Detection of Virulence Factors \textit{hla} and \textit{hlb} of \textit{Staphylococcus aureus} using PCR Technique

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Abstract
Thirty isolates were collected from patients sputum and throat swabs who admitted at Teaching General Hospital in Hill city during a period of two months lasting from (February to March 2014). The morphological characterization and biochemical reactions showed 18 isolates diagnosed as \textit{Staphylococcus aureus}, of which obtain only six isolates, two from sputum samples (25%) and four from throat swabs (0.4%) have \textit{hlb} gene and only four isolates, two from sputum samples(25%) and two from throat swabs (0.2%) have \textit{hla} gene using PCR techniques.

Keywords: \textit{S. aureus}, virulence factors, PCR, Antibiotic.

Introduction
\textit{Staphylococcus aureus} is a Gram-positive, facultative anaerobic and non-spore forming spherical bacterium that belongs to the \textit{Staphylococcus} genus and characterized by individual cocci which divide in more than one plane to form grape-like clusters [1].

\textit{Staphylococcus aureus} known as golden staph, when viewed through a microscope appear as large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates [2].

\textit{Staphylococcus aureus} subspecies distinguished from \textit{Staphylococcus aureus} by lack of pigment and clumping factor and by the inability to carry out anaerobic fermentation of mannitol, to grow at 45°C, to produce acetoin from glucose to reduce nitrate, to produce b-glucosidase, and to produce acid from galactose, lactose, mannose, mannitol, ribose, and trehalose [3].

\textit{Staphylococcus aureus} is a dangerous human pathogen in both community-acquired and nosocomial infections. \textit{Staphylococcus aureus} which can be found as part of the normal skin flora and in anterior nares of the nasal passages and it is the most common species of \textit{Staphylococcus} to cause Staph infections and is a successful pathogen due to a combination of nasal carriage and bacterial immuno-evasive strategies [4].

A fundamental biological property of this bacterium is its ability to asymptotically colonize healthy individuals [5].

\textit{Staphylococcus} produce many potential virulence factors such as toxins like alpha, beta and gamma toxin...
leukocidin, toxic shock syndrome toxin and different types of enterotoxins. Alpha and beta toxins are exotoxins hemolysins in nature cause lysis of erythrocytes by pore formation. A wide range of cell types is affected by alpha toxins including erythrocytes, monocytes, lymphocytes, macrophages and epithelial cells [6].

Staphylococcus aureus a-toxin a polypeptide encoded by hla is a pore-forming cytotoxin that is produced by the majority of S. aureus strains and targets a broad range of host cell types. Like most staphylococcal extracellular proteins, a-toxin is not expressed constitutively but is tightly regulated by an array of extracellular and intracellular signals [7].

However α-toxin is secreted as a water-soluble monomer that undergoes a series of conformational changes to generate a heptameric, β-barrel structure in host membranes [8]. On the other hand β-toxin is a neutral sphingomyelinase secreted by certain strains of Staphylococcus aureus. This virulence factor lyses erythrocytes in order to evade the host immune system as well as scavenge nutrients [9].

β-Toxin (hlb) among S. aureus toxins, the function of β-toxin in pneumonia and lung injury. This β-toxin is a Mg2+-dependent neutral sphingomyelinase that hydrolyzes sphingomyelin of the host cell plasma membrane to generate phosphocholine and the bioactive secondary messenger [10].

β-toxin does not lyse most types of host cells but leaves them susceptible to a number of other lytic agents, such as α-toxin and Panton-Valentine leukocidin, the cytotoxic effect of β-toxin is cell type-specific and species-specific, suggesting that its primary virulence activity is to modulate host processes that affect pathogenesis, rather than to directly kill host cells [11].

Materials and Methods

Collection of specimens:
The study was conducted at Teaching General Hilla Hospital in Babylon Governorate. 30 samples collected from patients sputum and throat swabs during the period from February to March 2014. Only 18 isolates of Staphylococcus aureus were obtained from patients with tonsillitis, pharyngitis, sinusitis and otitis by standard bacteriological methods.

Bacterial identification:
The samples were processed on blood agar, macCkonkey agar and selective media (Salt milk agar) were incubated at 37°C performed by standard biochemical methods (catalase test, oxidase test, coagulase test, urea hydrolysis test, hemolysin, produce of lipase produce of phosphatase, reduce of nitrate to nitrite, ferment mannitol and gelatin liquefaction) according to Bergy’s Manual for Determinative Bacteriology [12].

DNA extraction for gram positive bacteria:
DNA extraction was carried out according to the genomic DNA purification kit supplemented by manufactured company (Promega, USA).

