ARTICLE / INVESTIGACIÓN

Phenotypic and Molecular Investigation of *Streptococcus pneumoniae* pneumolysin

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Abstract: The current study aimed to detect the prevalence of Streptococcus pneumoniae by identifying Pneumolysin and determining the gene (*ply*) using polymerase chain reaction (PCR). The study aimed to highlight the isolation and identification of *Streptococcus pneumoniae* using morphological, biochemical and Vitek, as well as investigation about pneumolysin phenotypically and molecularly through ply gene and sent the PCR products to sequencing by sanger method. Fifty sputum specimens were collected from patients at AL Salam Hospital, Iben Sina/ Mosul/ Iraq, from August 2021 to March 2022. The isolated bacteria were identified depending on morphology and biochemical properties; Vitek and the ply gene were detected by PCR technique. Five isolates of *Streptococcus pneumoniae* showed the ability to produce pneumolysin when tested by a double agar layer. When PCR reaction was performed on the *Streptococcus pneumoniae* pneumolysin gene, the results on gel electrophoresis showed three bands with 238 bp, and the ratio of the presence of the *ply* gene was 80%. PCR products were then submitted to sequencing by the Sanger method, and the *ply* sequencing result showed Point mutations that nucleotide and amino acid change with location.

Key words: *Streptococcus pneumonia*, pneumolysin, *ply* gene, PCR sequencing.

Introduction

S.pneumoniae is gram-positive immotile diplococci or short-chain and facultative anaerobic bacteria; it grows better with a 5% of CO_2 and gives alpha-hemolysis when grown on blood agar media; they are catalase and oxidase negative with colonies 1-2 mm in diameter[1]. It can be distinguished from the types of other Streptococci through its optochin susceptibility[2]. The pneumococcus colonizes the upper respiratory tract, which includes the nose, pharynx, nasal cavity and larynx as microbiota without symptoms. Still, since it is considered an opportunistic bacteria, it can cause infections of pneumococcal diseases such as pneumonia, otitis, bacteremia and meningitis[3]. Pneumonia occurs through inhalation of bacteria when direct contact between people and the migration of bacteria to the mucous membrane of the lungs that lines the bronchi, which is filled with fluids and pus such as serum exudates; fluid this liquid represents a source of nutrients for bacteria leads to reduces oxygen uptake and also to breathing pain[4]. *S.pneumoniae* can penetrate the mucous membrane of the respiratory tract to reach the bloodstream and thus spread to other parts, such as bone or cerebrospinal fluid, by penetrating the blood barrier in the brain[5]. The access of *S.pneumoniae* to the organs helps to possess several virulence factors, which is one pneumolysin it is one of the pore-forming toxin βPFT related to Class III bacteriocin[6]. At the same time, (7) and (8) referred that it has a protein nature with a molecular weight of 53.7 KDa, which plays an essential role in causing infections because of its ability to destroy membranes of lung cells and transition the bacteria from lung to bloodstream and may cause inflammation to essential organs such as heart and brain, as well pneumolysin perforates cholesterol-rich lipid cell membranes which form pores in the membrane by oligomerize undergo a structural transition that enhances the pore-forming[9].

Materials and methods

Collection of specimens

Fifty sputum specimens were collected from inpatients and outpatients at AL-salam and Ibn-sina hospitals in Mosul/Iraq from August 2021 to March 2022 using sterile containers to avoid external contamination.

Bacterial isolation and diagnosis

The specimens were cultured on the blood agar and chocolate agar, incubated at 37°C with 5% CO_2 for 24 hours; the isolates were identified morphologically depending on the shape of the colony as well as microscopic examination[10], sensitivity test of the optochin[2] and vitek identification[11].

Investigation of pneumolysin by using agar double-layer method

A double agar layer detected the ability of the isolates to produce pneumolysin depending on the method of (12).

DNA Extraction and PCR amplification

Total DNA was extracted for five bacterial isolates by using Geneaid.

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Genomic DNA Purification

A Nanodrop device measured the concentration of DNA, and the ply gene was amplified by using a specific primer F. (GGCGCCAAGTCTATCTCAAG) and R (GCTGTAAAGCGACTGCCTTC)

Designed according to (13), the PCR premix master mix components (Geneaid, Accupower®Profi Taq PCR Premix) placed in standard (Accupower®Profi Taq PCR Premix) that containing all other components which needed for PCR reaction Top DNA polymerase, dNTP, Tris-HCl, pH 9, KCl MgCl2 stabilizer and tacking dye as shown in Table 1.

