Investigation of the presence of some virulence factors of the *Streptococcus pneumoniae* isolates among patients in Basra Governora

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Abstract

Extensive studies have been conducted on the microbial properties of *S. pneumoniae* all over the world, but there are few studies in Iraq on the most important factors of virulence possessed by *S. pneumoniae* isolates found in Iraq. 195 of sputum specimens were collected from patients with pneumonia acquired from the community who were clinically diagnosed by specialized doctors depending on symptoms and Radiography of Chest. Eighteen isolates of *S. pneumoniae* were diagnosed by special traditional methods that used in the phenotypic identification. All isolates (100%) have been given positive results for the optochin test, bile solubility test, latex agglutination. Genetically, the study of virulence factors was limited to only 11 (61.11%) isolates by using the Polymerase Chain Reaction (PCR) technique, four genes were investigated responsible for virulence factors deemed necessary for *S. pneumoniae* to colonize and invade the host. The results showed that the isolates of *S. pneumoniae* in Basra city were fierce; where the results of PCR amplification showed that the genes CpsA, LytA and Ply were found in all isolates (61.11%) while the Psa gene was present in only 9 (50%) isolates within the current study.

Keywords: *Streptococcus pneumoniae*, Virulence factors, capsule, Pneumococci surface adhesion, autolysin, pneumolysin.

التحري عن وجود بعض عوامل الضراوة لعزلات العقدية الرئوية بين مرضى محافظة البصرة

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الخلاصة

لقد أجريت دراسات مستفيضة على الخصائص الميكروبية لبكتريا العقدية الرئوية في جميع أنحاء العالم، ولكن هناك القليل من الدراسات في العراق حول أهم عوامل الضراوة التي تمتلكها عزلات العقدية الرئوية الموجودة في العراق. جمعت 195 عينة قشع من المرضى المصابين بالالتهاب الرئوي المكتسب من المجتمع، الذين تم تشخيصهم سريرياً، من قبل أطباء متخصصين اعتماداً على الأعراض والتصوير الشعاعي للصدر، حيث شملت 18 عينة تعود للعقدية الرئوية باستخدام الطريقة الروتينية الخاصة في التشخيص المفتوح. أظهرت جميع العزلات (100%) نتائج موجبة لاختبارات الاكتئاب والتشخيص الشعاعي للمصابين، وائلياً (2017) اقتصرت على دراسة عوامل الضراوة في 11 عينة فقط باستخدام تقنية تفاعل البلمرة المشبعة المتسلسل (PCR) ثم التحري عن وجود أربعة جزيئات مسؤولة عن عوامل الضراوة التي تعتبر ضرورية للعقدية الرئوية في...
1. Introduction

Streptococcus pneumoniae (Pneumococcal) is one of the common causes of a wide range of diseases such as sinusitis and middle ear infection or may cause pneumococcal diseases invasive and dangerous, including meningitis, bacteremia and Pneumonia [1]. Approximately 1.6 million persons die each year due to pneumococcal infection, especially children under 5 year of age. It was indicated that it caused pneumonia acquired from community by 30-50% of the total number of cases [2]. S. pneumoniae has the ability to express a number of complex virulence factors [3]. Pneumococci surface adhesion A (PsA A) is an extracellular protein found on the surface of the cell, the presence of antibodies to PsA A reduces the adhesion of different serotypes of pneumococci with epithelial cells in host [4]. Capsule is an important virulent factor for pneumococci, it has been reported that strains containing capsules are more harmful than those lack capsule [5]. The capsule is highly negative charged, inhibiting the interaction between the complement factor C3b and the complement receptor on the surface of the bacterial cell [6]. LytA is a major autolysin enzyme that act to break down the peptidoglycan of cell wall and thereby lead to release pneumolysin and other materials that cause inflammation [7]. Pneumolysin is an intracellular toxin, consisting of a single 53 kDa peptidoglycan chain, it is produced by all clinical isolates of pneumococci and has several infection specially in early stage of infection [8].

