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Isolation and Identification of *Streptococcus pneumoniae* Isolated From Pneumonia Patients In Thi-Qar Province/Iraq

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Abstract:

Pneumococcal pneumonia is an infection of the lung tissue, frequently, it is described as lung parenchyma/alveoli inflammation causes to alveoli fill up with fluid or pus and tissue lysis this is a result from the toxic activity of *Streptococcus pneumoniae* toxins, this can lead to trouble breathing, and in progressive cases even death. The aim of this study is Isolation and identification of *Streptococcus pneumoniae* bacterium from the pneumonia patients at Thi-Qar province by using PCR technic by using *lytA* gene. Hundred sputum samples were collected from patients suffering from pneumonia infection (age range between 15-85 years) from both sexes attending Al-Hussein teaching hospital in Thi-Qar province during the period from November (2017) to February (2018). The samples were cultured on Blood agar with gentamicin, then the positive samples were subcultured and diagnosed according to morphological characteristics, hemolysis on blood agar, optochin susceptibility, biochemical tests and using the Api 20 Strep system. Where the initial results were revealed that; only 27/100 (27%) gave positive growth for *S. pneumoniae*. The final diagnosis to confirm the isolates depended on present the *lytA* gene in all 27 isolates which are considered specific for the detection of *S. pneumoniae* by using Polymerase Chain Reaction (PCR) technique.

Keywords: *Streptococcus pneumoniae*, *lytA* gene, pneumonia, PCR.

1. Introduction

Pneumonia is an acute respiratory illness can result from a variety of causes, including bacteria, viruses, fungi, or parasites, it is generally defined as an inflammation of the pulmonary parenchyma, in which consolidation of the affected parts and a filling of the alveolar air spaces with exudates, inflammatory cells, and fibrin (Mizgerd, 2008). *S. pneumoniae is the most predominant pathogen causing community-acquired pneumonia (CAP) counting for about 20% to 60% of all community-acquired bacterial cases of pneumonia (CAPs) in adults (Cilloniz et al., 2015), studies also suggest it causes between 13% to 38% of CAP in children, pneumococcal CAP frequently progresses to invasive pneumococcal disease which is linked with high morbidity and mortality rates worldwide* (Janbeck *et al*., 2016).

Pathogen, host and environmental factors combine to allow the reproduction of pneumococci in the alveolar space, the local threat and the related threat of bacterial invasion resulting in sepsis are met with activating a response from the epithelium and alveolar macrophages resulting in massive neutrophil entry and loss of alveolar integrity (Cilloniz *et al*., 2017). In 2017, the WHO included *S. pneumoniae* as one of 12 priority pathogens, the continued high burden of disease and rising rates of resistance to penicillin and other antibiotics have renewed interest in prevention (Weiser *et al*., 2018).

In the present study, diagnosis of *S. pneumoniae* not only depend on traditional methods in identification which are usually determine from phenotypic characteristics such as colony morphology, hemolysis, carbohydrates fermentation, and analytic profile index (API) 20 Strep testing, but we also used the PCR technique to confirm the existence of the *lytA* gene in all isolates.

lytA gene often considered crucial to virulence in humans, in addition to diagnosis role of *lytA* gene the major autolysin (LytA) of pneumococci encoded by its, LytA responsible for the cleavage of peptidoglycan, is also considered a virulence factor, LytA may be important both directly and indirectly in the pathogenic process by mediating inflammation and by allowing the release of other non exported virulence factors from the cell (Mellroth *et al.*, 2014), such as pneumolysin (Ply) that encoded by *ply* gene which is considered an important virulence factor of *S. pneumoniae*, in addition to its ability to cause direct tissue damage, Ply can modulate the immune system in several ways, including by the stimulation of polymorphonuclear leukocytes and complement activation (van Pee *et al*., 2017). The polysaccharide capsule is a key determinant of *S. pneumoniae* virulence, promoting adhesion to epithelial surfaces and playing a crucial role in the escape from host defences by complement-dependent and -independent phagocytosis (Blumental *et al*., 2015).