Ingredients	Volum µ1
DNA template	6
Forward primer	2
Reverse primer	2
PCR water	10
PCR premix	Lypholyse
Total volume	20

Table 1. PCR premix master mix Volume.

PCR tubes were prepared according to manufacture instructions, then transferred into spin and vortex centrifuge at 300 rpm for 3 minutes at 3000 rpm, then placed in PCRThremocycler PCR complication condition shown in Table 2.

Gene	Initial denaturation	No.Of Cycle	Denaturation	Annealing	Extension	Fal Extention
PLY	95°C for 4 min	30 cycle	95°C for 35 sec	58°C 1min	72°C for 1min	72°C for 7 min

Table 2. PCR condition.

PCR products were resolved on 1% agarose gel, as shown by UV illumination and photographed[14].

Sequence analysis

Ply PCR products were sent to Macrogene Korea for sanger sequencing.

Comprehensive phylogenetic tree

The evolutionary history was inferred using the UPGMA method[15]. The optimal tree with the sum of branch length = 6.56300659 was shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[16] and are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. The ply sequencing was compared with similar sequences using NCBI-BLAST (https://www.ncbi.nlm.nih.gov/). There was a total of 200 positions in the final dataset. Evolutionary analyses were conducted in MEGA6, and nucleotide and amino acids were analyzed in Genes program[17].

Results

Isolation and Identification of Streptococcus pneumoniae

Microscopic examination of isolates showed gram-positive diplococci lancet-shaped cells Figure 1. In contrast, on blood agar, the isolates exhibited α-hemolysis Figure 2 as well it was susceptible to optochin when submitted to the optochin susceptibility test Figure 3.

Result of investigation on pneumolysin

The results of the investigation of pneumolysin by

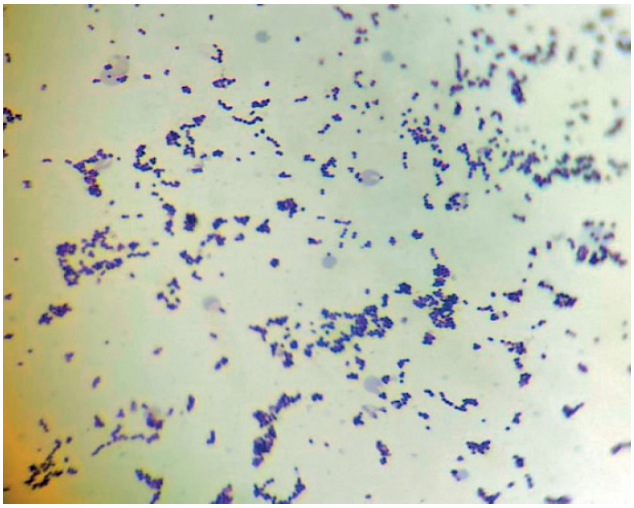

Figure 1. *S.pneumoniae* under the light microscope (100X)

the agar double layer method showed that *S.pneumoniae* has the ability to produce pneumolysin, as no growth was appeared on the surface of the culture medium after inoculation with bacterial types which were sensitive to pneumolysin including gram positive bacteria *S.aureus* figure 4 and gram negative bacteria *E.coli* figure 5.

Investigation of *ply* gene by using PCR technique

DNA primers amplify a specific ply gene by polymerase chain reaction (PCR). DNA was extracted, and using a Nanodrop device, the concentration of DNA was measured, which ranged between (51-112 ng/µ1), and its purity was determined with (1.8-2). The ply gene was amplified to the size of 238 base pairs compared to the DNA ladder 100bp, and the result matched with the standard isolate Streptococcus pneumoniae ATCC 49619. The results showed that 3 isolates from 5 (60%) contained ply gene, as in Figure 5.

DNA ladder starts from 100 base pairs, the path N represents the negative control, P represents positive control stander isolate *Streptococcus pneumoniae ATCC* 49619, columns representing the Gene *ply* of the *Streptococcus pneumoniae* isolates and their size 238 base pairs.

. *ply* sequencing

Ply sequences were obtained by Sanger sequencing

Genetic variation of *ply* gene

Allele of *ply* (n=7) was identified compared to 18 known *ply* alleles. Three amino acid changes gave three mutations, Table 3. The Allele subtype found that nucleotide sequences varied more than amino acid sequences.