Because of the lack of studies in Iraq and in particular in the city of Basra on pneumococcal pneumonia, the current study aimed at screening the most important virulence factors which possessed by strains of pneumococcal that spread in Iraq, which causes pneumonia.

2. Material and methods

2.1. Collection of sputum samples:

The current study included the collection of sputum samples from 195 patients who are expected to have acquired pneumonia from community (CAP) who were admitted to the Consultant Respiratory and Chest Diseases at the General Basra Hospital and the Center for Chest Diseases in the city of Basra and who have been diagnosed by specialist doctors depending on symptoms and Radiography of Chest during the period from March 2016 to October 2017. The study included adult patients of both sexes for ages from 14 to 85 years. Sputum samples were collected early in the morning and before any treatment is taken in sterile containers and then transferred to the laboratory [9]. In addition, 40 saliva samples (control group) were collected from healthy people, from both sexes for age 22 to 50 years.

2.2. Sputum examination

A part of the sputum has been pigmented by using the Ziehl-Neelson stain (acid fast stain) to ensure not infected with Mycobacterium tuberculosis as cording to Macfaddin [10]. Another part of sputum has been stained by gram stain and examined microscopically to confirm the presence of immune cells that indicate bacterial pneumonia, otherwise the sample is considered to be contaminated with oral cavity bacteria and the sample is rejected, as according to WHO [9].

2.3. S. pneumoniae isolation and identification:

The sputum samples were homogenized with an appropriate amount of normal saline and mixed by shaker for 30 second [11]. Then the samples were inoculated on blood agar (with 5% of human blood) and chocolate agar under 2-5% CO₂ (candle jar) for 18-24 hours at 35°C [9].

2.3.1 phenotypic characteristics

Initial identified methods for pneumococcal isolates have been adopted such as morphology of colony, alpha hemolytic on blood agar plate with flattened and grayish draughtsman colony [11]. Gram positive diplococci, lancent shap [9].

2.3.2. Optochin test

A pure isolate of expected pneumococcal was streaked on the blood agar plate, then the optochin disc (5µg) is placed in the middle of the plate and incubated at 35-37°C with 2-5% CO₂ (candle jar) for 18-24 hours. The test is considered positive when the diameter of inhibition zone was more than 14 mm, this test is used to differentiate between S. pneumoniae and other types of Streptococci [9].

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2.3.3. Bile solubility test
This test is performed after preparation of bile salt solution at concentration (2%) by adding 0.2g of sodium deoxycholate to 10 ml of distilled water. Some droplets of solution are added directly to the bacterial colonies at the age of 18-24 hours and incubated at 35-37°C for 15 minutes. The test is considered positive when bacterial colonies disappear from the plate [9].

2.3.4. latex agglutination
An immunological test to investigate the antigen of the capsules by using the latex agglutination Wellcogen S.pneumoniae Kit, this test has been applied according to manufacturer instructions (Thermo Scientific, UK).

2.4. Maintain the survival of pneumococcal isolates
During the study period, two type of media have been used to preserve and maintain S.pneumoniae isolates included, Tryptone Soya broth (TSB) with 15% glycerol and Skim milk-Tryptone-Glucos-Glycerol (STGG) broth. The last medium was prepared depending on O'Brien, et al. [12].

2.5. DNA Extraction
DNA was extracted for 11 (61. 11%) isolates of S.pneumoniae by a boiling manner, as reported in Leung [11]. The pure isolate of S.pneumoniae was cultured on Blood agar for 18-24 hours at 35°C with 5% CO2. Disposable loops 10µl were used to collect the growth of S.pneumoniae colonies from two plates of blood agar for obtained a heavy suspension of bacteria in 50µl (1X ) of Phosphate Buffer Saline (PH=7.2) in eppendorf tubes, boiling in a water bath at 95°C for 5 minutes to induce bacteria to lysis, centrifuged at 10.000 xg for 5 min. The supernatant which containing DNA of S.pneumoniae was diluted with 90µl of PBS or PCR nuclease and stored in -20°C.