2. Materials and Methods

2.1 Isolation of *S. pneumoniae*

A total of hundred sputum samples were collected from the patients (age range between 15-85 years) from both sexes suffering from pneumonia in Al-Hussein teaching hospital in Thi-Qar province during the period from November (2017) to February (2018). The specimens were cultured individually by transferring a loop-full or swab of samples to *Streptococcus* selective medium used for isolation of *Streptococcus spp*, this medium was prepared to be consist of gentamicin antibiotic to inhibit the growth of other bacteria particularly *Staphylococcus spp* (Facklam,1980), then the plates were incubated anaerobically by using an anaerobic jar for 24 hrs. at 37ºC. After estimation of positive samples on the surface of the selective medium, small colonies were subcultured on the surface of blood-agar plates for further purification and incubated anaerobically for 24 hrs. at 37 ºC. The following methods were used for initial characterization of the isolates:

• Morphology of the colony on blood agar with gentamicin and blood agar.
• Gram-staining and microscopic examination.
• Catalase test.
• Optochin sensitivity
• Bile solubility
• Rapid API 20-Strep.

2.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to confirm the presence or absence of the *lytA* genes in the positive isolates. One colony of each bacterium from an agar plate was extracted of DNA used as the template. Genomic DNA was extracted from *S. pneumoniae* bacterial isolates by using Genomic DNA Mini Bacteria Kit. All primers used in detection *lytA* genes were designed by using NCBI Gene-Bank and Primer one online and provided by (Bioneer Company, Korea). Forward primer (5′CGGTTGGAATGCTGAGACCT3′) and Reverse primer (5′AGTACCAGTTGCCGTCTGTG3′). PCR was performed with 5µL of template DNA in a total reaction volume of 20 µl consisting of 1.5µL of Forward Primer, 1.5µL Reverse Primer (12µL) Nuclease-free water placed in PCR Thermocycler (MyGene, Bioneer. Korea). The PCR program consisted of initial denaturation (95ºC for 5 min), 30 cycles of denaturation (95ºC for 30 sec.), annealing (58ºC for 30 sec.), and extension (72ºC for 1 min) and a final extension step at 72°C for 5 min to amplified *lytA* gene. The positive result of the *lytA* gene was confirmed by 1% agarose gel electrophoresis, then electric current was performed at 100 volt and 80 AM for 1hour. PCR products were visualized by using UV Transilluminator (Sato *et al*., 2003).

3. Results

In the present study, a total of hundred clinical samples of sputum from patients infected with pneumonia have been collected and tested during the period from November (2017) to February (2018). Only twenty-seven samples (17 males, 10 female) are given growth for *S. pneumoniae* this is about 27% of patients as in table and figure (1).

Gender	Infected by *S.pneumoniae* (%)	Other cause(%)
Male	17 (63.0%)	38 (52.1%)
Female	10 (37.0%)	35 (47.9%)
Total	27 (100.0%)	73 (100.0%)
Calculated X^2=0.948, df= 1, P≤0.05		

There is no significant difference because the tabulated X^2=3.84 at df= 1, P≤0.05

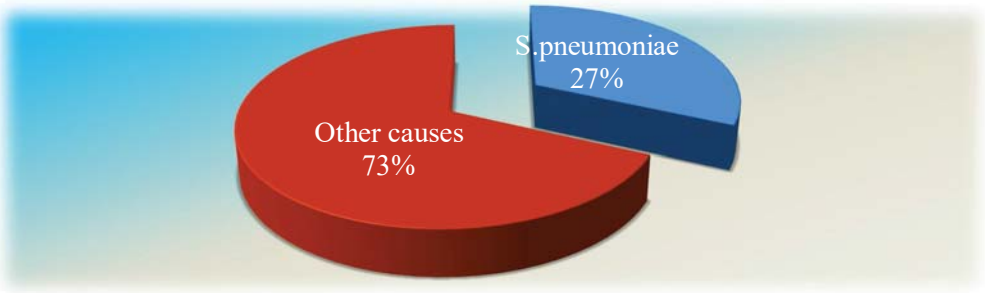

Figure (1): The percentage of *S. pneumoniae* isolated from hundred sputum samples.

3.1 Morphology of colonies

One the selective blood agar with gentamicin plates, *S. pneumoniae* colonies appeared grey in color about 0.5-2mm in diameter, Most of *S. pneumoniae* colonies had a depression at the middle of the colony (draughtsman morphology in older colonies). colonies are translucent with alpha-haemolysis and had one of the common morphology, such as flat, raised, irregular, mucoid or elongated. Some colonies appeared as irregular colonies with rough surface appearance (rough colonies), while others appeared with smooth surfaces colonies (smooth colonies) as in figure (2).

Figure (2): *S. pneumoniae* colonies growth on blood agar with gentamicin.

3.2 Catalase test

The results of this test have been showed that all *S. pneumoniae* (27 positive sample) were negative as a figure (3).

Figure (3): Catalase test of *S. pneumoniae* showing the negative result.

3.3 Microscopic Examination

This test has been showed that all *S. pneumoniae* (27 positive sample) were gram-positive, ovoid or lancet-shaped, typically in pairs, sometimes singly or in short chains as in figure (4).

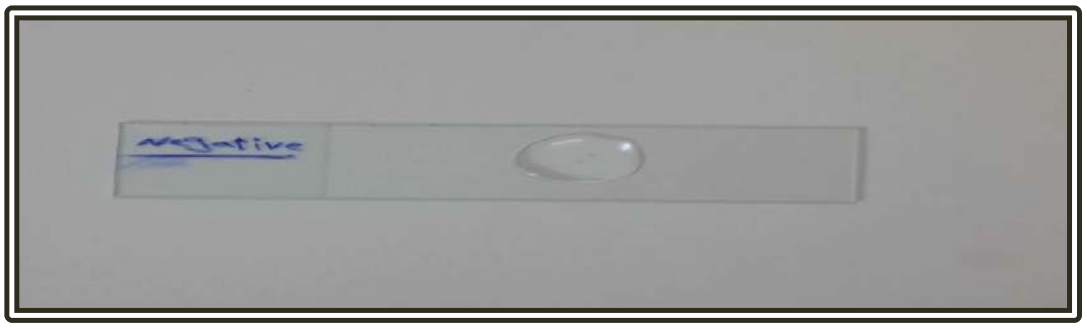

Figure (4): Gram's stain of *S. pneumoniae* cells showing the gram-positive stain (1000x magnification).

3.4 Optochin sensitivity test

The results of this test have been showed that all *S. pneumoniae* isolates were sensitive to optochin as a figure (5).

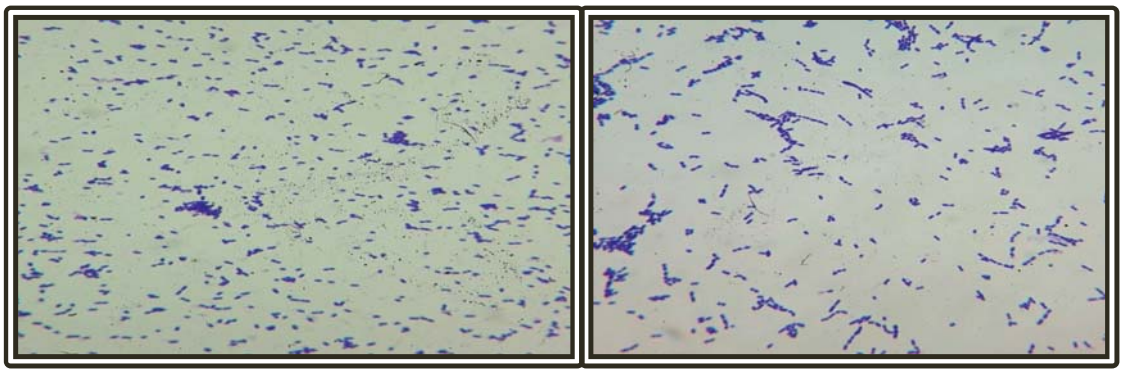

Figure (5): Optochin sensitivity test shows inhibited *S. pneumoniae* growth around the optochin disk

3.5 Bile solubility test

S. pneumoniae is bile soluble whereas all other alpha-hemolytic streptococci are bile resistant. All 27 samples were positive for this test. Sodium deoxycholate (2% in water) will lyse the pneumococcal cell wall as a figure (6).

