DOI:10.24996/ijs.2018.59.3A.8

ISSN: 0067-2904

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

Halah M. AlHajem*[1], Mohammed A. almazini[1], Bassam Y. Khudaier[2]
[1]Department of Biology, College of Science, Basra University, Basra, Iraq
[2] Department of microbiology, College of Veterinary Medicine/ Basra University, Basra, Iraq

Abstract

Extensive studies have been conducted on the microbial properties of *Streptococcus 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 18 (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 11 (٦١.11%) while the *Psa* gene was present in only 9 (50%) isolates within the current study.

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

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

هاله معين الحاجم[١]، محمد عبدالامام المازني[١]، بسام ياسين خضير[٢]
[١]قسم علوم الحياة ،كلية العلوم ، جامعة البصرة، البصرة، العراق
[٢]فرع الاحياء المجهرية ، كلية الطب البيطري ، جامعة البصرة، البصرة ، العراق

الخلاصة

لقد أجريت دراسات مستفيضة على الخصائص الميكروبية لبكتريا العقدية الرئوية في جميع انحاء العالم ، لكن هناك القليل من الدراسات في العراق حول أهم عوامل الضراوة التي تمتلكها عزلات العقدية الرئوية الموجودة في العراق . جمعت ١٩٥ عينة قشع من المرضى المصابين بالالتهاب الرئوي المكتسب من المجتمع و الذين تم تشخيصهم سريريا" من قبل اطباء متخصصين اعتمادا"على الاعراض والتصوير الشعاعي للصدر، شخصت ١٨ عزلة تعود للعقدية الرئوية بأستخدام الطرق الروتينية الخاصة في التشخيص المظهري ، أعطت جميع العزلات 18 (١٠٠%) نتيجة موجبة لاختبار الاوبتشين و اختبار الذائبية و اختبار التلازن . وراثيا" ،اقتصرت على دراسة عوامل الضراوة في ١١ عزلة فقط بأستخدام تقنية تفاعل البلمرة المتسلسل (PCR) تم التحري عن وجود أربعة جينات مسؤولة عن عوامل الضراوة التي تعتبر ضرورية للعقدية الرئوية في

*Email: Halh_alhachem@yahoo.com

استعمار وغزو المضيف ، أظهرت النتائج ان عزلات العقدية الرئوية في مدينة البصرة كانت ذات ضراوة ، حيث أظهرت نتائج تضخيم PCR ان الجينات *CpsA* ، *LytA* و *Ply* موجودة في جميع العزلات ١١ (61.11%) في حين ان الجين *PsaA* موجود فقط في ٩ (50%) عزلات ضمن الدراسة الحالية .

1. Introduction

Streptococcus pneumonia (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 a extracellular protein found on the surface of the cell , the presence of antibodies to *PsaA* 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 22to 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% CO_2 (candle jar) for 18-24 hours at 35°C [9].

2.3.1 phenotypic characteristics

Initial identified methods for pneumoncoccal isolates have been adopted such as morphology of colony, alpha hemolytic on blood agar plate with flatted 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% CO_2 (candle jar) for 18-24 hours . The test is considered positive when the diameter of inhibition zone was more than 14mm, this test is used to differentiate between *S.pneumoniae* and other types of Streptococci [9].

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℃ 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-Glucous-Glycerol (STGG) broth. The last medium was prepared depending on O´Brien, *et al*. [12].

2.5. DNA Eetraction

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℃ 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 *PsaA* 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 1min and 30 seconds , and final extension at 72 ° C for 7min . For *PsaA* 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* 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.

genes	Primer sequences	Amplicon size (bp)	Reference
Cps F *Cps* R	5-ACGCAACTGACGAGTGTGAC-3 5-GATCGCGACACCGAACTAAT-3	353	[13]
Psa F *Psa* R	5-CTT TCT GCA ATC ATT CTT G-3 5-GCC TTC TTTA CCT TGT TCT GC-3	838	[14]
LytA F *LytA* R	5-CAA CCG TAC AGA ATG AAG CGG-3 5-TTA TTC GTG CAA TAC TCG TGC G-3	308	[15]
Ply F *Ply* R	5-ATT TCT GTA ACA GCT ACC AAC GA-3 5-GAA TTC CCT GTC TTT TCA AAG TC-3	329	[16]

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(polymorphounclear) 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 shap, 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.