2.6. Polymerase Chain Reaction (PCR) analysis
In the current study, four primers (BIONEER, Korea) were selected, specially for pneumococcal which responsible for virulence factors genes, including the CpsA gene (encodes synthesis of capsule), the PsA gene (encodes the adhesion), LytA (encodes the autolysin) and ply (encodes the pneumolysin) as show in table 1 . Each 25µl of PCR tube reaction containing 1 µl of each primer forward and reverse, 5 µl of DNA template, 11 µl of free nuclease water and 7µl PCR PreMix (BIONEER, Korea) , also negative control contains all components except DNA, vortex PCR tubes, then placed in the thermocycler PCR device (Fisher scientific) according to the following programs: for CpsA primer, initial denaturation at 94°C for 3 min and 35 cycles of 94°C for 1 min, then 53°C for 1 min and 72°C for 1 min and 30 seconds, and final extension at 72°C for 7 min. For PsA primer, initial denaturation at 94°C for 3 min then 30 cycles consisting of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 2 min, followed by a final step of extension at 72°C for 7 min. Also for LytA primer, initial denaturation at 94°C for 2 min and 30 cycles of 94°C for 15 seconds then 53°C for 15 seconds and 72°C for 15 seconds, followed by final extension at 72°C for 5 min. Finally, for Ply primer initial denaturation at 94°C for 2 min then 25 cycles consisting of 94°C for 10 seconds, 58°C for 15 seconds, and 72°C for 1 min, followed by a final step of extension at 72°C for 5 min. The product of PCR amplification are loaded on 1% agarose gel staining with ethidium bromide by electrophoresis, and compared with ladder 100 bp (BIONEER, Korea) with 70 volts for 45 min.

**Table 1:**-primers for virulent factors of S.pneumonia that used in present study.

<table>
<thead>
<tr>
<th>genes</th>
<th>Primer sequences</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cps F</td>
<td>5-ACGCAACTGACGAGTGTGAC-3</td>
<td>353</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>5-GATCGCGACACCGAACTAAT-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Psa F</td>
<td>5-CTT TCT GCA ATC ATT CTT G-3</td>
<td>838</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>5-GCC TTC TTATA CCT TGT TCT GC-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LytA F</td>
<td>5-CAA CCG TAC AGA ATG AAG CGG-3</td>
<td>308</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>5-TTA TCC GTG CAA TAC TCG TGC G-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ply F</td>
<td>5-ATT TCT GTA ACA GCT ACC AAC GA-3</td>
<td>329</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>5-GAA TTC CCT GTC TTT TCA AAG TC-3</td>
<td></td>
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</tbody>
</table>
3. Results and Discussion

After staining with Ziehl-Neelson stain and gram stain then microscopic examination of sputum samples, only 113 (57.95%) samples were suitable for culture, where the sample sputum is considered a high quality when the number of epithelial cells is smaller than 10 cells while immune cells (polymorphonuclear) greater than 25 when the examination under the magnification of 100X which indicates a bacterial infection [17]. From 113 samples (57.95%) the number of pneumococcal isolates in the current study was 18 isolates (15.92%), this result was consistent with the study of Al-Ali et al. [18], which indicated the isolation 18 (26%) isolates only in Jordan, as well as the study of Aljanaby [19] in the Najaf Governorate, which included an immunological study of 22 (24.44%) isolates of pneumococcal. However, the results did not correspond with the numbers mentioned in the study Mahdi [20] where the number of pneumococcal isolates was 31 (15.5%) isolated from the total number of 200 pneumonia patients in Baghdad. All isolates were diagnosed based on the phenotypic characteristics of isolates, where showed all isolates were α-hemolysis, gram positive, diplococci, lancent shape, sensitive to optochin, as shown in Figure 1. Optochin acts on inhibition the ATPase of S. pneumoniae but not interfere with other types of streptococci, therefore it is an important diagnostic test for S. pneumoniae [21].