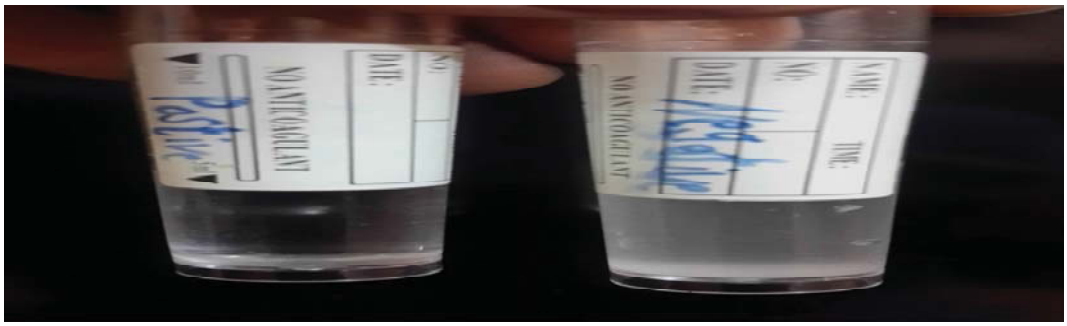

Figure (6): Results of the bile solubility test.

3.6 Api 20 Strep. System

The result of Api 20 strep. the test has revealed that only 27 isolates from 100 samples were identified as *S. pneumoniae* as in figure (7).

Figure (7): Calculate the numerical profile in Api-20 Strep. System(+): The test positive; (-): The test negative; (0051451) are the numerical profile.

3.7 Detection of *S. pneumoniae* by *lytA* gene

A total of twenty-seven isolates of *S.pneumoniae* which identified by conventional methods such as; morphology of colonies, microscopic examination, biochemical tests and API 20 strep. were subjected to DNA extraction and PCR assay for the presence of the *lytA* gene. The results demonstrated that 27 of isolates had *lytA* gene with band 461bp as in figure (8).

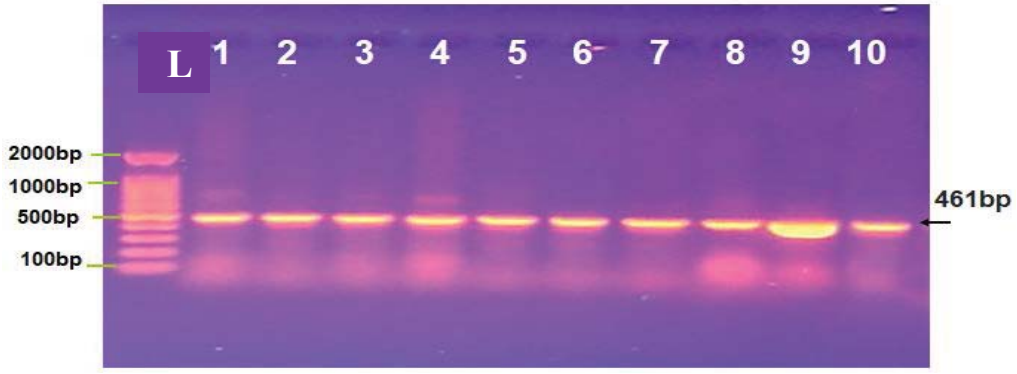

Figure (8): Agarose gel electrophoresis image that shown the PCR product of autolysin (*lytA*) gene in *S. pneumoniae* isolates.

Where L: Marker (2000-100bp), lane (1-10) some positive amplification at (461bp) PCR product.

4. Discussion

Pneumonia responsible for high morbidity and mortality worldwide, *S. pneumoniae* a gram-positive bacterium with over 90 serotypes, it is the most common as bacterial causative agents of pneumonia (Ahmed *et al.*, 2017). The present study deals with *S. pneumoniae* isolation and identification, these were done according to colony morphology, Gram stain morphology, haemolysis on blood agar, optochin susceptibility, solubility in sodium deoxycholate (bile), biochemical tests and using the Api 20 Strep system which is specific for identification of Streptococcaceae from other bacteria. In addition, the final identification was based on the presence of the *lytA* gene which is considered specific for the detection of *S. pneumoniae* (Abdeldaim *et al.*, 2010).