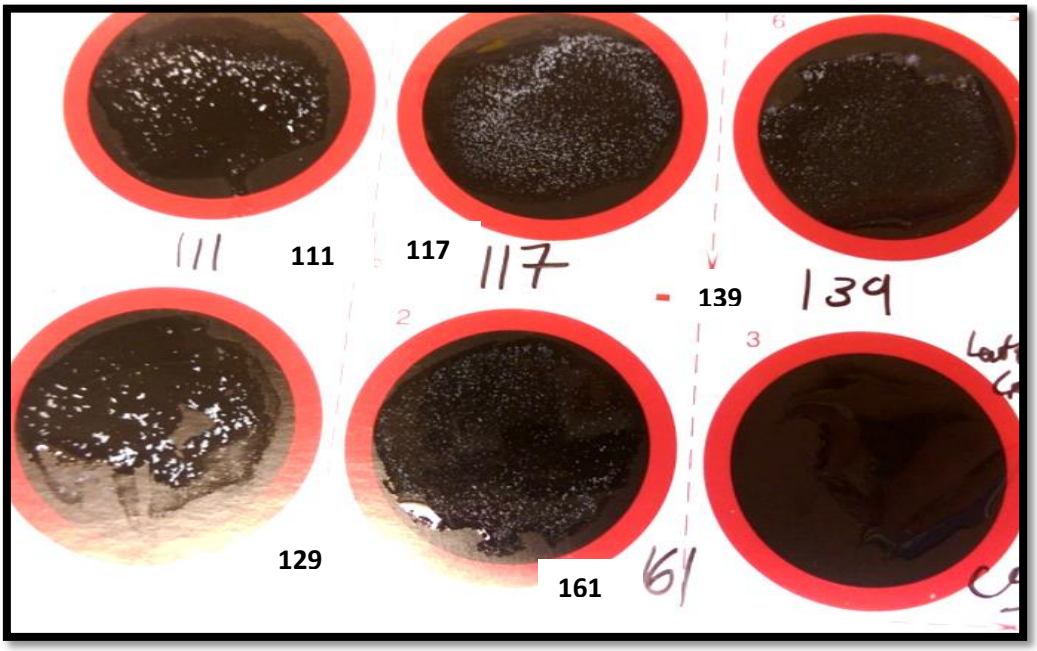

Figure 2-shows the positive result of Latex agglutination test.

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 indicter 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