Detection of some virulence gene markers by PCR:
The primers and PCR conditions used to amplify genes encoding virulence factors with PCR are listed in table (1). The primers includes hla and hlb genes, Each 25μl of PCR reaction contained 2.5μl of each upstream and downstream primer, 2.5μl of free nuclease water, 5μl of DNA extraction and 12.5μl of master mix. The PCR amplification product were visualized by electrophoresis on 1% agarose gels for 45min at 70v. The size of the amplicons were determined by comparison to the 100 bp allelic ladder (Promega, USA).
Table (1): Primers 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>
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</thead>
<tbody>
<tr>
<td>hlbF</td>
<td>GCC AAA GCC GAA TCT AAG</td>
<td>833</td>
<td>94°C 2min 1x</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>GCG ATA TAC ATC CCA TGG C</td>
<td></td>
<td>94°C 1min</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>54°C 1min 30x</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>72°C 1min</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>72°C 10min 1x</td>
<td></td>
</tr>
<tr>
<td>hlbR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hlaF</td>
<td>TTGGCTGGGGAGTTGAAGCACA</td>
<td>306</td>
<td>94°C 2min 1x</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>CGCCTGCCCAGTAGAAGCATT</td>
<td></td>
<td>94°C 1min</td>
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<td>55°C 1min 30x</td>
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<td>72°C 10min 1x</td>
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<tr>
<td>hlaR</td>
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Results

Staphylococcus aureus isolates were subjected to produce major cytotoxic agent (α-toxin and β-toxin). Molecular detection of Staphylococcal α-toxin (hla) and β-toxin (hlb) was done by using specific PCR primer. The results showed that only six of investigated isolates contained the (hlb) gene, two from sputum isolates (25%) and four from throat swabs (0.4%) as shown in figure (1-1).
Figure (1-1) : Gel electrophoresis of PCR product of hlb 
(1,2,3,4,5,6) isolates with positive result for hlb (1,2) isolates from sputum (3,4,5,6) isolates from throat swabs. L= ladder(1500-100). The electric current was allowed at 70 volt for 30 min.

Also the results revealed that only four isolate gave positive amplification for (hla),two from sputum samples (25%) and two from throat swabs (0.2%) as shown in figure (1-2).

Discussion
The majority of initial inflammatory responses to inhaled bacteria is signaled by mucosal cells lining the respiratory tract. Staphylococcus aureus has a potential to activate the host inflammatory response in several different ways through the adherence of intact bacteria to the host epithelial cells, by internalization of the bacteria and by direct interaction of
bacterial adhesins and toxins with the mucosal epithelium [15]. Many factors of Staphylococci are known but the most frequently mentioned and detected in this study by using PCR with primers specific genes includes: hla and hlb.

It was found that (hlb) is present in only six isolates. This result is correlated with the results done by [16] who investigated the presence of this gene among most S. aureus isolates.

Also the results revealed that only four isolate gave positive amplification for (hla). This result is agreement with the results done by [17] who investigated the presence of this gene among most S. aureus isolates.

This gives the important role of this gene in pathogenesis of S. aureus among hospitalized patients.

Other study investigate by [15] indicated that 96% of hla and hlb give positive isolates obtained from nasal carriage.

Harris et al., [18] and Cheung et al.,[19] reported that Hla is positively controlled by agr, sarA, and sae. It appears that agr activates hla at both the transcriptional and translational levels, whereas sarA exerts a complex positive impact on hla expression by both agr-dependent and agr-independent pathways.

In addition, sae appears to positively activate hla via an agr dependent pathway in vitro [20].

Suchart, and Jorgen [2] and Ryan and Ray [21] reported that α-toxin possesses additional biological functions such as binding to a putative glycoprotein receptor on host cells, activation of intracellular signaling, and modulation of several processes.

It was recently described, that α-toxin facilitates the secretion of newly synthesized chemokines into the airway and exaggerates neutrophil-mediated inflammatory lung injury [22].

Study of [23] and [24] uncovered a previously unknown in vivo function of β-toxin in pneumonia. β-toxin has been shown to maximize lung injury not through its cytotoxic activity, but rather through its capacity to enhance PMN infiltration in a syn-decan - 1-dependent manner.

The study show has been done by [25] the beta toxin is the hot-cold toxin because of its unique activity on sheep blood agar plates. At 37ºC, beta toxin interacts with sheep red blood cells but does not lyse them. If the red cells are then placed at 4ºC the cells lyse, this is observed as a lack of hemolysis on blood agar plates at 37ºC and then complete hemolysis at 4ºC.

A survey by [26] found that beta toxin was produced in 72% of bovine mastitis isolates, in 11% of healthy human nasal isolates, and in 13% of human septicemia isolates. Due to the likelihood of contamination from one or more cytolysins and the differential and species dependent susceptibility to beta toxin.

References: