High Frequency of Hemolysin Associated Genes among Staphylococcus Aureus Clinical Isolates in Iraq

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Abstract

In current study, 120 clinical isolates of Staphylococcus aureus were collected from different hospitals in Diyala. In addition of phenotypic method, all isolates diagnosed as S. aureus by genotypic method was done by Polymerase Chain Reaction (PCR) technique to detection nuc gene. PCR technique has been used to investigate hemolysin associated genes that encoding for hla, hlb, hlg and hld by using specific primers which showed the prevalence in percentage (95%, 79.17%, 90% and 96.67%) respectively. All isolates were found to be positive for at least two or more hemolysin genes and 69.17% of isolates were carrying all hemolysin genes.

Keywords: Staphylococcus aureus, Thermo nuclease gene, Virulence genes, Alpha, Beta, Gamma and delta hemolysin.

Introduction

Although Staphylococcus aureus is a commensally bacterium found in nearly 30% of the healthy population, However it is considered one of the most virulent bacterial pathogens either acquired from hospital or community [1]. The capability S. aureus to cause diseases is related with a number of virulence factors which allowing colonization, propagation in the host body and avoidance of the immune system. The set of virulence factors required to cause disease is likely to be highly dependent on the site of infection [2].

The production of cytolytic toxins is a main mechanism deployed by S. aureus to target host phagocytes [3]. These bacteria are capable of causing destruction within the human body, primarily by destroying red blood cells through the production of hemolytic toxins. Particularly, S. aureus secretes numerous exotoxins that invade host cell like hemolysins which classified into four different toxins including alpha, beta, gamma and delta which encodes by hla, hlb, hlg and hld genes respectively. These toxins induce lysis of a wide spectrum of cells mainly erythrocytes, platelets, monocytes and neutrophils, so they have a cytolytic effect [4]. A-toxin was the first bacterial exotoxin to be identified as a pore former and it considered the major cytotoxic agent produced by S. aureus. Pore formation on susceptible host cell membranes triggers changes in ion gradients then damage of membrane and cell death [5]. This pore forming toxin is secreted by majority of S. aureus clinical isolates and is effective against a broad range of mammalian cells, particularly remarkable activity against rabbit erythrocytes [6, 7]. Also this toxin induces the release of cytokines and chemokines [8, 9].

The dose of the α-toxin can effect in two different styles of activity. at Low concentrations bind to specific cell surface receptors and form a heptameric pore which lead to allows the exchange of monovalent ions, resulting in DNA fragmentation then apoptosis, while at high concentrations result in the toxin absorbing to the lipid bilayer nonspecifically and forming large, Ca2+-permissive pores which lead to massive necrosis and other secondary cellular reactions triggered by the uncontrolled Ca2+ influx[10,11]. α-toxin play an important role in the pathogenesis of staphylococcal diseases, therefore S. aureus mutants without hla gene reduced virulence in invasive disease models [12]. Certain strains of S. aureus also secrete, θ-toxin encoded by
the hlb gene. In contrast to α-toxin, β-toxin is highly hemolytic for sheep but not for rabbit erythrocytes [11]. S. aureus β-toxin is a Mg2+-dependent neutral sphingomyelinase (S Mase) that hydrolyzes sphingomyelin of the host cell plasma membrane to generate phosphocholine and the bioactive secondary messenger, ceramide. These ceramides may have a number of effects in eukaryotic cells based on the chain length of their fatty acids or the mode of metabolism, these effects includes stimulation of second messenger systems, activation of the mitogen-activated protein kinases (MAPKs), changes in cell shape, and even apoptosis[13].

Hemolytic activity of β-toxin is promoted after incubation at temperatures below 10°C, so this toxin is often called as the ‘hot-cold’ hemolysin [14]. β-toxin has lytic activity against to human monocytes, polymorph nuclear leukocytes and lymphocytes [15]. Even if it did not direct lysis the cells, it causes weak in cells making them vulnerable to attack other toxins [16].