In this study there was (27%) of pneumonia patients ages between (15-80 years), positive growth culture for *S. pneumoniae* this consistent with Aljanaby, (2010) in AL - Najaf city, he reported the rate of *S. pneumoniae* was (24.44%). Also, these results correspond to those previously by Hassen, (2005) in Baghdad city, he finds that the ratio of *S. pneumoniae* is (29%) and a study in Uppsala, Sweden the ratio was (27%) (Abdeldaim *et al.*, 2010). This is lower than the findings reported previously in a Babylon city (73.9%) (Al-Yassari, 2014), in Basra (43.9%) (Al-Ghizawi *et al.*, 2007), in Makkah and Medina, Saudi Arabia (53.8%) (Memish *et al.*,2014) and in Cairo city, Egypt (58%) (Mansour, 2016) but corresponded with study done in Baquba city (28.7%) (Fadhil *et al.*, 2018), and higher than a study done in Baghdad and Al-Anbar (Ramadi) governorates was (21.4%) (Al-Bayatee, 2012) and Najaf city (12.3%) (Motaweq and Naher, 2017).

In this study, it was found that the contrast of the pneumonia infections was higher in male patients than females, from the 27 patients there are 17 (63%) male and 10 (37%) female this agreed with Aljanaby, (2010) in AL - Najaf city that showed the ratio in males are (63.6%) and in females are (36.3%). While this result was different from other studies by Al-Bayatee, (2012) in Baghdad and Al-Anbar (Ramadi)

governorates who found the percentage of *S. pneumoniae* in infected males was (67%) and in females was (33%).

It is clear that there are a convergence and divergence between the percentage of *S. pneumoniae* in the current study and the proportions in previous studies. This may be due to similarity and difference in the method and isolation conditions of *S. pneumoniae* in addition to mediums which are used in these studies, as well as a method of sampling can change the ratio of bacteria isolation.

S. pneumoniae colonizes the mucosal surfaces of the upper respiratory tract which includes the nose, nasal cavity, pharynx, and larynx, although colonization within the nasal passage is often asymptomatic, but under permissive conditions it is access to the airways can result in lower respiratory tract infections (pneumonia); with further dissemination causing invasive pneumococcal disease (i.e. otitis media, bacteremia and meningitis) (Thevaranjan *et al*., 2016)

The most prevalent causal pathogen identified in CAP is *S. pneumoniae* reaching more than 10%, the lethality of hospitalized patients with pneumonia is relatively high despite potent antibiotic treatment, pathogen-host interaction in severe pneumonia may evoke an increase in pulmonary endothelial permeability, resulting in life-threatening acute lung injury, virulence factors of *S. pneumoniae*, including specific toxins as well as an uncontrolled host immune response, may induce lung barrier dysfunction (Gutbier *et al*., 2017).

Depending on molecular diagnostic methods, an amplification of the *lytA* gene from 27 isolates was performed to confirm bacterial identification. The result showed that all isolates of *S. pneumoniae* had *lytA* gene with band 461 bp.

Result presented in this work is an agreement with a result of a study done in Iran 100% by Hajia *et al.*, (2013) and the study was done by Sanz *et al*., (2017) in Spain showed the sensitivity for detection of the *lytA* in *S. pneumoniae* isolates was 100%. The result in this work is high than the result reported by Gholamhosseini, (2015) in Iran was 84.74%, and the result that reported by Thors *et al.,* (2016) in the United Kingdom was (97.5%).

lytA gene in addition to its benefit in diagnosis, it is encoded to autolysin (LytA) is an enzyme that degrades the peptidoglycan backbone of bacterial organisms, leading to bacterial autolysis (Gholamhosseini *et al*., 2015). This enzyme is located in the cell envelope and is involved in a variety of other physiological cell functions, LytA is responsible for the characteristic autolytic behavior associated with pneumococcus (Mellroth *et al*., 2012). It was reported that LytA potentially contributes to pneumococcal pathogenesis by catalyzing the release of intracellular toxins, such as pneumolysin (Ply), and generating pro-inflammatory cell-wall fragments (Mitchell and Dalziel, 2014).

However, the conventional methods for identification of microorganisms are sometimes inaccurate. These kinds of problems could be solved by the use of our present PCR method, which is simpler, more rapid, species-specific and accurate for identification of cariogenic species. Therefore, our PCR method would facilitate the process of identifying isolates from clinical samples and be more useful than the conventional methods used in previous studies.

