Distribution of Virulence genes in *Streptococcus pneumoniae* Isolated from Different Baghdad Hospitals

Dina Muthana Fahran[1], Basima Qasim Hasan Al-Saadi[2]

[1]Scholar Researcher, [2]Assist. Prof. Genetic Engineering and Biotechnology Institute for Postgraduate Studies University of Baghdad, Iraq

Abstract

Streptococcus pneumoniae causes serious infections (pneumonia, meningitis and bacteremia) with significant morbidity and mortality rates. The goal of this study is to isolate and identify *S. pneumoniae* from clinical samples in Baghdad City hospitals in order to explore the presence of three virulence genes (*lytA*, *ply* and *psaA*) in clinical isolates. A total of 120 separate clinical samples were obtained from inpatients and outpatients with upper and lower respiratory tract infections at different hospitals in Baghdad. Twenty-five isolates of *S. pneumoniae* were identified by using cultural, morphological and biochemical characteristics as well as the diagnosis by VITEK2 system. Clinical samples were including sputum 19 isolates (76%) and urine 6 isolates (24%). The results of collected specimens showed that male's percentage is highest than female's patients, which was out of 25 isolates are 15 (60%) males and 10 (40%) females, The results also showed that it is much more common in elderly people than in younger people. The genomic DNA of bacterial isolates was applied in a polymerase chain reaction (PCR) to amplify certain genes. The PCR results were: Six isolates (24%) show positive results for the presence of *lytA* gene. Five isolates (20%) showed positive results for the presence of *ply* gene. Twenty-three isolates (92%) show positive results for the presence of *psaA* gene. As concluded from the present study, that the results exhibited that the presence of the virulence genes can be considered as a serious issue.

Keywords: *virulence genes, Streptococcus pneumoniae, Baghdad hospitals*

Introduction

Streptococcus pneumoniae bacteria carries a plethora of factors that helps in disease pathogenicity and immune evasion. The most common habitat is the upper respiratory tract, which not only assist in colonization but also provide an opportunity to cause an invasive disease. It is gram–positive facultative anaerobic pathogen, alpha- hemolytic bacteria [(1)]. Estimation of recent data show that this bacteria is responsible for 14.5 million annual infections worldwide and more than 800,000 deaths in children less than five years of age [(2)]. The genome of *S. pneumoniae* is quite plastic that means they can readily acquire DNA from environment and can recombine with the incoming DNA thus acquiring virulence and new genes for adaptation so it has a horizontal gene transfer that leads to a wide variation in pneumococcal genome and explain the presence of a large number of virulence genes attributing to disease pathogenicity [(3)]. The virulence factors of pneumococcal include its` capsule, cell wall components, pili and recently identified pneumococcal proteins including pneumolysin (*ply*), autolysin lytic amidase (*lytA)*, pneumococcal surface adhesion A (*psaA*), choline-

Corresponding author:
Dina Muthana Fahran
Email: dinamuthana@gmail.com

binding proteins (CBPs), Neuraminidase, biofilm, IgA protease and Lipoteichoic acid [4]. Many risk factors for streptococcal infection such as age, race, immunodeficiency, other illness, previous antibiotic therapy and day–care attendance may play an important role in streptococcal diseases [5]. The epidemiological data of *pneumococcus* in Iraq is little at present time, therefore recent study aims to analyze distribution of virulence factors of isolates identified from Iraqi patients in Baghdad hospitals, to provide data support for development pneumococcal infection prevention strategies and vaccination.

Materials and Methods

Collection of samples

A total of 120 clinical samples were obtained, include: sputum samples that were collected from patients by asterial disposable cotton swaps. In addition, clean midstream urine samples collected from patients. The specimens sent to the laboratory, and examined.

Isolation and Identification of *Streptococcus pneumoniae*

The collected samples were inoculated on blood agar that already prepared and incubated at 37ºC for 24-48 hours with ~5% CO2 (in a candle-jar). Isolates were examined for their shape, size, color, pigments, and hemolytic activity [6]. The identification of *Streptococcus pneumoniae* done by microscopic examination (Gram stain) [7]. In addition to biochemical tests performed according to [8]. Including Catalase test [9], Optochin test [10] and diagnosis by VITEK2 device.

Bacterial isolates preserved using long-term preservation (20 ml of glycerol to 80 ml of Brain Heart infusion broth) [11].