The identity was high in alleles based on the nucleotide and amino acid sequences using pairwise comparisons of

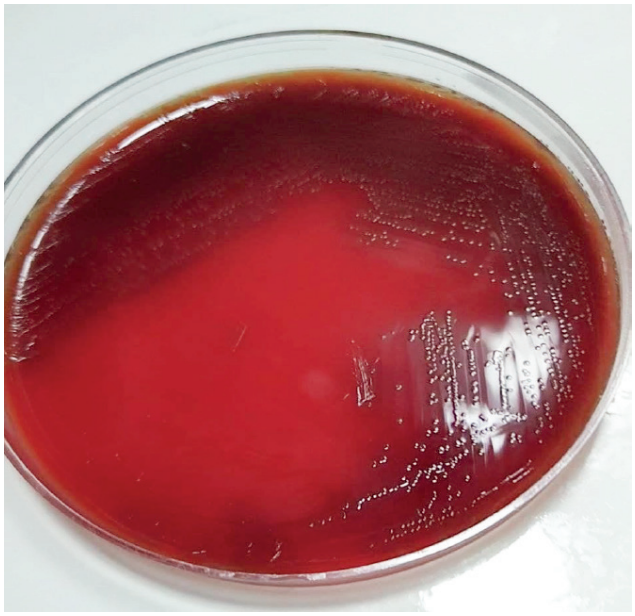

Figure 2. *S.pneumoniae* on blood agar.

Figure 3. Optochin sensitivity test of *S.pneumoniae*.

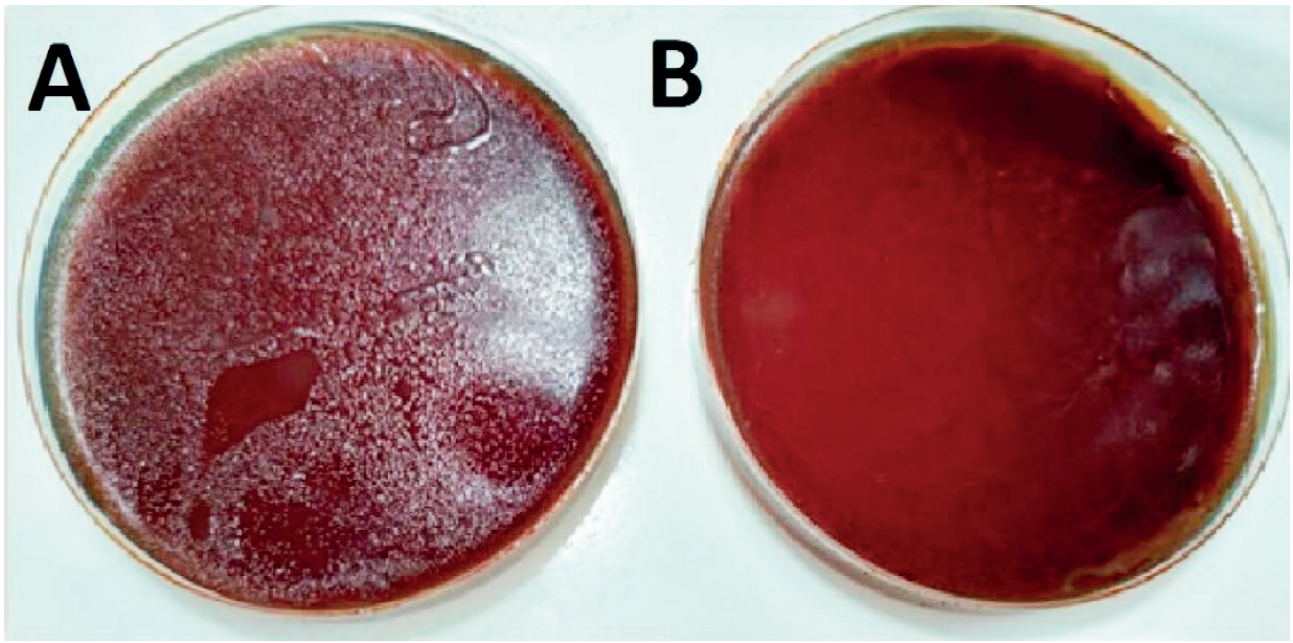

Figure 4. *E.coli* susceptibility to pneumolysin. A: control plate. B: test plate.

our isolate and EF413936.1; the identities were 99%. Our isolate had genetic variation at the *ply loci* allele (n=7). This locus may be replaced with foreign DNA through gene transfer mechanisms like transformation, conjugation, transduction and lysogenic conversion. This mutation changes the amino acid arginine to valine, a missense mutation.

Transvertion

The local isolate showed a 99% match with the 17 globally recorded isolates while showing a 94% match with standard isolate *S.pneumoniae* 49619 ATCC fig (6).

