MOLECULAR DETECTION OF CLASSICAL STAPHYLOCOCCAL SUPERANTIGENIC TOXIN GENES IN *STAPHYLOCOCCUS AUREUS* ISOLATES AND EFFECT OF PARTIAL PURIFIED TOXIN ON B6 MELANOMA CANCEROUS CELLS

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ABSTRACT

The current study aimed to molecular detection of classical staphylococcal super antigenic toxin genes by identify *Staphylococcus aureus* isolated from stool samples in Iraqi governorate, Babylon city. A total of 100 specimens were collected from patients, between July 2016 and September 2016. 20 strains were isolated and identified by microbiological tests, At least one type of *S. aureus* classical enterotoxin gene (SEs) was harboring in all 20 (100%) of the isolates. The Polymerase chain reaction (PCR) used for detection of virulence genes that coding to enterotoxin, exfoliative toxin and toxic shock syndrome toxin of *S. aureus* isolates, the result show that they were positive for, *sea, seb, tst, eta, etb* and *agr* with number and percentage, 8(40%),7(35%),20(100%),13(65%), 9(45%),14(70%) and 19(95%) respectively. many *S. aureus* isolates carry at least one of the enterotoxin genes and (95%) strains harbored more than one toxin coding genes. Also the results show effect of partially purified of *S.aureus* toxin on B16 melanoma as a model for human skin cancers, It has been revealed that *S. aureus* enterotoxin have an inhibiter effect on these cells.

KEYWORDS: *S. aureus, sea, seb, tst, eta, etb* and *agr.*

INTRODUCTION

*Staphylococci* are facultative anaerobes capable of generating energy by aerobic respiration, and by fermentation which yields mainly lactic acid. *Staphylococcus aureus* carries numerous
surface proteins named “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs) that mediate attachment to host tissues and initiate colonization leading to an infection.\[1\]

Although not all of \textit{S.aureus} food intoxication cases are recorded, yet \textit{S.aureus} is still considered the third worldwide cause amongst the food-borne illnesses reported cases.\[2\]

\textit{Staphylococcus aureus} a bacterium produces enterotoxins, which are causative agents of food borne intoxications. There are twenty serological types of enterotoxins, They are classified into classical and novel enterotoxins. Enterotoxins are single chain polypeptides and have a molecular weight of about 26-28 kDa and 228-239 amino acid residues.\[3\]

\textit{Staphylococcus aureus} is a major human pathogen that produces a wide array of toxins, thus causing various types of disease symptoms. Staphylococcal enterotoxins (SEs), a family of nine(9) major serological types of heat stable enterotoxins, are a leading cause of gastroenteritis resulting from consumption of contaminated food. In addition, SEs are powerful superantigens that stimulate non-specific T-cell proliferation. SEs share close phylogenetic relationships, with similar structures and activities.\[4\]

Bacterial toxins can kill cells or at reduced levels alter cellular processes that control proliferation, apoptosis and differentiation. These alterations are associated with carcinogenesis and may either stimulate cellular aberrations or inhibit normal cell controls.\[5\]

Cell-cycle inhibitors, such as cytolethal distending toxins (CDTs) and the cycle inhibiting factor (Cif ), block mitosis and are thought to compromise the immune system by inhibiting clonal expansion of lymphocytes. In contrast, cell-cycle stimulators such as the cytotoxic necrotizing factor (CNF) promote cellular proliferation and interfere with cell differentiation; bacterial toxins that subvert the host eukaryotic cell cycle have been classified as cyclomodulins.\[6\]

**MATERIAL AND METHODS**

\textit{S. aureus} isolates were identification according to.\[7\] This study includes a total of 100 feces specimen from patients who admitted to Hilla General teaching hospital and Merjan medical city for during the period from July 2016 to September 2016. Stool specimen were collected from patient that admitted of emergency room with specific symptom as diarrhea and
vomiting that are suspected to food poisoning according to physician by disposable sterile clean, leak-proof container proper way to avoid any possible contamination.