Molecular detection of virulence genes

DNA extraction of *S. pneumoniae* isolates done by a genomic DNA extraction kit supplemented by the manufacturing company (Promega, USA).

Polymerase Chain Reaction (PCR) amplification analysis

In the current study, four primers were selected, especially for pneumococcal which responsible for virulence factors genes, including the *LytA* (encodes the autolysin), *ply* (encodes the pneumolysin) and *PsaA* gene (encodes the adhesion). Each 25 PCR tube reaction contains 1.5 µl of each forward and reverse primer, 3 µl of DNA template, 14 µl of deionized water, and 5 µl of PCR PreMix (BIONEER, Korea), as well as a negative control that contains all components except DNA. Vortex PCR tubes, and then placed in the thermocycler PCR device according to table 1.

Table 1: Optimum of PCR reaction for *lytA*, *ply* and *psaA* genes detection.

Target gene	PCR conditions	Temperature (ºC)	Time	No. of cycles
lytA ply	Initial denaturation	95	3 min.	1
	denaturation	95	30 sec.	30
	Annealing	58	30 sec.	
	extension	72	30 sec.	
	Final extension	72	7 min.	1
Target gene	PCR conditions	Temperature (ºC)	Time	No. of cycles
psaA	Initial denaturation	95	3 min.	1
	denaturation	95	30 sec.	30
	Annealing	55	30 sec.	
	extension	72	30 sec.	
	Final extension	72	7 min.	1

Results and Discussion

Isolation and identification of *Streptococcus pneumoniae*

A total of 120 separate clinical samples (sputum and sterile urine samples) were obtained from inpatients and outpatients at different hospitals in Baghdad. About 30 samples showed negative growth of bacteria while 90 samples showed positive growth of bacteria. 50 isolates where non-*Streptococcus spp.* and 40 isolates where belonging to the *Streptococcus spp.* but only 25 isolates confirmed to be *Streptococcus pneumoniae*, , this expectation was observed after studying the shape of the colonies, size, color and surface texture after it was developed in the traditional differentiate. Selective and modified media cultures and in addition to the biochemical tests and VITEK2. Twenty-five isolates were recovered on subculture and confirmed as *S. pneumoniae* from different clinical sources including: sputum 19 isolates (76%) and urine 6 isolates (24%). Out of 25 isolates are 15 (60%) males and 10 (40%) females, the results of collected specimens showed that male's percentage is highest than female's patients. Distribution according to age groups demonstrated in figure 1.

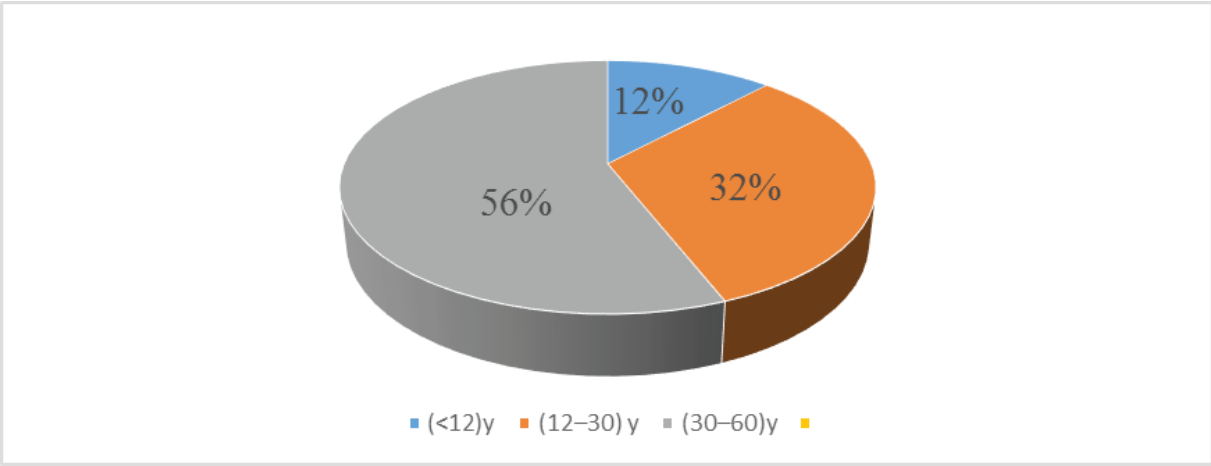

Figure 1: Patients distribution according to the age groups

This result approximately agree with Motaweq and Naher, (12) that the age group 51-60 years had the highest prevalence, with (19.7%), followed by age groups over 60 years (14%), 31-40 years (11%) and 1-10 years with (10.8%).