Source: Streptococcus pneumoniae							
Type of substitution	**Location**	**Nucleotide**	**Nucleotide change**	**Amino acid change**	**Predicted effect**	**Sequence ID with compare**	**Identities**
Transition	954	T\C	AGT\AGC	Serine\ Serine	Silent	ID: EF413936.1 gene="ply"	99%
Transvertion	955	C\G	CGC\GTC	Arginine\ Valine	Missense		
Transvertion	956	G\T	CGC\GTC	Arginine\ Valine	Missense		

Table 3. Nucleotide and amino acid change with location.

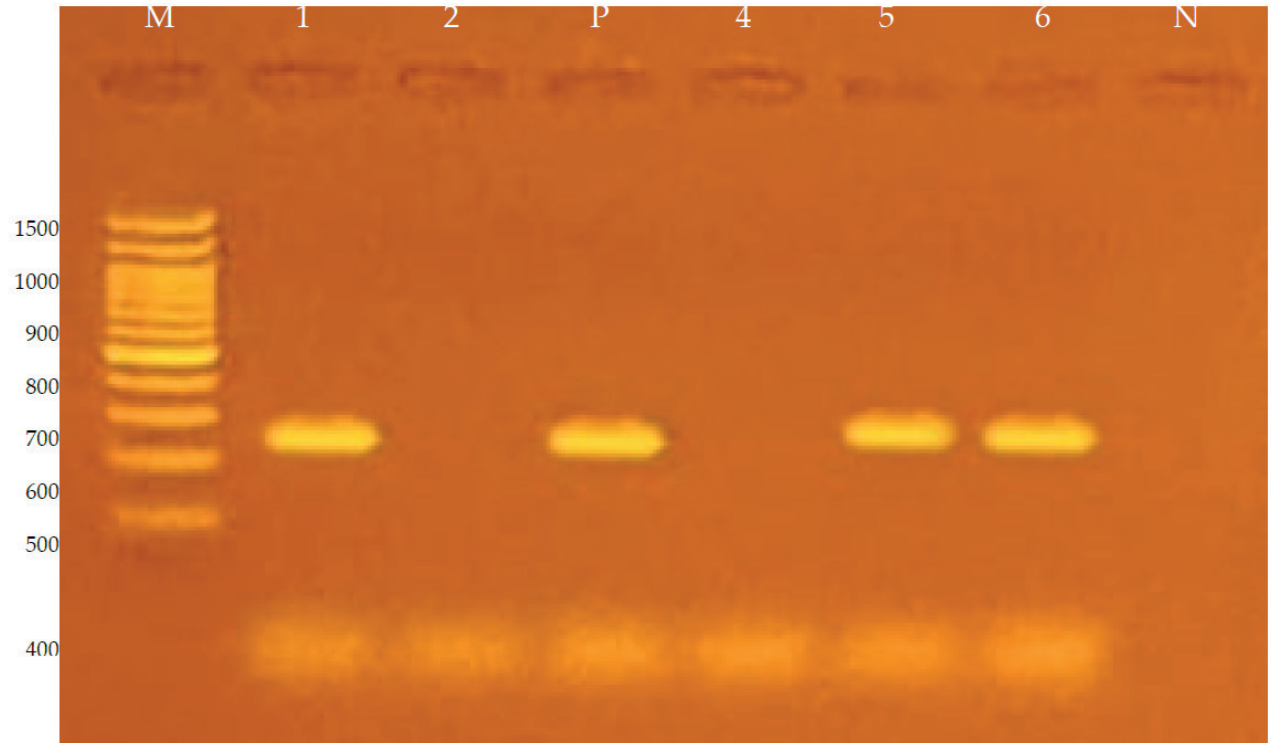

Figure 5. Electrophoresis for amplification products of *ply* in a 1% agarose gel with 65 volts and for an hour and a half using M path.