Γ-hemolysin is a two component pore-forming toxins present in almost all human S. aureus isolates. It able to lysis many varieties of mammalian erythrocytes and also toxic for leukocytes. This toxin can be genotypic ally detected because it is not identifiable on blood agar plates due to the inhibitory effect of agar on toxin activity [17,18]. Staphylococcal γ-hemolysin (Hlg) consists of polypeptides designated as S (slow, HlgA or Hlg C) and F (fast, HlgB), which cooperatively lysis target cells, where the S components are suggested to effect cell type susceptibilities to these toxins [19]. Their toxicity requires the synergistic combination of a class S and a class F component , two non-associated exoproteins. These proteins after recognition of their cell targets undergo conformational changes and form oligomeric complexes.

This process leading to trans membrane-pore formation and the result is cell death [19,20]. This toxin target polymorph nuclear cells, monocytes, macrophages and erythrocytes [2]. γ-hemolysin, belongs to a group of genes that code for both hlgA and hlgC as the S (slow) component, or hlgB as the F (fast) component which is located in the core genome [21]. Δ-toxin is the member of the phenol-soluble modulin (PSM) family that does not require a receptor for its hemolytic activity which has multiple functions in staphylococcal pathogenesis [22].

It is encoded by the hld gene within RNAIII, the regulatory molecule of the accessory gene regulator (agr) quorum-sensing system that activates transcription of a number of virulence factors and represses the transcription of other [16]. Most of S. aureus isolates produce β-toxin and it lysis erythrocytes, a variety of mammalian cells and sub-cellular structures such as membrane bound organelles, spheroplasts and protoplasts [23].

Methods and Materials
Isolation of S. Aureus from Clinical Samples
In this study one hundred twenty isolates were primarily diagnosed as S. aureus obtained from different hospitals in Diyala governorate including, Baquba Teaching Hospital, Specialized Hospital Al Batool Maternity & Children, General Hospital Al Khales and General Hospitals Baladrus during 2016. They were isolated from different clinical sites including, burn infections, ear infection, throat infection, nasal discharge, surgical wound from hospitalized patient, midstream urine from patient suffering urinary tract infections and boils under medical attention. Each isolate was cultured on MacConkey agar, Blood agar and Mannitol salt agar and biochemical tests and Gram stain were performed for all isolates.

Phenotypic Detection of S.aureus
The colonies were identified as S. aureus by biochemical properties; gram staining and cultural characteristics were transferred by sterile loop and directly inoculated on Chromagar Staph. aureus and incubated at 37°C for 24 hr. On this medium, the growing colonies that appeared distinctive pink to mauve color diagnosed as S.aureus.

DNA Extraction and Genotypic Detection of S. aureus
Genomic DNA was extracted according to boiling method described by klingenberg [24]. The purity and concentrations of the DNA were estimated by spectrophotometers at 260 and 280 nm. Primers for identifying and classifying bacteria nuc was used, giving products of 270 bpTable (1). Uniplex PCR was performed in a 25-μl volume that was prepared by adding 12.5μl of Go Taq® Green
master Mix (2X) promega, 5μl template DNA, 1.5μl from each forward and reverse primers with final concentration 1 poml /μl, finally volume was completed to 25μl by adding nuclease free water. PCR condition illustrated in Table (2) and PCR products were detected in 1 % agarose gel for 1 hr. at 75 V, stained with ethidium bromide and visualized by transilluminator.

Detection of Hemolysin Genes
The prevalence of hemolysin genes (hla, hlb, hlg and hld) among S. aureus local isolates in this study was detect by using uniplex PCR with specific primers and amp icon size Table 1. PCR mixture was prepared by adding 12.5μl of Go Taq® Green master Mix (2X) promega, 5μl template DNA, 1.5μl from each forward and reverse primers with final concentration 1 poml/μl, finally volume was completed to 25μl by adding nuclease free water.

PCR condition illustrated in Table (2) and PCR products were detected in 1 % agarose gel for 1 hr. at 75 V, stained with ethidium bromide and visualized by trans illuminator.