5. References:

[1]. Abdeldaim, G. ; Herrmann, B. ; Mölling, P. ; Holmberg, H. ; Blomberg, J. ; Olcén, P. and Strålin, K. (2010). Usefulness of real-time PCR for lytA, ply, and Spn9802 on plasma samples for the diagnosis of pneumococcal pneumonia. Clinical Microbiology and Infection. 16(8): 1135-1141.

[2]. Ahmed, A. R. ; Townsend, L. ; Tuite, H. and Fleming, C. (2017). A Peculiar Case of Invasive Streptococcus pneumoniae. Case reports in infectious diseases, 2017.
[3]. Al-Bayatee, N. T. A. (2012). Molecular Study on Streptococcus pneumoniae Isolated from Patients Suffering from Respiratory Tract Infection Using RAPD-PCR Technique. MSc Thesis. College of Science , AL-Nahrain University.
[4]. Al-Ghizawi, G. J. ; Al-Sulami, A. A. and Al-Taher, S. S. (2007). Profile of community-and hospital-acquired pneumonia cases admitted to Basra General Hospital, Iraq. Eastern Mediterranean health journal. 13(2): 230-242.
[5]. Aljanaby, A. A. A. J. (2010). Bacteriological and Immunological study of Streptococcus pneumoniae isolated from patients infected with respiratory tract infection in AL-Najaf Governorate (Doctoral dissertation, Kufa University).
[6]. Al-Yassari A. S. (2014). Real-Time PCR for Direct Detection of Streptococcus Pneumoniae in Patients Suffering from Upper Respiratory Tract Infection in Babylon Province. Medical Journal of Babylon. 11(3): 712-718.
[7]. Blumental, S.; Granger-Farbos, A.; Moïsi, J. C.; Soullié, B.; Leroy, P.; Njanpop-Lafourcade, B. M.; and Koeck, J. L. (2015). Virulence factors of Streptococcus pneumoniae. Comparison between African and French invasive isolates and implication for future vaccines. PloS one. 10(7): e0133885.
[8]. Cilloniz, C. ; Ewig, S. ; Gabarrus, A. ; Ferrer, M. ; Puig, C. J. ; Mensa, J. and Torres, A. (2017). Seasonality of pathogens causing community-acquired pneumonia. Respirology. 22(4): 778-785.
[9]. Cilloniz, C.; Albert, R. K.; Liapikou, A.; Gabarrus, A.; Rangel, E.; Bello, S.; and Torres, A. (2015). The effect of macrolide resistance on the presentation and outcome of patients hospitalized for Streptococcus pneumoniae pneumonia. American journal of respiratory and critical care medicine. 191(11): 1265-1272.
[10]. Facklam, R. R. (1980). Isolation and identification of streptococci. CDC USHEW.centers for disease control. Atlanta. USA.
[11]. Fadhil, R. M. (2018). Asthma aggravating factors. *European Journal of Pharmaceutical and Medical Research*. 5(5): 643-65
[12]. Gholamhosseini-Moghaddam, T. ; Rad, M. ; Mousavi, S. F. and Ghazvini, K. (2015). Detection of lytA, pspC, and rrgA genes in Streptococcus pneumoniae isolated from healthy children. Iranian journal of microbiology. 7(3): 156.
[13]. Gutbier, B.; Jiang, X.; Dietert, K.; Ehrler, C.; Lienau, J.; Van Slyke, P.; Kim, H.; Hoang, V. C.; Maynes, J. T.; Dumont, D. J.; Gruber, A. D.; Weissmann, N.; Mitchell, T. J.; Suttorp, N. and Witzenrath, M. (2017). Vasculotide reduces pulmonary hyperpermeability in experimental pneumococcal pneumonia. Critical Care. 21(1): 274
[14]. Hajia, M.; Farzanehkhah, M.; Imani, M.; Dehkharghani, A. D.; Farzami, M.; Saburian, R.; Hashemi, B. and Rahbar, M. (2013). Real-Time Assay as A Tool for Detecting lytA Gene in Streptococcus pneumoniae Isolates. Cell Journal. 16(2): 141.
[15]. Hassen, T. F. (2005). Outbreak of Streptococcus pneumoniae disease in children in Baghdad city. Journal of Thi-Qar University. 4(2): 27-35
[16]. Janbeck, F. B.; Takano, T.; Maus, R.; Stolper, J.; Jonigk, D.; Tarrés, M. T.; Fuehner, T.; Prasse, A.; Welte, T.; Mattie, S. M.; Stocker, B. L.; Nakanishi, Y.; Miyamoto, T.; Yamasaki, S. and Maus, U. A. (2016). C-type Lectin Mincle Recognizes Glucosyl-diacylglycerol of Streptococcus

pneumoniae and Plays a Protective Role in Pneumococcal Pneumonia. PLoS Pathogens. 12(12): e1006038.

