COMPARISON OF SUPERANTIGENIC TOXINS GENES BETWEEN MRSA AND MSSA ISOLATED FROM CLINICAL SPECIMENS IN IRAQ

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ABSTRACT
One hundred fifteen isolates of Staphylococcus aureus were collected from different hospitals in Iraq. For preliminary design isolation of S. aureus culture media was used, and depending on features on cultures, Biochemical tests and (Vitek2 system). Different methods for screening to methicillin resistance S. aureus were used phenotypic method, disc diffusion method, the results showed that a 30/115(26.1%) of MRSA isolates, 79/115(68.7%) MSSA and 6/115(5.2%) intermediate resistance to cefoxitin, in genotypic methods PCR were used to detect housekeeping gene femA responsible to confirm species and mobile genetic element mecA in order to confirm resistance of isolates to methicillin. The results revealed that all S. aureus isolates 100% contained femA gene while 61% isolates contained mecA gene (MRSA) and 39% isolates devoid mecA gene (MSSA). The most prevalence gene was sea 28.12% gene; seg 15.62% gene; sei 10.93% gene; tstl 3.12% gene; seb, seh and selp 1.56% genes. Results showed a higher prevalence of the sea gene in MSSA isolates 32% compared with MRSA isolates 25.64%, seg gene in MRSA isolates 17.94% compared with MSSA isolates 12%, sei gene in MSSA isolates 12% compared with MRSA isolates 10.25%, tstl gene in MRSA isolates 5.12% compared with MSSA isolates 0%, seh gene in MRSA isolates 25.6% compared with MSSA isolates 0%, seb gene in MRSA isolates 0% while seb gene in MSSA isolates 4% compared with MRSA isolates 0%. Higher prevalence of Superantigenic toxin genes in MRSA isolates 64.1% compared with MSSA isolates 60%. Superantigenic genes distributed in various clinical samples was: 22% in wound, 11% in burn, 7.8% in nasal, 6.3% in tonsil, 4.7% in urine, 4.7% in boils, 3.1% in ear, 1.6% for each of eye and pimples. The most common genotype S. aureus was sea 54.83% followed by (seg, sei) 16.12%, seg 9.67% and (seg, sei, tstl), (sea, seg, sei), seb, selp, seh, tstl 3.22%.

Keywords: Superantigenic Toxins, MRSA, MSSA, security, Multiplex, mec A, fem A, sei.

INTRODUCTION
Staphylococcus aureus is a major cause of multiple infection, these infections range from superficial skin infection to deeper infection of hair follicles, abscess and deep tissue infection, systemic infections including hair, lung, blood and bones (Elazhari et al., 2011). S. aureus is benign both communal and common pathogens in human, the infections can be acquired through both hospital and community setting (Adwan et al., 2013). S. aureus commonly defined as methicillin-susceptible S. aureus (MSSA) and methicillin-resistance S. aureus (MRSA). MSSA strains are common human colonizers. Associated MRSA have remained a problem since the 1970; on the other hand, starting in the 1990 new strains of MRSA mentioned to as community-associated MRSA (CA-MRSA) appeared in community residents (Shukla et al., 2010). MRSA is especially troublesome in hospital, prisons and nursing homes, where patients with open wounds invasive devices, and weak immune systems are at more risk of infection.

Staphylococcal super antigens (SAgs) including Staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1) (Park et al., 2011). These toxins have been classified as member of the pyrogenic toxins super antigen family because of their biological actions and structural relatedness. Super antigen avoids normal antigen presentation and have tough T-cell mitogenic activity as a consequence of direct binding to the region of specific T-cell and the major histocompatibility complex class II molecules of antigen presenting cell (Hu et al., 2008). S. aureus is a dangerous bacterium which has proved flexible in developing resistance to antimicrobials and acquiring virulence factors. Significant efforts have been taken on to clarification the importance that specific molecular causes have in defining S. aureus virulence and regulatory systems governing expression of the virulence genes, which is considered now an active field of research aiming at the development of new methods for therapy against infectious diseases (Saleem, 2016; Alsoufi, and Aziz, 2017).

More than 20 distinct super antigenic toxins are known to be produced by S. aureus, both MRSA and MSSA can harbor one or more super antigenic toxin gene. The pathogenic mechanism and virulence factors are assumed to be different between MRSA and MSSA (Wongboo et al., 2013). The aim of this work was toxins genotypic characterization of MRSA and MSSA which the last one acts as a potential source for a new strain of MRSA and to make a comparison between both of them.
MATERIALS AND METHODS

Sample collection: Total of one hundred fifteen clinical isolates of *S. aureus* were collected during the period between September 2016 to December 2016 from different hospitals in Baghdad, Babylon and Karbala governorates. The source of isolates distributed as 33 isolates from wounds infections (all male ware in the army and security forces), 19 isolates from burn patients, 15 isolates from nasal of medical staff, 10 isolates from tonsil infection, 9 isolates from urine, 9 isolates from blood, 6 isolates from nasal infection, 5 isolates from boils, 3 isolates from pimples, 3 isolates from eye infection, 2 from ear infection and 1 isolates were from vagina. The bacterial isolates were stored for long time in Brain Heart Infusion (BHI) broth containing 20% glycerol at -20°C (deep freezing).