More *S. pneumoniae* were isolated from males 15 (60%) than females 10 (40%) This may be due to the fact that smoking is more common in males [12]. Despite variations in percent of isolates, Al-Taaie [13] found that *pneumococci* had a total incidence among those aged over 49 years.

Pneumococcal pneumonia is much more common in elderly people than in younger and middle-aged people, according to Pejcic *et al*. [14]. *Pneumococcus* is the most common organism found in hospitalized elderly patients suffering from CAP (community acquired pneumonia), according to Gupta *et al*. [15].

The high prevalence of pneumococcal isolate in the 51-60 age group may be attributed to immune system impairment, and the majority of elderly people are infected with chronic diseases [16]. Females had a higher number of cases than males in the age group 31-40. Most females in this age group are likely to be taking a variety of medications and hormones for pregnancy-related issues, putting them at risk of infection due to lowered immunity [17]. In contrast to our results, increased infection rates with pneumonia

in the 1-10 age group could be due to low immunity and increased exposure to contaminated materials. Pneumonia is also the leading cause of death from infectious disease around the world. It killed almost 1.6 million children under the age of five in 2008 [18].

Molecular detection of *S. pneumoniae* genes by PCR

Detection of Autolysin *(lytA)* gene

The extracted DNA was amplified for *lytA* gene by PCR. Amplified product size was 319bp as shown in figure 2. Six isolates (24%) show positive results for the presence of *lytA* gene.

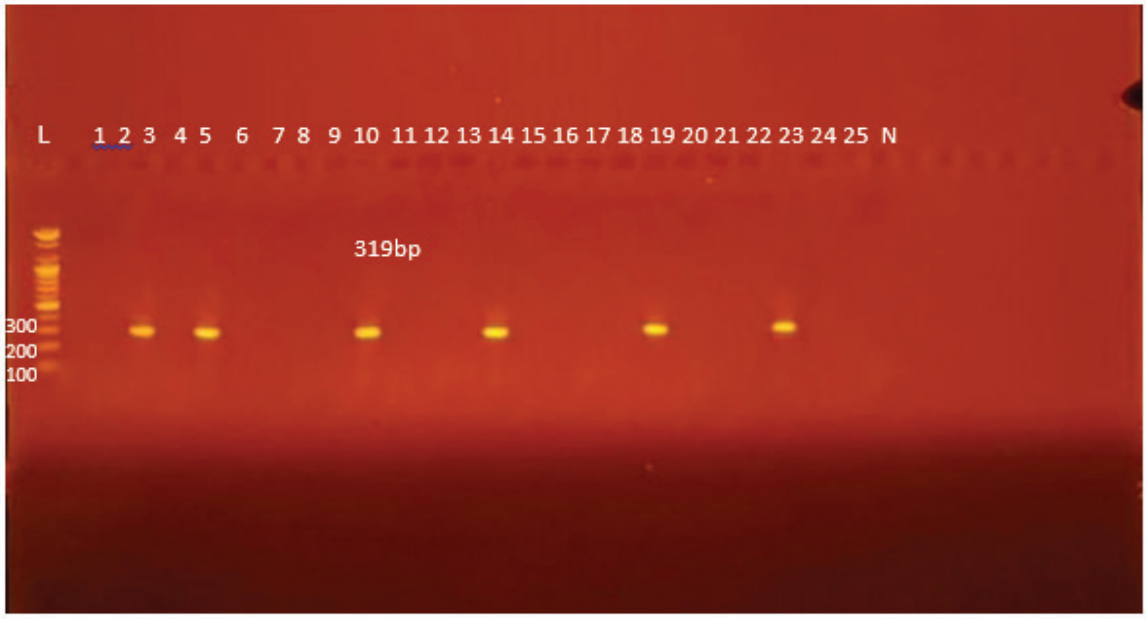

Figure 2: Gel electrophoresis for PCR product of *lytA* gene for *S. pneumoniae* using 1.5% agarose gel at 60volt for 15 minutes and then at 90volt for another 15 minutes. Lane L: 100bp DNA ladder, Lane (1-25): results for *lytA* gene; Lane N: Negative control

This result is identical with the result obtained by Asadi *et al*. [19] which found that *lytA* gene detected in (23%) from Middle ear effusion samples. Moreover, this result in disagreement with Abdul-Lateef *et al*. (20) results which found that *lytA* gene is present only in 4 isolates out of 8 (50%) and Irajian *et al*. [21] study, which found that *lytA* was present in (100%) isolates when using multiplex PCR with other virulence genes. Also, disagree with the results obtained by Kurola and Paula [22], that found the prevalence of *lytA* is (51%) of isolates by PCR or with Motaweq *et al*. [23], which showed that the *lytA* gene was (89.2%).