No.	No. of *S.pneumoniae* isolates n=18	STGG broth mediun							TSB medium						
		Days +4°C			Months -20°C				Days +4°C			weeks -20°C			
		1-4	5	6	1	2	3	4	1	2	3	1	2	3	4
1	*S.pneumoniae* 5	+	-	-	-	-	-	-	/	/	/	-	-	-	-
2	*S.pneumoniae* 8	+	-	-	-	-	-	-	/	/	/	-	-	-	-
3	*S.pneumoniae* 30	+	-	-	-	-	-	-	/	/	/	/	/	/	/
4	*S.pneumoniae* 46	+++	++	-	+++	+++	+	-	+	-	-	+	+	-	-
5	*S.pneumoniae* 66	++	-	-	-	-	-	-	/	/	/	-	-	-	-
6	*S.pneumoniae* 76	+	-	-	-	-	-	-	/	/	/	-	-	-	-
7	*S.pneumoniae* 111	+++	+	-	+++	+++	++	-	+	-	-	+	+	-	-
8	*S.pneumoniae* 117	+++	+	-	+++	+	-	-	-	-	-	-	-	-	-
9	*S.pneumoniae* 121	+	-	-	-	-	-	-	-	-	-	-	-	-	-
10	*S.pneumoniae* 123	+++	+	-	+++	+++	++	-	+	-	-	+	+	-	-
11	*S.pneumoniae* 129	+	-	-	-	-	-	-	+	-	-	+	-	-	-
12	*S.pneumoniae* 130	+++	+	-	+++	++	+	-	+	-	-	+	+	-	-
13	*S.pneumoniae*137	+++	+	-	+++	++	-	-	-	-	-	+	+	-	-
14	*S.pneumoniae* 139	+++	+	-	+++	+	-	-	-	-	-	+	+	-	-
15	*S.pneumoniae* 153	+++	+	-	+++	++	+	-	-	-	-	+	+	-	-
16	*S.pneumoniae* 157	++	+	-	+++	+++	++	-	/	/	/	+	+	-	-
17	*S.pneumoniae*161	+++	+	-	+++	++	-	-	/	/	/	+	+	-	-
18	*S.pneumoniae* 185	++	+	-	+++	+++	++	-	/	/	/	+	+	-	-

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(50%) 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].

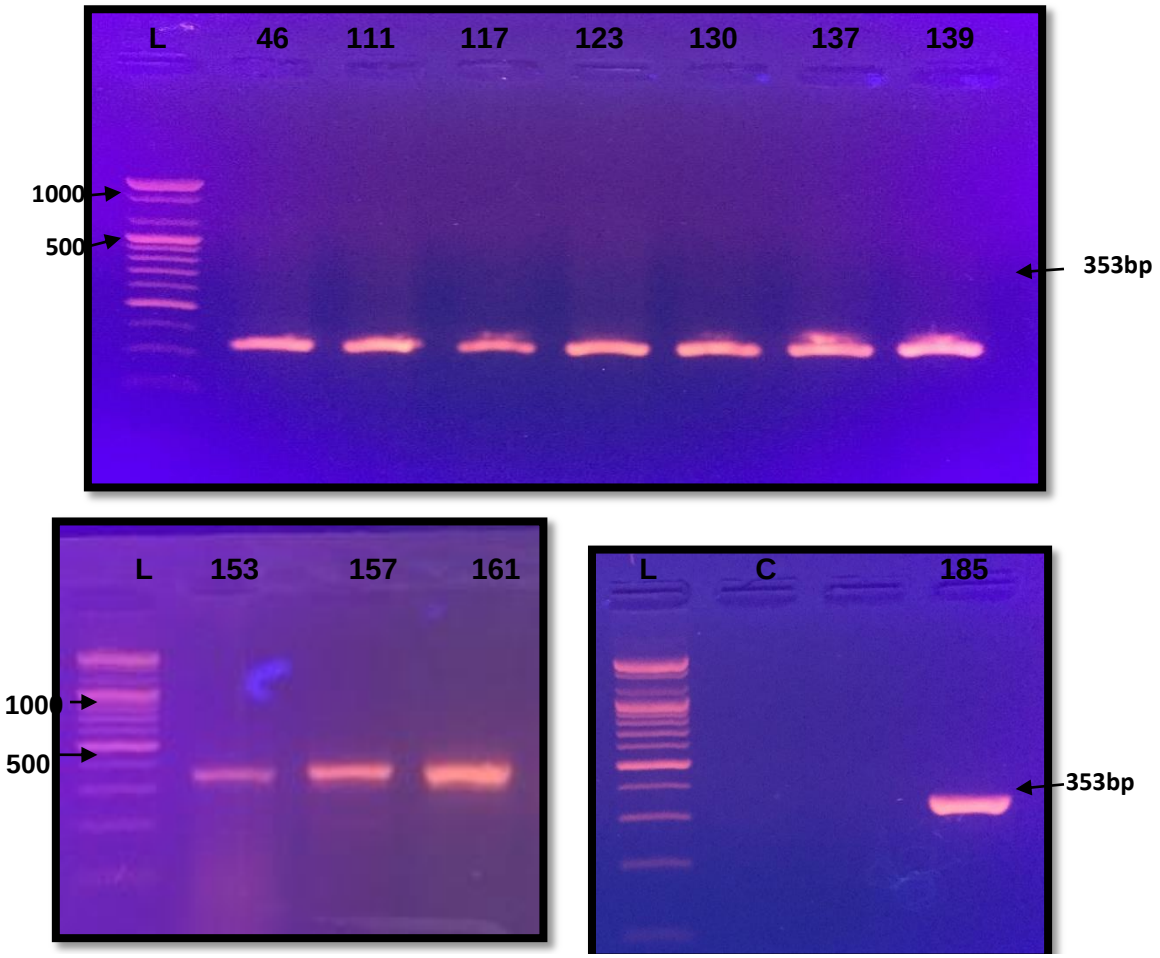

Figure 3-shows the Gel electrophorsis of amplification PCR product of the *Cps* gene (353 pd).Lane (L) (100bp DNA ladder), Lane (no. 46-185) as a positive result of *S.pneumoniae* , Lane (C) as a negative control .