1. **Study effect partial purification of Enterotoxin on Cell-line of human skin cancers**

The B16 melanoma is a model for human skin cancers. The B16 cell line was kindly obtained and cultured at cancer research lab / college of medicin - University of Babylon. The viable cells number was determined by using crystal violet assay before and after using the toxins. Two types of toxins were used in the present study. The first test was isolate number (6) and the second was isolate number (8). We cultured 96 wellplate with B16 cell line with different volumes of toxins. Then these 96 wellplate were incubated at 37°C for 24 hours. We divided the plated cells into five groups for toxin No 1: group 1 as a control group (not treated); group 2 (add 10 µl of toxin 1); group 3 (add 15 µl of toxin 1); group 4 (add 20 µl of toxin 1) and group 5 (add 25 µl of toxin 1). Toxin 2 was added by using the same method. The final cells volumes with toxin were 200 µl. The plated cells were incubated at 37°C for 24 hours before counting.

2. **Crystal Violet Assay**

The cultured cells were washed with 100 µl of Phosphate buffer saline (PBS) and 200 µL of the crystal violet staining solution (0.5% crystal violet in 20% methanol) was added to the cells in the 96 well plates. These cells were fixed and stained for 20 minutes at room temperature. Next plate was generously rinsed and allowed to dry overnight. The next day the dried and stained cells were measured at an absorbance of 570 nm by microplate reader.

3. **DNA Extraction**

Whole bacterial DNA was extracted from *S.aureus* isolates using Geneaid genomic DNA purification kit (BIONEER, USA) according to the manufacturers protocol. DNA was electrophoresed in a 1% agarosegel. and primer sequences for (*sea*, *seb*, *tst*, *eta*, *etb*, *agr*) genes design according to.[8]

4. **PCR amplification**

Bacterial DNA was used as a template for detection of, *seg*, *seh*, *sei*, *coa*, genes. A pair of specific primers were used for the amplification of gene was showing in table (1-1).
Table (1-1): The primer sequences and PCR condition

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5’-3’)</th>
<th>Size of product bp</th>
<th>PCR condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sea F</td>
<td>GGTTATCAATGTGCGGGGTG CGGCACCTTTTTCTTTCCGG</td>
<td>188</td>
<td>94 °C 5min 1x, 94 °C 2 min, 57 °C 2 min, 72 °C 1 min, 72°C 5 min, 1x</td>
<td>[8]</td>
</tr>
<tr>
<td>sea R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>seb F</td>
<td>GTATGGTGTTGTAATCGAGC CCAAATAGTGACGAGGTTAGG</td>
<td>100</td>
<td>96 °C 5 min, 1x, 94 °C 2 min, 72 °C 1 min, 72°C 5 min, 1x</td>
<td>[8]</td>
</tr>
<tr>
<td>seb R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tst F</td>
<td>ACCCCTGTTCCCTATCATC TTTCACTATTTGTAACGCC</td>
<td>147</td>
<td>94°C 4 min, 55°C 1 min, 35x</td>
<td>[8]</td>
</tr>
<tr>
<td>tst R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eta F</td>
<td>GCAGGTGTGGATTTAGCATT AGATGTCCCTATTTTCTTG</td>
<td>93</td>
<td>94°C 5 min, 94 °C 1 min, 57°C 1 min, 72°C 1 min</td>
<td>[9]</td>
</tr>
<tr>
<td>eta R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>etb F</td>
<td>ACAAGCAAAAGAATACAGCG GTTTTGGCGTCTTCTTCTTG</td>
<td>867</td>
<td>94°C 4 min, 55°C 1 min, 35x</td>
<td>[9]</td>
</tr>
<tr>
<td>etb R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>agr F</td>
<td>GTGCCATGGGAATACCTCC TTCC TGTTACCTCAACTTCTCCA TTATG</td>
<td>976</td>
<td>94°C 3 min, 94 °C 30 sec, 55°C 4 sec, 30x</td>
<td>[8]</td>
</tr>
<tr>
<td>agr R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RESULT

Molecular Detection of Enterotoxin genes

1. A. Classical Enterotoxin genes

2. sea and seb genes

The results of this study was found that (40%) of S. aureus isolates are bored of the sea enterotoxin coding gene, followed by seb enterotoxin coding gene which constituted (35%) of all isolates by using PCR technique with specific primers with molecular length (100,102)bp respectively when compared with allelic ladder as shown in figures (1-1) and (1-2).