Table 1: Primers and amplified PCR products used in study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’→3’)</th>
<th>size (bp)</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuc</td>
<td>F- GGATATGATGTTAGACCGT</td>
<td>270</td>
<td>Alpha</td>
<td>25</td>
</tr>
<tr>
<td>hla</td>
<td>F- CTTGATCTACGGAATTCAG</td>
<td>209</td>
<td>Alpha</td>
<td>26</td>
</tr>
<tr>
<td>hlb</td>
<td>F- GGTGACCTGATCAGATTCG</td>
<td>309</td>
<td>Alpha</td>
<td>26</td>
</tr>
<tr>
<td>hlg</td>
<td>F-GCCATCGTCTGGATAGGAAG</td>
<td>937</td>
<td>Alpha</td>
<td>27</td>
</tr>
<tr>
<td>hld</td>
<td>F-AAGAATTTTTATCTTTATT</td>
<td>111</td>
<td>Alpha</td>
<td>26</td>
</tr>
</tbody>
</table>

Table 2: PCR condition to genes used in study

<table>
<thead>
<tr>
<th>Amplified gene</th>
<th>Initial denaturation</th>
<th>No. of cycles</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Elongation</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuc</td>
<td>95°C 5 min</td>
<td>30</td>
<td>94°C 1 min</td>
<td>55°C 1 min</td>
<td>72°C 1 min</td>
<td>72°C 1 min</td>
</tr>
<tr>
<td>hla</td>
<td>95°C 5 min</td>
<td>35</td>
<td>94°C 1 min</td>
<td>57°C 1 min</td>
<td>72°C 1 min</td>
<td>72°C 1 min</td>
</tr>
<tr>
<td>hlb</td>
<td>95°C 5 min</td>
<td>35</td>
<td>94°C 1 min</td>
<td>60°C 1 min</td>
<td>72°C 1 min</td>
<td>72°C 1 min</td>
</tr>
<tr>
<td>hlg</td>
<td>95°C 5 min</td>
<td>35</td>
<td>94°C 1 min</td>
<td>56°C 1 min</td>
<td>72°C 1 min</td>
<td>72°C 1 min</td>
</tr>
<tr>
<td>hld</td>
<td>95°C 5 min</td>
<td>35</td>
<td>94°C 1 min</td>
<td>58°C 1 min</td>
<td>72°C 1 min</td>
<td>72°C 1 min</td>
</tr>
</tbody>
</table>

Results and Discussion
Phenotypic Identification of S. aureus
In this study, all one hundred and twenty clinical isolates grew in blood agar and no growth was appeared in Mac Conkey agar. In manitol salt agar medium, all isolates caused fermentation of manitole and changed the color of colonies to yellow.

All isolates were catalase positive and oxidize negative and was identified as gram- positive cocci in grape like clusters under microscope.

All isolates were cultured on CHRO Magar Staph aureus media, the growing colonies appeared as pink to mauve due to the hydrolysis of chromomeric substrates including in media which confirms that they are colonies of S. aureus according to manufacturer’s instruction. Regarding the source of isolates, 30(25%) from burns, 30(25%) from urine, 24(20%) from wound, 14(11.7%) from boils, 9(7.5%) from throat infection, 7(5.8%) from nasal infection and 6(5%) from ear infection.

Genotypic Identification of S. aureus
All S. aureus isolates were tested for nuc gene by PCR. The results revealed that all (120) S. aureus isolates were yielded this gene by PCR. Figure (1) shows the genetic detection of nuc gene to different isolates of the bacteria under study and nuc gene was approximately equal to 270bp.