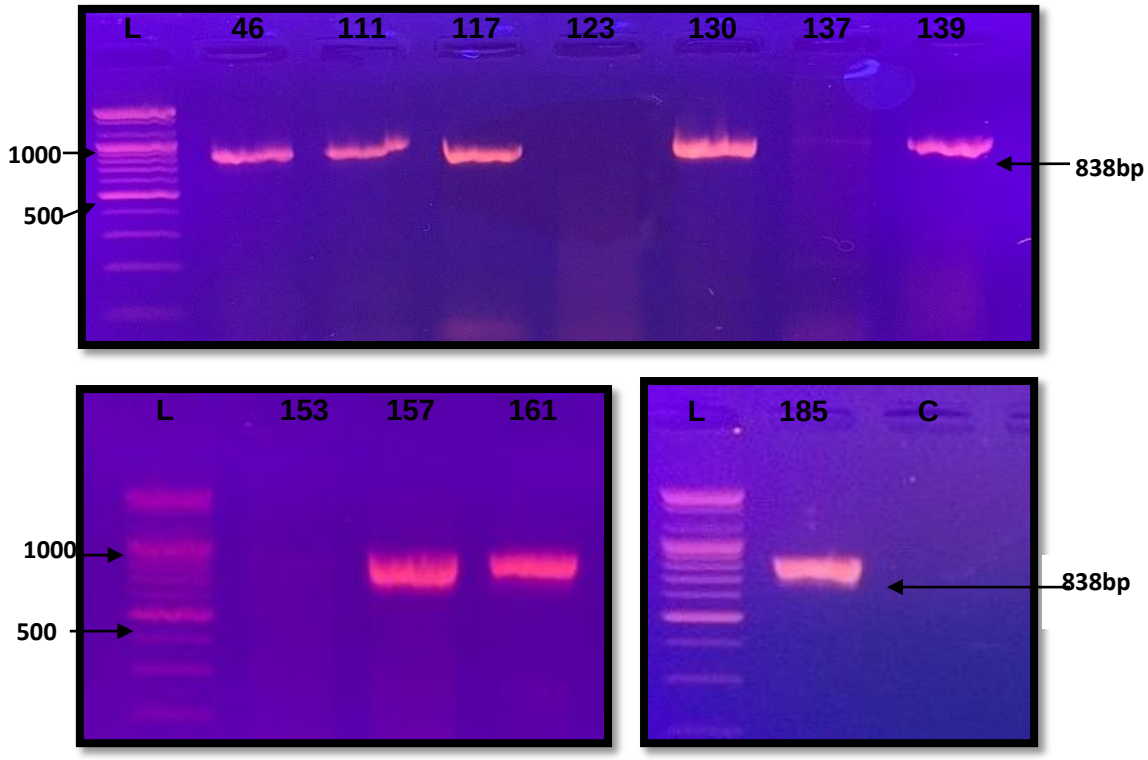

Figure 4-shows the Gel electrophorsis of amplification PCR product of the *Psa A* gene (838 pd), Lane (L) (100bp DNA ladder), Lane (no. 46-117, 130-139,157-185) as a positive result of *S.pneumoniae*, Lane (no. 123,153) as a negative result.

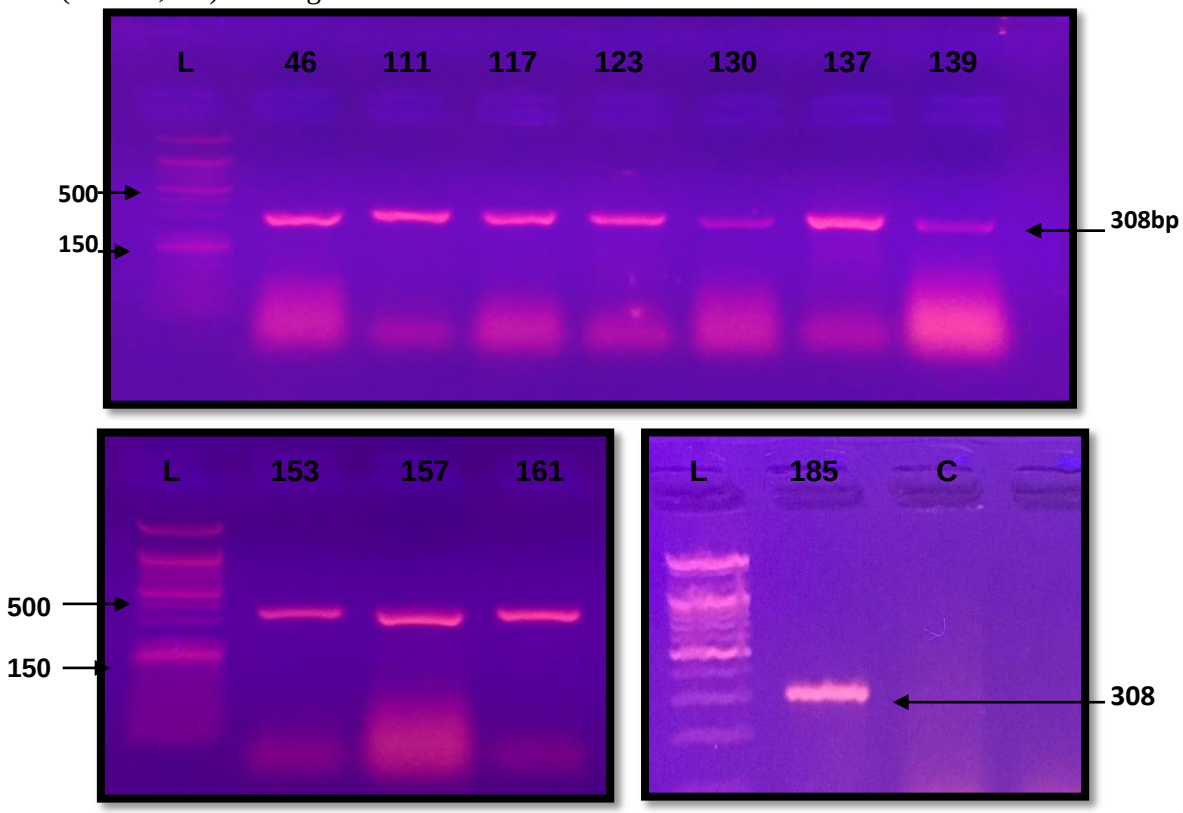

Figure 5-shows the Gel electrophorsis of amplification PCR product of the *lyt A* gene (308 pd). Lane (L) (100bp DNA ladder), Lane (no. 46-185) as a positive result of *S.pneumoniae*, Lane (C) as a negative control

The results of amplification of the *Ply* gene were found in all isolates 11 (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].