Figure (1-1) agarose gel electrophoresis at 70 volt for 50 min. for sea gene in S. aureus. PCR product visualized under U.V light at 280nm. After staining with ethidium
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bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 were positive for this gene with amplicon size 102 bp.

Figure (1-2) agarose gel electrophoresis at 70 volt for 50 min. for seb gene in S. aureus. PCR product visualized under U.V light at 280nm. After staining with ethidium bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 were positive for this gene with amplicon size 100 bp.

3. Exfoliative genes detection

3.1. eta and etb genes

Primers of staphylococcus Exfoliative toxin detection genes were used for detection the presence of (eta, etb) genes in S. aureus, it has been found that the percentages of detection are (45% and 70%) respectively with length of (93bp and 867bp) base pairs respectively when compared allelic ladder as show in figure (1-3) and (1-4).

Figure (1-3) - Agarose gel electrophoresis at 70 volt for 50 min. for eta gene in S. aureus. PCR product visualized under U.V light at 280nm. After staining with ethidium bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 were positive for this gene with amplicon size 93 bp.
Figure (1-4) agarose gel electrophoresis at 70 volt for 50 min. for etb gene in *S. aureus*. PCR product visualized under U.V light at 280nm. After staining with ethidium bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 were positive for this gene with amplicon size 867 bp.

4. Toxic shock syndrome toxin *(tst)* gene detection

when compared with Primers of staphylococcal toxic shock syndrome toxin gene were used for detection the presence of (*tst*) gene in *S. aureus*, it has been found that the percentage of detection is (100%) with length of (147) base pairs, when compared with allelic ladder as shown in figure (1-5).

Figure (1-5) - Agarose gel electrophoresis at 70 volt for 50 min. for *tst* gene in *S. aureus*. PCR product visualized under U.V light at 280nm. After staining with ethidium bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 were positive for this gene with amplicon size 147 bp.

5. Molecular detection of the *agr* Accessory gene Regulator

The result of PCR techniques showed that (19 of 20) of *S. aureus* isolates contain *agr* gene in percentage (95%) with 976 bp as shown in Figure (1-6).
Figure (1-6) Agarose gel electrophoresis at 70 volt for 50 min. for agr gene in *S. aureus*. PCR product visualized under U.V light at 280nm. After staining with ethidium bromide. L: Ladder with 2000 bp. 1, 2, 3, 4, 5, 6, 7, 8, were positive for this gene with amplicon size 976 bp.

6. The effect of partially purified of *S. aureus* toxin on B16 melanoma as a model for human skin cancers

Bacterial infections are more common in patients with skin lymphoma as their skin in more fragile and that is due to their illness, so many new researches revealed that the infection may slow down the growth of cancer cells and from this hypothesis we design this experiment and the result show that there is an effect of partially purified of *S.aureus* toxin on B16 melanoma as a model for human skin cancers as showed in figures (1-7).

figure (1-7) effect of partially purified of S.aureus toxin on B16 melanoma as a model for human skin cancers.
DISCUSSION

The result agrees with that of\textsuperscript{10} which indicated that sea enterotoxin gene prevalent(41%). and Also identical with\textsuperscript{11} who found the most frequently encountered gene was sea (36.7%).

The result disagrees with the\textsuperscript{12} who found that the percent of sea gene are (62.5%) .also the result agreed with the\textsuperscript{13} who found that the rate (31.9 %) of the isolates produced seb enterotoxin coding gene. while disagreement with that of\textsuperscript{10} who indicated that seb was (7.7%) the frequent enterotoxin gene prevalent.