The nuc gene encodes the thermo nuclease enzyme in S. aureus and detection of nuc gene by PCR is potential for rapid diagnosis of S. aureus infection [28].The nuc is S. aureus-specific gene, so this gene is always being used for the identification of S. aureus as a molecular target [29, 30]. Molecular techniques like (PCR) have become most important technique for more rapid and precise for detection bacteria that causes diseases in species level [31].
Screening of Hemolytic Genes

PCR amplification of the hla, hlb, hlg and hld genes were done for all isolates to detect hemolysin genotypes of S. aureus. In this study all isolates exhibited carriage of at least two hemolysin genes. The PCR results showed that hld gene was the most frequent and was carried by 116 isolates (96.67%) followed by hla in 114 isolates (95%), hlg in 108 isolates (90%) and hlb in 95 isolates (79.16%). Figure (2) shows positive results to amplified fragment of hla, hlb ,hlg and hld genes.

There are many studies done around the world to detect prevailing hemolysin genes of S. aureus in those countries. The results were obtained from Egyptian study which done by El-baz [18], studying 85 S. aureus isolates obtained from variety of clinical specimens in Egypt, who found the most common hemolysin coding gene in their study was hld with a prevalence of 88.2%, followed by hlg, hlb and hla with 81.1%, 64.7% and 30.5% prevalence respectively. In the Iranian study carried out by Hoseini Alfatemi [32] performed on 146 Methicillin Resistant Staphylococcus aureus(MRSA) isolates, the most common hemolysin coding gene were hla and hld with 93.15% and 84.24% prevalence respectively. In the Japanese study carried out by Maeda [33] performed on MRSA isolates which causes bacteremia, who found the frequency rates of hla and hld genes were 100% for each followed by hlg and hlb with 99% and 97% prevalence respectively.

High frequency of hla gene in present study probably due to its association with the accessory gene regulator (agr) the agr locus consist RNAIII which operon encodes a δ-haemolysin [34]. In the study done in Iraq by Saleem [35] about prevalence of agr in local clinical MRSA isolates, the results showed 97.35% of isolates under study were carrying agr genes. This is very close to the findings of the current study on the prevalence of hld gene. Through the results of the current study, it should be noted the most common
gene profile was \textit{hla}, \textit{hlb}, \textit{hlg}, \textit{hld} in other word all hemolysin associated genes which was harbored by 83 (69.17%) isolates and 27 (22.5%) isolates were carried three hemolysin genes with two profiles. The first pattern combine 19 (15.83%) isolates with \textit{hla}, \textit{hlg}, \textit{hld} genes while the second pattern combine 8 (6.67%) isolates with \textit{hla}, \textit{hlb}, \textit{hld} genes. Only 10 (8.33%) isolates under this study have two hemolysin genes with two profiles, 6 (5%) isolates with \textit{hlg}, \textit{hld} pattern and 4 (3.33%) isolates with \textit{hla}, \textit{hlb} pattern.

Overall, 90% (108) of the all \textit{S. aureus} isolates carried same hemolysis associated genes (\textit{hla}, \textit{hlg} and \textit{hld}). In a study conducted by Ando [36], who found combination between \textit{hla} and \textit{hlb} enhanced bio film formation. This combination reached to 79.17% in \textit{S. aureus} isolates under current study, which indicates to their ability to resist antimicrobial. Bio film plays an important role in increasing antibiotic resistance, as is known at present, these bacteria have capability to resistant most antibiotic types [37]). Also the results of this study shows that the \textit{hlb} gene is associated with the presence of \textit{hla} gene and the same words apply to a relationship \textit{hlg} gene with \textit{hld} gene, suggesting the possibility of a molecular relationship between them.

It could be conclude that there is one strain of these bacteria has a high level of genes prevalent within the geographical area under study with the possibility of presence strain or more but less spread. As well as, the frequency of these genes is greater as compared to other toxins genes in \textit{S. aureus} according to the study conducted on these bacteria in Iraq [38]. The high frequency of these genes can leads to reduce the prevalence of genes encoding toxins and acquired genetic elements carrying genes are located on chromosome hemolysin. Finally, the high level of the hemolysin genes between \textit{S. aureus} local clinical isolates indicates to their ability to invade host tissue and colonization as well as the high ability to resist and avoid immune cells.

References