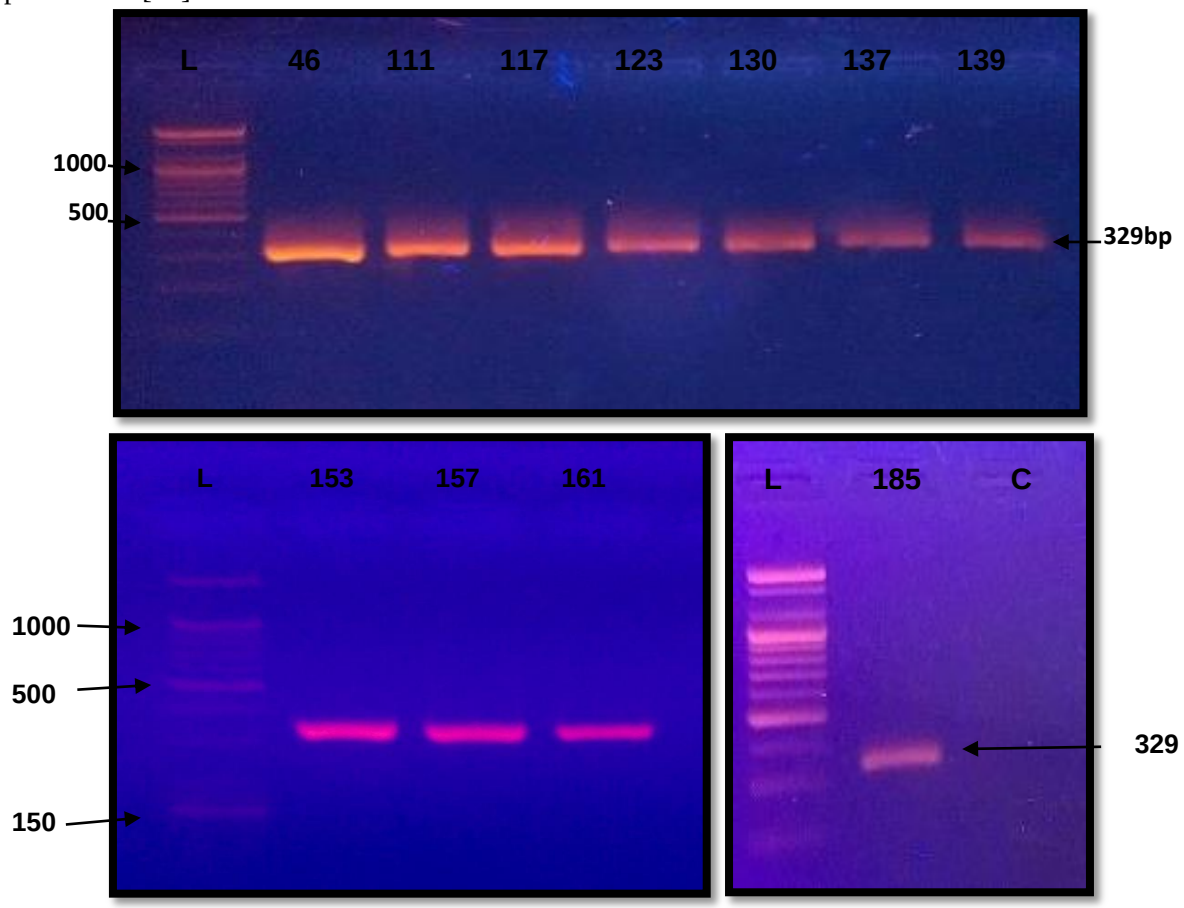

Figure 6-shows the Gel electrophorsis of amplification PCR product of the *Ply* gene (329 pd). Lane (L) (100bp DNA ladder), Lane (no. 46-185) as a positive result of *S.pneumoniae*, Lane (C) as a negative control

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.