The results indicated that sea gene is the most isolated gene and associated with staphylococcus food poisoning other than seb, sea genes is important because the SEA is toxic in low concentration.

The sea gene which is responsible for production of entrotoxin A and associated with food poisoning since it is toxic at low concentration, and it produced at the beginning of the exponential phase of growth and its expression is not regulated by agr while seb gene are regulated by agr gene.

It is well known that enterotoxin genes are not putative genes, and they are true virulence genes because they have the ability to cause food poisoning. However, the presence of one gene or two genes may confer the ability of bacteria to cause food poisoning.\textsuperscript{14}

The toxin of non-producing sea and seb genes strain of S. aureus are due to low level production of enterotoxin or to mutation in coding region or in regulatory region.\textsuperscript{15}

Also the results revealed that (15%)of isolate carried both the sea and seb genes , The strain that not contain the classical enterotoxin (sea and seb genes)may due to it contain other classical genes which are responsible for the production of enterotoxin that cause staphylococcus food poisoning like sec and sed genes.

Also many factors affected of S. aurues enterotoxin production, such as presence of specific amino acid, glucose, PH, and bacterial growth.\textsuperscript{16}
The variation in the toxicity of *S. aureus* isolate might be contributed to genetic variation of enterotoxin genes which reflect geographical difference, which may be further affected by the different ecological origins of the isolated strains.[17]

The above result agrees with other studies found that the exfoliative toxin (ET)-producing in *Staphylococcus aureus* strains was (51%) of all isolates, also the result disagrees with the[18] who observed *eta* gene which was (18%) of all isolates and with[19] who observed that the percentage of *etb* gene was (11.4%) of all isolates under the study.

High rate of the expressions of (*eta, etb*) as a virulence factors secreted by *S. aureus* are depended on the activity of the accessory gene regulator *agr*, a regulon responsible for the alteration of gene expression in response to immune reactions and dependent on quorum sensing, strong activities of *agr* activity, resulting in high levels of produced exotoxins.[20]

The Different rate of *eta* Exfoliative gene and *etb* gene produced by certain strains of staphylococci, may be due to *eta* is which genes encoded on the chromosome, while *etb* gene originates from plasmid. Exfoliative toxins and the majority of toxin-encoding genes are located on mobile genetic elements (MGEs), resulting in a pronounced heterogeneity in the endowment with toxin genes of individual *S. aureus* strains.[21]

Absence of toxin genes may be due to growth conditions and environmental factors that effect on regulatory gene, production below assay detection level or mutation in regulatory or gene coding region.[22]

Also the strain that not contain the classical exfoliative toxin (*eta, etb* genes) may due other classical genes which are responsible for the production of toxin like *etc* and *etd* genes that cause *staphylococcus aureus* diseases.

In human isolates the different isoforms (ETA, ETB) which occurrence is strongly dependent on the geographic region.[23]

The above result agreed with the[24] who found that the *tst* gene presents in (83%) *S. aureus* isolates, found that the *tst* gene present in (25%) of total isolates and with the results obtained by[18] who observed *tst*-1 gene with percent (36%) of all isolate under the study.
The justification presence of gene *S. aureus* strains isolated from patients characterized with toxic shock syndrome TSS because the *tst* gene presents in up to 70% of the *S. aureus* strains isolated from patients with TSS according to.[25]

Presence of *tst* gene that found in *S. aureus* strains isolated from patients with staphylococcal food-poisoning. The variable distribution of *S. aureus* superantigenic toxin genes in different areas may be explained by the fact that the superantigenic toxin genes are mostly carried by mobile genetic elements, resulting in a pronounced heterogeneity in the endowment with toxin genes of individual *S. aureus* strains.[26]

Also accounting for differences in the geographical distribution of *S. aureus* superantigenic toxin genes presences of *tst gene in* highly rate due to a clear association between virulence genes and disease symptoms of toxic shock syndrome (TSS) has been established or is strongly suspected only for some potent *S. aureus* toxins.[27]