Samples were inoculated on blood agar and then to mannitol salt agar, after incubation for 24 hrs. The plates were examined for colony characteristics. Isolates stained with gram stain, catalase test, oxidase test, coagulase test, mannitol fermentation and Vitek2 system were used to identify the organism.

Cefoxitin Sensitivity Test: The phenotypic detection of Methicillin Resistance *S. aureus* (MRSA) was used disc diffusion methods with antimicrobial discs of Cefoxitin 30µg and Cefepime 30µg according to the method described by Rasheed et al., (2014). The result compared with the Clinical Laboratory Standards Institute (CLSI) (2013) and decided as susceptible (S), resistant (R) and intermediate (I). Two reference strains were used as quality control strains. The first strain: (MSSA)- ATCC 29213 (Sherlock et al., 2009; Puah et al., 2016). The second strain is (MSSA)-ATCC 25923 (Kong et al., 2016; Herrera et al., 2016).

DNA Extraction: Genomic DNA was extracted from *S. aureus* by using (Geneaid GBB100, Korea). DNA extraction was done according to company instruction. Nanodrop framework was utilized to assess DND fixation and immacu-lateness (Sambrook and Russell, 2001). Then it was stored at -20 °C until perform PCR assay.

Primers selection and preparation: The sequence of oligonucleotide forward and reverse primers which were used to detect *sea*, *sec*, *sed*, *see*, *seg*, *seh*, *selk*, *sell*, *selm*, *seln*, *selo*, *selq*, *selr*, *tst1*, *femA* and mecA are listed in the Table 1. The primers which were providing as lyophilized form were dissolved to give a final concentration of 100 picomole /µl conferring to recommendation of provider. The volume of primers was 2 µl which were added to reaction mix with final concentration 0.2 µM of each primer. The stock solutions of the primers were stored at -20 °C. The primer sets used to detect *sea* to mecA were defined by Mehrotra et al., (2000), Omoe et al., (2005) and Park et al., (2011). The primer which are specific to *S. aureus* was used to amplify femA gene (Mehrotra et al., 2000; Perez-Roth et al., 2001). The primer which are specific to *S. aureus* (MRSA) was used to amplify mecA gene. To set up a multiplex PCR system, four sets (Set 1: *sea*, *seb*, *sec*, *sed*, *see*; Set 2: *seg*, *seh*, *selk*, *selq*, femA; Set 3: *selk*, *selm*, *selo*, *tst1*, mecA; Set 4: *sell*, *seln*, *selq*, *selr*) (Omoe et al., 2005).

Multiplex PCR reaction condition: Multiplex PCR of every groundwork set was performed with GoTaq Green Master Mix PCR Kit as indicated by maker’s guidelines. Every reaction, mix 50µl comprised of 25 µl of 2X PCR Master Mix, 2µl of each primer and 2µl (10–100 ng) of DNA template. DNA amplification was completed with the accompanying warm cycling: an initial denaturation of DNA at 95°C for 15 min was trailed by 35 cycles of amplification (95°C for 30 s, 57°C for 1.30 min, and 72°C for 1.30 min), finishing with a final extension at 72°C for 10 min and soak at 4°C for 5 min. PCR items were settled by electrophoresis in 2% agarose gel in 1X TBE buffer for 60 min., recolored by 5µl/100ml of Red Safe and pictured on a transilluminator (Omoe et al., 2005).