Detection of Pneumolysin *(ply)* gene

The extracted DNA was amplified for *ply* gene by PCR. Amplified product size was 347bp as shown in figure 3. Five isolates (20%) show positive results for the presence of *ply* gene.

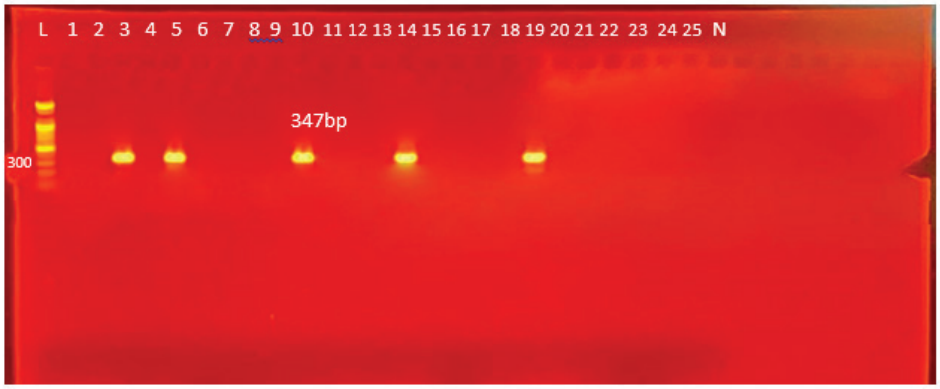

Figure 3: Gel electrophoresis for PCR product of *ply* gene for *S. pneumoniae* using 1.5% agarose gel at 60volt for 15 minutes and then at 90volt for another 15 minutes. Lane L: 100bp DNA ladder, Lane (1-25): results for *ply* gene; Lane N: Negative control

This result in disagreement with Abdul-Lateef *et al*. [20] results which found that *ply* is present only in 4 isolates out of 8 (50%). Also, disagree with the results obtained by Sourav *et al*. [24] that found the isolates gave (71%) PCR positive result for pneumolysin (17 out of 24 isolates). Moreover, this result totally disagrees with the result obtained by Kurola and Paula [22], which found that (85%) *ply* gene, during the testing of *S. pneumoniae* isolates. These variations may be due to a variation in the marker's sequence. However, all *S. pneumoniae* isolates found to generate pneumolysin extracellularly in blood agar, implying that pneumolysin encoded by several genetic loci, and that other bacterial exotoxins may have the potential to guide cell lysis and thus aid in microbial spread through tissues by causing significant damage to the extracellular matrix or eukaryotic cells' plasma membrane [25].

Detection of Pneumococcal surface adhesion A *(psaA)* gene

The extracted DNA was amplified for *psaA* gene by PCR. Amplified product size was 512bp as shown in figure 4. Out of 25 only 23 isolates (92%) show positive results for the presence of *psaA* gene.