Figure 1—shows the positive result of the optochin test and bile solubility test on blood agar plate.
The results showed that all isolates had a solubility at concentration 2% of bile test, as shown in Figure-1. The solubility is due to the presence and effectiveness of the autolysin enzyme. As well as the occurrence of agglutination in all isolates of *S.pneumoniae* in current study as phenotypical characteristic indirecter to present capsule antigen, as shown in Figure-2.

The results showed that *S.pneumoniae* isolates were fastidious (very sensitive) bacteria to dehydration and heat, where characterized by rapid decomposition after the period of incubation 18-24 hours in laboratory conditions because of the autolysin enzyme is synthesized first ineffective form but become effective during the stationary phase [22]. The results showed that the isolates could be preserved at 4 °C for 1-4 days, at -20°C for two months (8 weeks) and only 7 (38.88%) isolates were able to stay for three months with a very clear decrease in the number of bacterial colonies when preserved in STGG broth but 7 isolates (38.88%) were lost due to repeated subculture and effectiveness of autolysin enzyme while in the TSB medium the results showed the ability to preserved bacteria only for 24 hours at 4 °C and only for 1-2 weeks at -20 °C when repeated culture on blood agar plates, as shown in table 2. These results were similar to those reported by O’Brien, *et al*. [12], which indicated that the pneumococcal isolates can be conserved for a short period approximately 9 weeks, while long-term conservation is preferred at -70° C. The study of O’Brien, *et al*. [12] recommended using STGG broth as the medium of transport and storage of samples in epidemiological studies, as well as being an inexpensive medium and remain stable for 6 months after preparation and sterilization. Leung [11] study also suggested that more comprehensive analyzes could be carried out later without significant losses in the number of isolates when using STGG broth to store pneumococcal.
Table 2 - showing the media used in the conservation of pneumococcal isolates

<table>
<thead>
<tr>
<th>No.</th>
<th>No. of S. pneumoniae isolates n=18</th>
<th>STGG broth medium</th>
<th>TSB medium</th>
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<tr>
<td></td>
<td></td>
<td>Days +4°C</td>
<td>Months -20°C</td>
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<tr>
<td></td>
<td></td>
<td>1-4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>S. pneumoniae 5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>S. pneumoniae 8</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>S. pneumoniae 30</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>S. pneumoniae 46</td>
<td>+++</td>
<td>++</td>
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<tr>
<td>5</td>
<td>S. pneumoniae 66</td>
<td>++</td>
<td>-</td>
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<tr>
<td>6</td>
<td>S. pneumoniae 76</td>
<td>+</td>
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<tr>
<td>7</td>
<td>S. pneumoniae 111</td>
<td>+++</td>
<td>+</td>
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<td>8</td>
<td>S. pneumoniae 117</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>9</td>
<td>S. pneumoniae 121</td>
<td>+</td>
<td>-</td>
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<tr>
<td>10</td>
<td>S. pneumoniae 123</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>S. pneumoniae 129</td>
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<td>12</td>
<td>S. pneumoniae 130</td>
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<td>14</td>
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<tr>
<td>16</td>
<td>S. pneumoniae 157</td>
<td>++</td>
<td>-</td>
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<tr>
<td>17</td>
<td>S. pneumoniae 161</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>S. pneumoniae 185</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

Mitchell and Mitchell [3] study noted that the virulence factors of S. pneumoniae including capsules, enzymes and surface proteins had a role in adding complications to prevent the control of pneumococcal infection, also strains that lock one of the important virulence factors become weak strains.
Genetically, the study was limited to 11 isolates of *S.pneumoniae*. The results showed that all the 11 (61.11%) isolates of the present study possess CpsA gene, Figure-3, thus consistent with study Motaweq *et al.*[23], Abdul-Lateef *et al.*[24] and Irajian *et al.*[25], which indicated in their research that all isolates (100%) of *S.pneumoniae* have capsule gene. In the study Park *et al.*[13], it has been noted that the use of CpsA as a special gene to distinguish pneumococcal from close related streptococci. The capsule allows the bacteria to escapes from the phagocytosis as well as to remove it from the surface of the mucous membranes of the host [26]. Some previous studies have indicated that CpsA gene is the housekeeping gene and its use in molecular diagnosis but subsequent studies have shown that it is a regulatory gene and may not be present in some serotypes [27].