### Table 1: Sequence and size of the primer

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer</th>
<th>Oligonucleotides sequence 5…………………..3</th>
<th>PCR product (bp)</th>
<th>PCR Set</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sea</em></td>
<td>SEA-F</td>
<td>CCTTGGAAACGTTAAACGC</td>
<td>127</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SEA-R</td>
<td>TCTGAACTTTCCCATCAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Seb</em></td>
<td>SEB-F</td>
<td>TCGCATCACAATTGACACAA</td>
<td>477</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SEB-R</td>
<td>GCGGTACTTAACTGTTGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sec</em></td>
<td>SEC-F</td>
<td>CTCAAGAAGTACATTCATTAAAGGTCTAG</td>
<td>271</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SEC-R</td>
<td>TCAAAATCAGGATTTACATTACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sed</em></td>
<td>SED-F</td>
<td>CTAGTGGTTGTAATTACCTCTTTTAAC</td>
<td>319</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SED-R</td>
<td>TTAAGTGTATCATATCTTATAGGTTAACAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>See</em></td>
<td>SEE-F</td>
<td>CGATGACATTGATAATAGTAAAGTAA</td>
<td>178</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SEE-R</td>
<td>TAACTTCAGTTGACACCCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Seg</em></td>
<td>SEG-F</td>
<td>AAGTAGACATTTTGGCGTTTC</td>
<td>287</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>SEG-R</td>
<td>AGAACCATCAAACGTGTATAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>She</em></td>
<td>SHE-F</td>
<td>GTCTATATGGAGGTTACACT</td>
<td>213</td>
<td>2</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Isolation and identification of *Staphylococcus aureus*: Out of 453 clinical samples only 115 samples were *S. aureus* depending on colony morphology, Gram stain, catalase test, oxidase test, coagulase test, Vitek2 system and PCR technique was used to detect housekeeping gene *femA* responsible to confirm species.

Screening for Methicillin Resistance *Staphylococcus aureus* Isolates (MRSA)

1-Phenotypic method: The traditional method used in most of laboratories to detect MRSA is disc diffusion method using methicillin, oxacillin and/or cefoxitin antibiotic disc (Mendes et al., 2016). In this study, cefoxitin and cefepime disc have been used to screen about MRSA. The results showed that the isolate of *S. aureus* was divided to three groups: 30 (26.1%) isolates showed resistance to cefoxitin disc thus they considered MRSA, 115 (68.7%) isolates showed sensitivity to cefoxitin disc and these isolates were classified as MSSA, while some isolates 61 (13.5%) showed intermediate sensitivity to cefoxitin. This test was conducted to all isolates by using disc diffusion test to two types of antibiotics included cefoxitine and cefepime. The results were interpreted according to the recommendation of CLSI (2013).

In this study some of *S. aureus* isolates appeared intermediate sensitivity to cefoxitin 5.2%, this group of isolates was confused because some researchers considered them as resistance isolates while other researchers consider it as sensitive isolates (David and Daum, 2010). In this study these isolates were considered as sensitive isolates. The highest percentage of MRSA ware isolate from wound 8.7%, burn and urine 4.35% for each.

2-Genotypic method for screening of MRSA isolates: Another way to detecting MRSA was done depending on a mobile genetic element *mecA*. The PCR assay was used for direct detection of methicillin (*mecA*) antibiotics resistance gene in *S. aureus* isolates (Jubair and Khlebos, 2016; Ahmed, 2016). On the basis of specific amplifications of the *mecA* and *femA* genes Figure 1, the multiplex PCR procedure allowed the specific identification of *S.aureus* and the determination of its susceptibility to β-lactam antibiotics (Vannuffel et al., 1995). The results revealed that the 61% isolates contained *mecA* as MRSA versus 39% isolates did not harbor *mecA* (as MSSA). The study results revealed that the 100% isolates contained *femA* thus they are *S. aureus* isolates.

The *femA* gene encodes a factor which is essential for methicillin resistance and is universally present in all *S. aureus* isolates. The *femA* gene product, a protein, has been implicated in cell wall metabolism and is found in large amounts in actively growing cultures (Mehrotra et al., 2000). The *mecA* gene is the structural gene located in the chromosome of *S.aureus*. This gene encodes the modified penicillin-binding protein PBPs which has a low affinity for beta-lactams due to a distorted active site (Deurenberg and Stobberingh, 2008).

The results of this study show difference between the phenotypic and genotypic detection. Generally, there are several methods for the detection of MRSA However, the genotypic methods were better
than phenotypic methods and PCR technique to detect of mecA gene is still considers a golden standard tool to confirmation of MRSA (Cabrera et al., 2010).

**Superantigenic toxin genes distribution among Staphylococcus aureus isolates:** Amplification of genes by multiplex PCR technique to detect sea, seb, sec, sed, see, seg, seh, sei, selk, sell, selm, seln, selo, selp, selq, selr and tst1 genes. Results showed Table 2 that 28.12% carry sea gene, 15.62% carry seg gene, 10.93% carry sei gene, 3.12% carry tst1 gene, 1.56% carry seb, seh and selp genes Figures 1, 2 and 3, while sec, sed, see, selk, sell, selm, seln, selo, selq and selr genes did not appear in any isolate, 48.5% isolates was positive for one or more toxin gene.

