysis separate the isolates into different groups which are genotypically related to each other. DNA sequence analysis of the meca gene provides an unambiguous, portable dataset that simplifies information sharing between laboratories and facilitates creating a large scale database for studying global and local epidemiology. Amplification of specific gene and sequencing of meca gene gives insight into pharmaceutical aspects to design new effective drugs for treatment of methicillin resistance S. aureus. In conclusion the current study of isolation, molecular identification and sequencing of the meca gene from MRSA strain will give insight in to the molecular diagnosis of S. aureus and to find better synthetic drugs (analogs) or organic molecules by computational biologist in pharmaceutical companies to control expression of antibiotic genes in different pathogenic organism in general and in particular MRSA strains.

Table 1: Source, location, number of isolates and methicillin resistant isolates.

<table>
<thead>
<tr>
<th>Hospital</th>
<th>Location</th>
<th>Samples</th>
<th>Isolates</th>
<th>MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Khalid</td>
<td>Koya</td>
<td>62</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>Rizgary</td>
<td>Erbil</td>
<td>93</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>Azadi</td>
<td>Duhok</td>
<td>86</td>
<td>21</td>
<td>11</td>
</tr>
</tbody>
</table>

**Total** 241 64 31

Table 2: Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target gene</th>
<th>Forward 5'----------------3'</th>
<th>Reverse 5'-----------------3'</th>
<th>Size(bp.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>meca</td>
<td>PBP2a</td>
<td>GTAGAAATGACTGAACGT CCGATGA</td>
<td>CCAATTCACATTGTTTCG GTCTAA</td>
<td>310 (Merza 2009)</td>
</tr>
<tr>
<td>nuc</td>
<td>Nuclease</td>
<td>GCGATTGTGTTGATACGGTGTTGTTTTCG GTCTAA</td>
<td>AGCCAAGCCCTGACGAACCT AAAGC</td>
<td>280 (Merza 2009)</td>
</tr>
</tbody>
</table>
### Table 3: Sequence Identity Matrix of *S. aureus mecA* gene partial sequence.

<table>
<thead>
<tr>
<th>Seq-&gt;</th>
<th>Kurdistan/ Du5</th>
<th>Kurdistan/ Er17</th>
<th>Kurdistan/ Sul24</th>
<th>Turkey/ HM5</th>
<th>Iran/ M795</th>
<th>Egypt/1</th>
<th>India/TN/ CN/1/12</th>
<th>S. Korea/ YSSA/ 11</th>
<th>UK/NT CC124/93</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kurdistan/ Du5</td>
<td>ID</td>
<td>0.975</td>
<td>0.985</td>
<td>0.961</td>
<td>0.957</td>
<td>0.933</td>
<td>0.954</td>
<td>0.961</td>
<td>0.961</td>
</tr>
<tr>
<td>Kurdistan/ Er17</td>
<td>0.975</td>
<td>ID</td>
<td>0.971</td>
<td>0.957</td>
<td>0.961</td>
<td>0.926</td>
<td>0.943</td>
<td>0.957</td>
<td>0.957</td>
</tr>
<tr>
<td>Kurdistan/ Sul24</td>
<td>0.985</td>
<td>0.971</td>
<td>ID</td>
<td>0.961</td>
<td>0.957</td>
<td>0.933</td>
<td>0.950</td>
<td>0.961</td>
<td>0.961</td>
</tr>
<tr>
<td>Turkey/ HM5</td>
<td>0.961</td>
<td>0.957</td>
<td>0.961</td>
<td>ID</td>
<td>0.996</td>
<td>0.939</td>
<td>0.967</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Iran/ M795</td>
<td>0.957</td>
<td>0.961</td>
<td>0.957</td>
<td>0.996</td>
<td>ID</td>
<td>0.935</td>
<td>0.964</td>
<td>0.996</td>
<td>0.996</td>
</tr>
<tr>
<td>Egypt/1</td>
<td>0.933</td>
<td>0.926</td>
<td>0.933</td>
<td>0.939</td>
<td>0.935</td>
<td>ID</td>
<td>0.967</td>
<td>0.939</td>
<td>0.939</td>
</tr>
<tr>
<td>India/TN/CN/1/12</td>
<td>0.954</td>
<td>0.943</td>
<td>0.950</td>
<td>0.967</td>
<td>0.964</td>
<td>0.967</td>
<td>ID</td>
<td>0.967</td>
<td>0.967</td>
</tr>
<tr>
<td>S. Korea/ YSSA/ 11</td>
<td>0.961</td>
<td>0.957</td>
<td>0.961</td>
<td>1.000</td>
<td>0.996</td>
<td>0.939</td>
<td>0.967</td>
<td>ID</td>
<td>1.000</td>
</tr>
<tr>
<td>UK/NT CC124/93</td>
<td>0.961</td>
<td>0.957</td>
<td>0.961</td>
<td>1.000</td>
<td>0.996</td>
<td>0.939</td>
<td>0.967</td>
<td>1.000</td>
<td>ID</td>
</tr>
</tbody>
</table>