The results showed that the PsaA gene was present in only 9 of 11 (81.81%) isolates, as shown in Figure-4. This results was not consistent with Abdul-Lateef *et al.*[24], which indicated that the PsaA gene is present in only 2 of 8 isolates (25%), and Anthony *et al.*[14], which stated that PsaA gene was present in 30% of pneumococcal isolates. PsaA (surface adhesion A of pneumococci) is an extracellular protein linked to lipid with size 37kDa, which is an important virulence factor and acting as a bacterial transport system for the transport of Mn$^{2+}$ and Zn$^{2+}$ in to bacterial cell [6].

The results of DNA amplification has been showed that the LytA gene is found in all 11 (61.11%) isolates with size 308bp in the present study Figure-5., this was agreement with Irajian *et al.*[25] study, which found that the LytA gene was present in 40 isolates (100%) when using multiplex PCR with other virulence genes, but they were not agreement with Motaweq *et al.*[23], which indicated that the LytA gene was 89.2% and Abdul-Lateef *et al.*[24] study, which found that the LytA gene is present in 50% of the pneumococcal isolates. Autolysin is a virulence factor in pneumococci as it plays a role in the release of pneumolysin and cell wall components that stimulate responses to inflammation in the host [7].

**Figure 3** shows the Gel electrophorsis of amplification PCR product of the Cps gene (353 pd). Lane (L) (100bp DNA ladder), Lane (no. 46-185) as a positive result of *S.pneumoniae*, Lane (C) as a negative control.
Figure 4 shows the Gel electrophorsis of amplification PCR product of the *Psa A* gene (838 pd). Lane (L) (100bp DNA ladder), Lane (no. 46-117, 130-139,157-185) as a positive result of *S. pneumoniae*, Lane (no. 123,153) as a negative result.

Figure 5 shows the Gel electrophorsis of amplification PCR product of the *lyt A* gene (308 pd). Lane (L) (100bp DNA ladder), Lane (no. 46-185) as a positive result of *S. pneumoniae*, Lane (C) as a negative control.
The results of amplification of the Ply gene were found in all isolates (61.11%), as shown in Figure-6, this results consistent with Motaweq et al.[23] study, which was found in (97.3%) and with Irajian et al.[25] study, which showed that the Ply gene is present in all pneumococci isolate. Pneumolysin dose not only play a role in disease events but has a role in disrupting the function of epithelial cells of the lung as well as the ability of pneumococci to invade the bloodstream [28]. In addition, pneumolysin works to inhibit the beating the cilia of the epithelial cells lining the respiratory tract and thus increase the accumulation of pneumococci in the lungs resulting in the occurrence of pneumonia [29].

Figure 6-shows the Gel electrophorsis of amplification PCR product of the Ply gene (329 pd). Lane (L) (100bp DNA ladder), Lane (no. 46-185) as a positive result of S.pneumoniae, Lane (C) as a negative control

Conclusion

_S.pneumoniae_ is very sensitive bacteria and difficult to deal with it because of its rapid degradation in the culture media thus it requires special practice. The STGG broth showed a high efficiency in keeping the bacterial isolates for a longer period to be studied intensively compared to the TSB medium. Pneumococcal isolates that isolated from patients with pneumonia in the province of Basra were characterized by their having different virulence factors which had a role in colonizing the host and thus causing infection.

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References