[17]. Mansour, R. H. B. H. (2016). Microbiological and immunological studies on major prevalent serotypes of resistant Streptococcus pneumoniae in Egypt. M.Sc. thesis. Faculty of Pharmacy Cairo University.

[18]. Mellroth, P. ; Daniels, R. ; Eberhardt, A. ; Rönnlund, D. ; Blom, H. ; Widengren, J. and Henriques-Normark, B. (2012). LytA, the major autolysin of Streptococcus pneumoniae, requires access to the nascent peptidoglycan. Journal of Biological Chemistry. M111.

[19]. Mellroth, P. ; Sandalova, T. ; Kikhney, A. ; Vilaplana, F. ; Hesek, D. ; Lee, M. ; Mobashery, S. ; Normark, S. ; Svergun, D. ; Henriques-Normark, B. and Achour, A. (2014). Structural and functional insights into peptidoglycan access for the lytic amidase LytA of Streptococcus pneumoniae. mBio. 5(1): e01120-13.

[20]. Memish, Z. A. ; Almasri, M. ; Turkestani, A. ; Al-Shangiti, A. M. and Yezli, S. (2014). Etiology of severe community-acquired pneumonia during the 2013 Hajj—part of the MERS-CoV surveillance program. International Journal of Infectious Diseases. 25: 186-190.

[21]. Mitchell, T. J. and Dalziel, C. E. (2014). The biology of pneumolysin. In MACPF/CDC Proteins-Agents of Defence, Attack and Invasion. (pp. 145-160). Springer, Dordrecht.

[22]. Mizgerd, J. P. (2008). Acute lower respiratory tract infection. The New England Journal of Medicine. 358(7): 716-727.

[23]. Motaweq, Z. Y. and Naher, H. S. (2017). Antimicrobial susceptibility of Streptococcus pneumoniae isolates causing LRTI in Najaf, Iraq. Environmental & Socio economic Studies. 5(2): 10-18

[24]. Sanz, J. C. ; Ríos, E. ; Avial, I. R. ; Ramos, B. ; Marín M. and Cercenado, E. (2017). Identification of Streptococcus pneumoniae lytA , plyA and psaA genes in pleural fluid by multiplex real-time PCR. Enfermedades Infecciosas y Microbiología Clínica. 7(7): 1-3.

[25]. Sato, T. ; Matsuyama, J. ; Kumagai, T. ; Mayanagi, G. ; Yamaura, M. ; Washio, J. and Takahashi, N. (2003). Nested PCR for detection of mutans Streptococci in dental plaque. Letters in Applied Microbiology. 37: 66-69.

[26]. Thevaranjan, N. ; Whelan, F. J. ; Puchta, A. ; Ashu, E. ; Rossi, L. ; Surette, M. G. and Bowdish, D. M. E. (2016). Streptococcus pneumoniae colonization disrupts the microbial community within the upper 2 respiratory tract of aging mice. Infection Immunity. A-01275

[27]. Thors, V. ; Morales-Aza, B. ; Pidwill, G. ; Vipond, I. ; Muir, P. and Finn, A. (2016). Population density profiles of nasopharyngeal carriage of 5 bacterial species in pre-school children measured using quantitative PCR offer potential insights into the dynamics of transmission. Human vaccines & immunotherapeutics. 12(2): 375-382.

[28]. Van Pee, K. ; Neuhaus, A. ; D'Imprima, E. ; Mills, D. J. ; Kühlbrandt, W. and Yildiz, Ö. (2017). CryoEM structures of membrane pore and prepore complex reveal cytolytic mechanism of Pneumolysin. eLife. 6: e23644.

[29]. Weiser, J. N. ; Ferreira, D. M. and Paton, J. C. (2018). Streptococcus pneumoniae: transmission, colonization and invasion. Nature Reviews Microbiolgy. 18(7): 1-13.