**Figure (1):**

- **A.** Illustrates the gel electrophoresis of amplified DNA fragments generated by species-specific PCR amplification (nuc primer), and **B** illustrates the amplified DNA fragments for detection of *mecA* gene.
Figure 2. Restriction map of Kurdistan isolates of *S. aureus* (mecA) gene partial sequence that show the restriction enzymes which can be used to differentiate between these isolates. The process was done by using ChromasPro (version 1.5) online software.

![Restriction map of Kurdistan isolates of S. aureus (mecA) gene](image)

Figure 3. Multiple sequence alignment of the sequenced part of *S. aureus* (mecA) gene of Kurdistan isolates with other selected isolates from GenBank using (CLUSTAL 2.0.12 multiple sequence alignment) showing partial homology to *S. aureus* strains penicillin binding protein 2a (mecA) gene, partial cds. Gaps indicate in (-).

![Multiple sequence alignment](image)
Figure: 4. Phylogenetic tree of Kurdistan isolates and other selected strains from GenBank representing different geographic regions shows the relationship among the mecA gene sequence of Kurdistan isolates with other selected isolates from GenBank.

References:

UK SaNTCC1249mecA
Turkey SaHM5-mecA
S.Korea SaYSSA11-mecA
India SaTN/CN/1/12-mecA
Kurdistan SaSul24-mecA
Kurdistan SaDu5-mecA
Iran SaM795-mecA
Kurdistan SaEr17-mecA
Egypt Sa-mecA
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تحليل تسلسل الكليوتيدي لـ Staphylococcus aureus المقاومة ل Methicillin في كوردستان العراق

الخلاصة

في هذه الدراسة تم جمع 241 عينة سريرية مهمة من ثلاث مستشفيات مختلفة في أقليم كوردستان. تم حصول على 64 عينة لجرحية المكورات العنقودية الذهبية كـ Staphylococcus aureus التي تمثل 26.5% من عدد العينات. تم تأكيد الجيني للنتائج باستخدام nuc تقنية nuc المتخصص لجين nuc بواسطة بادئ PCR. أظهرت النتائج أن معدل انتشار مقاومة Methicillin في كوردستان كانت مرتفعة جدا، حيث وجدت أن 31 من هذه العينات (48.4%) كانت مقاومة ل Methicillin باستخدام بادئ nuc المالح. تم تأكيد مقاومة هذه العينات ل Methicillin بادئ باستخدام بادئ mecA. التجربة على عينة ثلاث عزلات (S. aureus SaSul24, SaEr17 و SaDu5) التي انتجت حزمة متضاربة بوزن جزيئي 110 كيلوجرامات لجين mecA المقاومة ل Methicillin لانحلل تسلسل الكليوتيدي لجين mecA لم دقع العلاقة بين هذه العينات والعزلات العالمية المحترفة من بنك mecA بين العينات كوردستان تراوحت بين 97.5% من البنك الجيني وانحلل تسلسل جين mecA بين العينات كوردستان والعزلات العالمية المحترفة من بنك البنك الجيني تراوحت بين 96.3%. كل البوات مقاومة بان تسلسل الكليوتيدي لجين mecA (India/TN/CN/1/12, Turkey/HM5, UKNTCC124, South Korea/YSSA11) و عزلات (SaSul24, SaEr17, SaDu5) مع عينات متناقضة مع تقريب جهة واعتبار عينات عام في ظل الاختلافات في مستوى المضادات الحيوية في الكائنات الحية المسببة للأمراض المختلفة بشكل عام وعلى وجه الخصوص سلالات MRSA