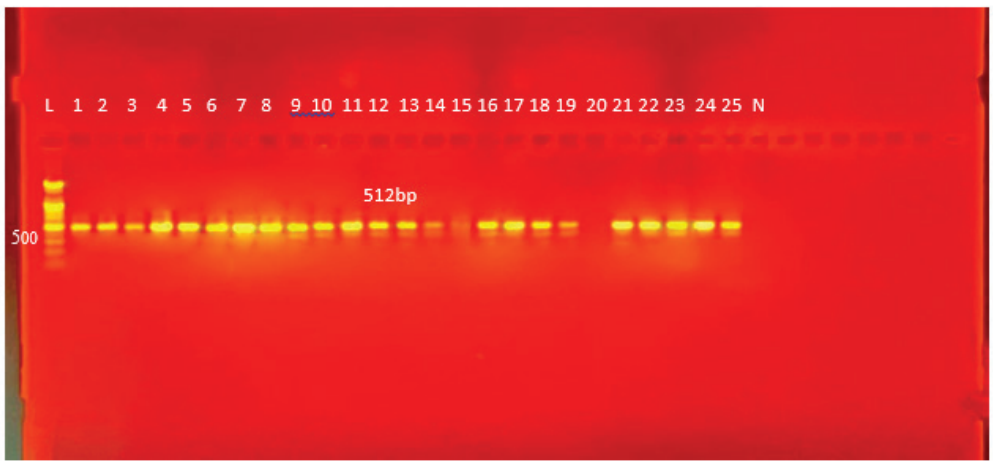

Figure 4: Gel electrophoresis for PCR product of *psaA* gene for *S. pneumoniae* using 1.5% agarose gel at 60volt for 15 minutes and then at 90volt for another 15 minutes. Lane L: 100bp DNA ladder, Lane (1-25): results for *psaA* gene; Lane N: Negative control

This result is in agreement with the result obtained by Morrison *et al*. [26], which found *psaA* gene detected in all isolates of *S. pneumoniae* from clinical samples. However, this result is in disagreement to the result obtained by Abdul-Lateef *et al*. [20] that *psaA* is present in 2 isolates out of 8 (25%) and Alhajem *et al*. [27] result that showed that the *psaA* gene was present in only 9 out of 11(50%) isolates.

Conclusion

S. pneumoniae is a very sensitive bacterium that is difficult to handle due to its quick degradation in culture conditions. The distribution of the three virulence genes of *S. pneumoniae* isolates in our study showed that, the number of different virulence factors in different clinical isolates, S3, S5, S10, S14, S19 have the three types of virulence factors. While, other isolates such as S15, S20 do not have any of them. The other isolates are variable. Which played a role in colonizing the host and thereby generating illness.

Conflict of Interest: None

Funding: self

Ethical Clearance: Not required

References

1. Brooks LR, Mias GI. Streptococcus pneumoniae's virulence and host immunity: aging, diagnostics, and prevention. Frontiers in immunology. 2018 Jun 22;9:1366.
2. Blumental S, Granger-Farbos A, Moïsi JC, Soullié B, Leroy P, Njanpop-Lafourcade BM, Yaro S, Nacro B, Hallin M, Koeck JL. Virulence factors of Streptococcus pneumoniae. Comparison between African and French invasive isolates and implication for future vaccines. PLoS One. 2015 Jul 27;10(7):e0133885.
3. Yamaguchi M, Win HP, Higashi K, Ono M, Hirose Y, Motooka D, Okuzaki D, Aye MM, Htun MM, Thu HM, Kawabata S. Epidemiological analysis of pneumococcal strains isolated at Yangon Children's Hospital in Myanmar via whole-genome sequencing-based methods. Microbial Genomics. 2021 Feb 1;7(2):000523.
4. Seid U, Demeke A. Review on Major Virulence Factors of Pathogenic *Streptococcus Species*. *International Journal of Research Studies in Microbiology and Biotechnology*. 2018, 4(2): 11-37.
5. Mingoia M, Morici E, Brenciani A, Giovanetti E, Varaldo PE. Genetic basis of the association of resistance genes mef (I)(macrolides) and catQ (chloramphenicol) in streptococci. Frontiers in microbiology. 2015 Jan 6;5:747.
6. Vandepitte J, Verhaegen J, Engbaek K, Rohner P, Piot P, Heuck CC, Heuck CC. Basic laboratory procedures in clinical bacteriology. World Health Organization; 2003 Dec 31.
7. Yoo SY, Kim PS, Hwan HK, Lim SH, Kim KW, Choe SJ, Min BM, Kook JK. Identification of non-mutans streptococci organisms in dental plaques recovering on mitis-salivarius bacitracin agar medium. Journal of Microbiology. 2005;43(2):204-8.
8. Forbes BA, Sahm DF, Weissfeld AS. Diagnostic microbiology. St Louis: Mosby; 2007, 276-216.
9. Al-Rubaye MR, Yildiztugay E, Uysa A, Mohammed TK, Abdullah HN. Molecular detection of virulent exoU mutation of Pseudomonas aeruginosa isolated from wound and burn samples. EurAsian Journal of BioSciences. 2020;14(2):2811-6.
10. Messmer TO, Sampson JS, Stinson A, Wong B, Carlone GM, Facklam RR. Comparison of four polymerase chain reaction assays for specificity in the identification of Streptococcus pneumoniae. Diagnostic microbiology and infectious disease. 2004 Aug 1;49(4):249-54.
11. Vandepitte, J.; J. Verhaegen; K. Engbaek; P. Rohner; P. Piot; and C.C. Heuck. (2003). Basic Laboratory Procedures in Clinical Bacteriology.