Environmental factors such as nutrient availability, temperature, pH, osmolarity, and oxygen tension have the greatest potential to influence the expression of virulence factors. Protein synthesis can influence virulence gene expression, too. Therefore, virulence gene regulators could affect the expression of target genes directly, by binding to their promoters, or indirectly, via other regulators.[28]

Several global regulatory loci determine the production of *S. aureus* virulence factors: accessory gene regulator (agr), which controls at least 15 *S. aureus* products such as extracellular toxins and enzymes: alpha-, beta-, and delta-hemolysin, leucocidin, lipase, hyaluronate lyase, and proteases. For those reasons, *agr* system of *S. aureus* is an important virulence determinant.[29]

There is relationship between *agr* groups and the pattern of *S. aureus* disease and found a strong association between *agr* types and certain diseases. However, in most cases the association reflected the link between the disease types, the pattern of toxin genes, and the genetic back- round of the strains. For instance, the strains causing SE- mediated diseases (disease group C) belonged to *agr* group I or II and phylogenetic group, in most of the disease types considered (mainly toxin-mediated diseases).[30]

The results showed that there is an effect of toxin on cancerous cells in different volumeS of bacterial toxins. Bacterial toxins of *S. aureus* have to some extent already been tested for
cancer treatment. Bacterial toxins can kill cells or at reduced levels alter cellular processes that control proliferation, apoptosis and differentiation. These alterations are associated with carcinogenesis and may either stimulate cellular aberrations or inhibit normal cell controls.\textsuperscript{[31]} It has been discovered that \textit{S. aureus} enterotoxin may be used as anti-cancer and anti-metastatic advantages and that may due to their ability to modify cell immunity processes as well as cancer cell signaling pathway which alteration of the cell signaling genes involved in cancer development and progression. As well as the researchers found that SEA, stimulate activation of signal transcription 3(STAT3) and up regulation of interleukin 17 in malignant T cells, and promote activation of an established oncogenic pathway.\textsuperscript{[32]}

Cell-cycle inhibitors, such as cytolethal distending toxins (CDTs) and the cycle inhibiting factor (Cif), block mitosis and are thought to compromise the immune system by inhibiting clonal expansion of lymphocytes. In contrast, cell-cycle stimulators such as the cytotoxic necrotizing factor (CNF) promote cellular proliferation and interfere with cell differentiation, bacterial toxins that subvert the host eukaryotic cell cycle have been classified as cyclomodulins.\textsuperscript{[33]}

Microbial pathogens have developed a variety of strategies to manipulate host-cell functions, presumably for their own benefit. term ‘cyclomodulins’ used to describe the growing family of bacterial toxins and effectors that interfere with the eukaryotic cell cycle. Inhibitory cyclomodulins, such as cytolethal distending toxins (CDTs) and the cycle inhibiting factor (Cif), block mitosis.\textsuperscript{[34]}

This might constitute powerful weapons for immune evasion by inhibiting clonal expansion of lymphocytes. Cell-cycle inhibitors might also impair epithelial-barrier integrity, allowing the entry of pathogenic bacteria into the body or prolonging their local existence by blocking the shedding of epithelia. Conversely, cyclomodulins that promote cellular proliferation, such as the cytotoxic necrotizing factor (CNF), exemplify another subversion mechanism by interfering with pathways of cell differentiation and development. The role of these cyclomodulins in bacterial virulence and carcinogenesis awaits further study and may delineate new perspectives in basic research and therapeutic applications.\textsuperscript{[35]}

**CONCLUSIONS**

1. The enterotoxin A genes \textit{sea} and \textit{sei} exhibited the highest prevalence in the present study.
2. It was found that the new described enterotoxin genes play a role in the toxigenic food poisoning.

3. The *agr* gene in the key regulator may control the types of enterotoxin production.

**REFERENCE**