The most common found gene in these isolates was the enterotoxin gene sea. MRSA isolates harbored sea gene and produced SEA more frequently than MSSA isolates. Ferry et al., (2005) suggested that enterotoxins generated by the sea gene, could create more severe immunological responses and consequently more tissue damages compared to other enterotoxins. Generally, SEs are similar in structural characteristics and biological activities, however, they are different in their mechanisms of actions. The study showed the most common genotype was sea 54.83% followed by (seg, sei) 16.12 %, seg 9.67%, (seg, sei, tst1), (sea, seg, sei), seb, selp, she and tst1 3.22% .

Figure 1: (set 2) Gel electrophoresis (2% agarose, 7v/cm² for 60 min.) for PCR products SEs genes lane M 100bp DNA Ladder, lanes 1-64 represent fem A bands (134bp), lane 18 represents seh bands (213bp), lane 49 represents of selp bands (396bp), lanes 6,8,24,25,32,57, 58 represent sei bands (454bp), and lanes 6,8,15, 20,24, 25,32,47,57,58 represent seg bands (287bp).

Figure 2: (set. 3) Gel electrophoresis (2% agarose, 7v/ cm² for 60 min.) for mec A and tst1 genes lane M 100bp DNA Ladder, lanes 2,3,4,6,7,8,10, 11,12,14,15, 17,18, 19,20,22,26,27,36,37,38,39,40,41,42, 44,47,48, 49, 50, 52, 53,54,55,57,58, 60,62,64 represents mec A bands (163bp), lanes 6,7 represents tst1 bands (447bp).

Figure 3: (set. 1) Gel electrophoresis (2% agarose, 7v/ cm² for 60 min.) for sea and seb genes lane M 100bp DNA Ladder, lanes 8,11,21,22,28,29,30, 33, 35, 37, 38, 42,46, 53,55,62,63,64 represents sea bands (127bp) and lanes 31 represents seb bands (477bp).
Super antigene toxin genes distribution in clinical samples: The study showed that the super antigenic toxins genes distributed in various clinical samples was: 22% in wound, 11% in burn, 7.8% in nasal, 6.3% in tonsil, 4.7% in urine and boils, 3.1% in ear, 1.6% for each of eye and pimples as shown in Figure 5.

Prevalence of super antegenic toxin genes MRSA and MSSA isolate: Our results displayed that 61% MRSA isolates screened by multiplex PCR technique were showed positive amplifications of the mecA gene. The detection of super antigenic toxin genes by PCR showed that 48.7% of MRSA isolates were positive for one or more of these genes, while 48% of MSSA isolates were positive for one or more genes.

The most prevalence of genes among MRSA isolated was sea 25.64%, seg 17.84%, sei 10.25%, tst1 5.12%, seh and selp 2.56% for each of genes. The prevalence of genes among MSSA isolated was sea 32%, seg 12%, sei 12%, seb 4%, seh, selp and tst1 0% for each of genes.

The percentage of toxin genes in MRSA isolates was higher 64.1% than in MSSA isolates 60%. Study by Wongboot et al., (2013) displayed Super antigenic toxin genes existing in MRSA and MSSA isolates were 79% and 53%. Respectively. The genes sea, seb and sei were more frequent in MSSA than MRSA isolates while seg, she, selp and tst1 more frequent in MRSA than MSSA isolates. Wongboot et al., (2013) showed sea was the most frequent found gene in S. aureus isolates from patient and carriers. Combination of the super antigenic toxin genes was noted seh, selp and tst1 genes distributed in MRSA isolates only. While seb gene was distributed in MSSA isolate only. Genes for exotoxins in S. aureus are coded by genetic elements, for example plasmids, transposons, phages and pathogenicity islands. MRSA was formed when MSSA gains the mobile genetic element SCCmec. A frequent horizontal transfer of SCCmec and numerous mobile genetic elements are the cause of the continuous genetic diversification of MRSA. There was a continuous evolution and interaction between MRSA and MSSA...
isolates. There was possibly a larger gene pool for resistance to antibiotics and virulence factors in MRSA isolates as compared to MSSA isolates, which confers to it the property of more severe and invasive infections (Taj et al., 2014). MRSA was formed when MSSA acquires a mobile genetic element, SCC mec. Toxin producing MSSA may also modify the pathogenicity of established MRSA via the transfer of virulence factors by plasmids or mobile elements (Hu et al., 2008).

Conclusion:
From this research, it could be concluded that the higher percentage of MRSA isolates in wounds, the genotypic method to confirm S. aureus to be MRSA was better than phenotypic method, hence identification of femA gene and mecA gene became a golden standard methods and the most frequent super antigen toxin gene was sea gene followed by seg and sei genes. The most common genotype of local S. aureus isolates was sea followed by seg, sei. The prevalence of super antigen genes in MRSA isolates was higher than those in MSSA isolates.

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