2nd.ed. World Health Organization, Geneva.

12. Motaweq ZY, Naher HS. Antimicrobial susceptibility of Streptococcus pneumoniae isolates causing LRTI in Najaf, Iraq. Environmental & Socio-economic Studies. 2017 Jun 27;5(2):10-8.
13. Al-Taaie M.J.K. (2013). Bacterial Causes of Typical Pneumonia and Immune Response of Laboratory Animals for Some Bacterial Spp. Isolated from Pneumonic Patients in Babylon Province. M. Sc. Thesis of Microbiology. College of Medicine. Univ. of Babylon.
14. Pejčić T, Đorđević I, Stanković I, Borovac DN, Petković TR. Prognostic Mortality Factors of Community-Aquired Pneumonia in the Elderly. Acta Facultatis Medicae Naissensis. 2011 Jun 1;28(2): 71-76.
15. Naher HS, Al-Taa'e MJ. Bacterial Profile and Risk Factors Associated with Typical Pneumonia. Age. 2013 Mar;18(33):34-48.
16. Domenech A, Ardanuy C, Calatayud L, Santos S, Tubau F, Grau I, Verdaguer R, Dorca J, Pallares R, Martin R, Liñares J. Serotypes and genotypes of Streptococcus pneumoniae causing pneumonia and acute exacerbations in patients with chronic obstructive pulmonary disease. Journal of antimicrobial chemotherapy. 2011 Mar 1;66(3):487-93.
17. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. Science. 2004 Sep 10;305(5690):1622-5.
18. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, Jha P, Campbell H, Walker CF, Cibulskis R, Eisele T. Global, regional, and national causes of child mortality in 2008: a systematic analysis. The lancet. 2010 Jun 5;375(9730):1969-87.
19. Asadi A, Goudarzi M, Goudarzi H, Houri H, Ebrahimi N, Bahri TD.. Investigation and antibiotic susceptibility of *Streptococcus pneumoniae* isolated from patients with otitis. *International Journal of Analytical, Pharmaceutical and Biomedical Sciences*. 2015, 4(2): 2278-0246.
20. Abdul-Lateef LA, Alturaihy SH, Al-Taai SA.. Molecular Characterization of Some Virulence Factors of Streptococcus pneumoniae Isolated from Children with Acute Otitis Media in Hilla, Iraq. Biotechnology Journal International. 2016, 10(3), 1–11.
21. Irajian G, Ahmadi A, Talebi M. The study of Streptococcus pneumoniae in invasive and non invasive infections and multiplex PCR detection of four virulence genes. Infection, Epidemiology and Microbiology. 2013 Sep 1;1(1):3-8.
22. Kurola, Paula. Role of pneumococcal virulence genes in the etiology of respiratory tract infection and biofilm formation. PL 310, 90101 Oulu Acta Univ. Oul. D1098. 2011.
23. MotaweqP ZY, NaherP HS, Al-DahhanP HA. Phenotypic and Genotypic characterization of some virulence factors in Streptococcus pneumoniae isolated from patients with LRTI in Najaf Province/Iraq. Gene. 2015;14:353.
24. Sourav S, Patricia A, Sharma S, Kanungo R, Jayachandran S, Prashanth K. Detection of pneumolysin and autolysin genes among antibiotic resistant Streptococcus pneumoniae in invasive infections. Indian journal of medical microbiology. 2010 Jan 1;28(1):34.
25. Shak JR, Ludewick HP, Howery KE, Sakai F, Yi H, Harvey RM, Paton JC, Klugman KP, Vidal JE. Novel role for the Streptococcus pneumoniae toxin pneumolysin in the assembly of biofilms. MBio. 2013 Sep 10;4(5):e00655-13.
26. Morrison KE, Lake D, Crook J, Carlone GM, Ades E, Facklam R, Sampson JS. Confirmation of psaA in all 90 serotypes of Streptococcus pneumoniae by PCR and potential of this assay for identification and diagnosis. Journal of

clinical microbiology. 2000 Jan 1;38(1):434-7.

27. Alhajem HM, Mohammed A, Khudaier BY. Investigation of the presence of some virulence factors of the Streptococcus pneumoniae isolates among patients in Basra Governora. *Iraqi J. Sci.*, 2018. *59*(3A), 1205–1215.