Acknowledgements

This study was supported by the University of Basra, the Department of Biology, Genetic Engineering Laboratories, and the Center for Thoracic and Respiratory Diseases in Basra Governorate.

References

1. Mandell, L.A. **2004.** Epidemiology and etiology of community-acquired pneumonia. *Infect. Dis. Clin. North*, **18**:761-76.
2. World Health Organization (WHO**) 2007**. Weekly Epidemiological Record. World Health Organization, **82**: 93-104.
3. Mitchell, A.M. and Mitchell, T. J**. 2010.** *Streptococcus pneumoniae*: virulence factors and variation. *CMI*, **16**: 411–418.
4. Romero-Steiner, S., Pilishvili, T., Sampson, J. S., Johnson, S. E., Stinson, A., Carlone, G. M. & Ades, E.W. **2003**,"Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-*PsaA* antibodies". *Clin. Diagn.Lab. Immun*., **10**: 246-251.
5. Nelson, A.,Roche, A., Gould, J. ,Chim, K., Ratner, A.& Weiser, J. **2007b.** "Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance". *Infect. Immun.,* **75**: 83-90.
6. Hirst, R. A., Kadioglu, A. and O'Callaghan, C. et al.**2004.** The role of pneumolysin in pneumococcal pneumonia and meningitis. *Clin. Exp. Immunol.* , **138**: 195–201.
7. Martner, A., Dahlgren, C., Paton, J. C. and Wold, A. E. **2008**, "Pneumolysin released during *Streptococcus pneumoniae* autolysis is a potent activator of intracellular oxygen radical production in neutrophils". *Infect. Immun*., **76**: 4079-4087.
8. Jedrzejas, M.J. **2001.** Pneumococcal virulence factors: structure and function. *Microbiol. Mol. Biol. Rev.,* **65**: 187-207.
9. World Health Organization (WHO) **2003**. Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World. Centers for Disease Control and Prevention, Atlanta, Georgia, USA
10. Macfaddin, J.F. **2000.** Biochemical tests for identification of medical bacteria .3rd ed., Lippincott Williium and Wilkins, U.S.A.
11. Leung, M. 2012. Phenotypic and genotypic diversity of streptococcus pneumoniae strains in Tanzania and the United Kingdom.PHD thesis, Department of Infection, University College London.
12. O'Brien, K., Bronsdon, M., Dagan, R., Yagupsky, P., Janco, J., Elliott, J., Whitney, C., Yang, Y., Robinson, L. & Schwartz, B. **2001**, "Evaluation of a medium (STGG) for transport and optimal recovery of *Streptococcus pneumoniae* from nasopharyngeal secretions collected during field studies" *Clin. Microbiol.*, **39**: 1021-1024.
13. Park, H.K., Lee, S.J., Yoon, J.W., Shin, J.W., Hyoung-Shik S., Kook,J., Myung, S. C. and Kim, W. **2010**. Identification of the *cpsA* gene as a specific marker for the discrimination of *Streptococcus pneumoniae* from viridans group streptococci .*Medical Microbiology*,.**59**: 1146–1152
14. Anthony, J., Scott, G., Marston, E.L., Hall, A.J. & Marsh, K. **2003**. Diagnosis of pneumococcal pneumonia by *psaA* PCR analysis of lung aspirates from adult patients in Kenya. *Journal of Clinical Microbiology*. **41**: 2554–2559.
15. Nagai, K., Shibasaki, Y., Hasegawa, K., Davies, T. A., Jacobs, M. R., Ubukata, K. and Appelbaum, P. C. **2001.** Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and beta-lactam resistance, and to detect common macrolide resistance determinants. *Antimicrob. Chemother.* **48**: 915–918.
16. Salo, P., Ortqvist, A. and Leinonen, M. **1995.** Diagnosis of bacteremic pneumococcal pneumonia by amplification of pneumolysin gene fragment in serum. *Infect. Dis.* **171**: 479–482.
17. Musher, D.M., Montoya, R. and Wanahita, A.**2004**. Diagnostic value of microscopic examination of Gram-stained sputum and sputum cultures in patients with bacteremic pneumococcal pneumonia. *Clin. Infect. Dis.* **39**: 165-9.
18. Al-Ali, M.K., Batchoun, R.G. and Al-Nour, T.M. **2006**. Etiology of community-acquired pneumonia in hospitalized patients in Jordan. *Saudi. Med.* **27**: 813-6.
19. Aljanaby, A.A. **2010.** *Bacteriological and Immunological study of Streptococcus pneumoniae isolated from patients infected with respiratory tract infection in Al-Najaf Governorate*. Ph.D. Thesis, Department of biology,College of Science, Kufa University ,Iraq.
20. Mahdi, A.S. **2007**. *Common bacterial causes isolated from patients with community –acquired pneumonia.*MSc. Thesis, Department of biology,College of Medicine , Mustansiriya University,Iraq.

21. Martin-Galiano, A.J., Balsalobre, L., Fenoll, A. and Campa, A.G. **2003.** Genetic characterization of optochin-susceptible viridians group streptococci. *Antimicrob. Agents. Chemother.* , **47**: 3187-94.

22. Tomasz, A. and Westphal, M. **1971.** "Abnormal autolytic enzyme in a pneumococus with altered teichoic acid composition". *Proc. Nat. Acad. Sci. U. S. A.,* **68**: 2627-2630.

23. Motaweq, Z.Y., Naher,H.S. and Al-Dahhan,H.A. **2015**. Phenotypic and Genotypic characterization of some virulence factors in *Streptococcus pneumoniae* isolated from patients with LRTI in Najaf Province/ Iraq. *International Journal of Scientific & Engineering Research*, **6**: 459-465.

24. Abdul-Lateef,L.A., Alturaihy, S.H. and Al-Taai, S.A. **2015**.Molecular Characterization of Some Virulence Factors of *Streptococcus pneumoniae* Isolated from Children with Acute Otitis Media in Hilla, Iraq . *British Biotechnology Journal*. 10: 1-11.

25. Irajian, G., Ahmadi, A. and Talebi, M. **2013.** The study of *Streptococcus pneumoniae* in invasive and non invasive Infections and multiplex PCR Detection of Four Virulence Genes . *Infect. Epidemiol. Med.* **1**: 3-8.

26. Hyams, C., Yuste, J., Bax, K., Camberlein, E., Weiser, J. and Brown, J.. 2010. "Streptococcus pneumoniae resistance to complement-mediated immunity is dependent on the capsular serotype". Infect. Immun., 78: 716-725.

27. Jourdain, S., Drèze, P.A., Vandeven, J., Verhaegen,J., Van Melderen, L. and Smeesters, P.R. **2011.** Sequential multiplex PCR assay for determining capsular serotypes of colonizing *S. pneumonia. BMC Infect. Dis.* **11**: 100.

28. Norman, E.M. **2015.** *Characterization of virulence in clinical isolates of Streptococcus pneumoniae* .Department of Microbiology ,Tumor and Cell Biology ,Karolinska Institutet, Stockholm,Sweden.

29. Boulnois, G. J., Paton, J. C., Mitchell, T., J. and Andrew, P. W. **1991.** Structure and function of pneumolysin, the multifunctional thiol-activated toxin of *Streptococcus pneumoniae. Molecular Microbiology*.**2**: 2611- 2616.