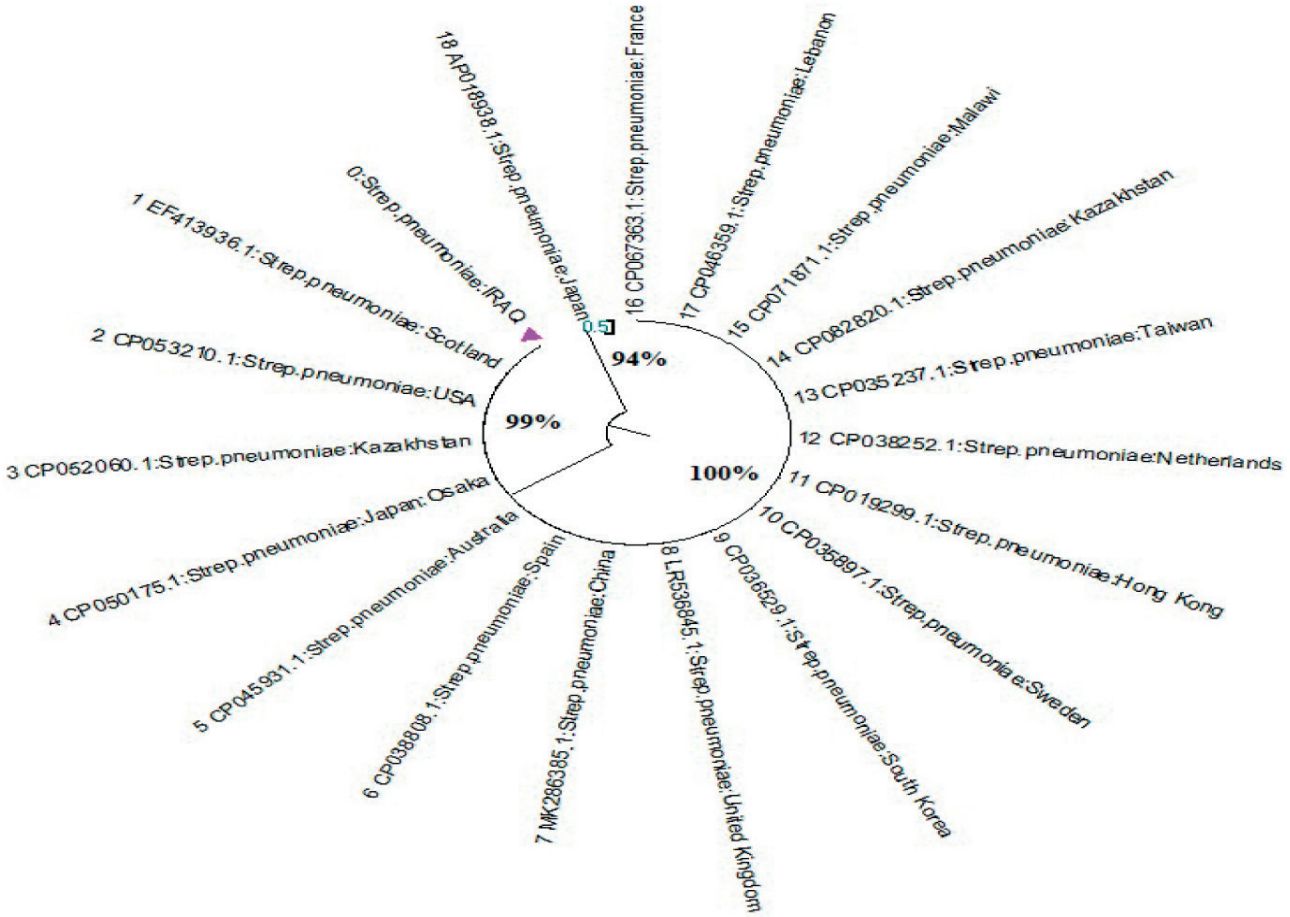

Figure 6. Phylogenetic tree of the *ply* variants of *S.pneumoniae* isolates.

Discussion

Five *Streptococcus pneumoniae* isolates were obtained from 50 sputum specimens with a 10% percentage. This percentage was lesser than the percentage obtained by Tamimi Zainab[18] where the ratio of isolation of this bacterium was 11%, but higher than the isolation rate of the study of (19), who found the isolation rate was 4%. The inhibitory activity of pneumolysin produced by *S.pneumoniae* on both gram-positive *S.aureus* and gram-negative *E.coli* were agreed with (20) who used the same bacterial types that were sensitive to pneumolysin gram-positive *S.aureus* and gram-negative *E.coli*. Nucleotides 954,955,956 were identified with position variation, (T954C),(C955G),(G956T) substitution replacement mutation in (T954C) without effect on encoding amino acid there for named silent) because the two codons (AGT) and (AGC) encode to Serine amino acid and no phenotypic will be changed as a result of this replacement, but the two-locus,(C955G),(G956T) were shown to affect encoding amino acid Arginine, nucleotide changes lead to amino acid change; as a result to change with the start codon and instead of encoding to produce Arginine will coding to Valine[21].

Conclusions

Due to the role of pneumolysin, which represents a key for infection with *S.pneumoniae* so this study aimed to highlight on isolation and identification of Streptococcus pneumoniae using morphological, biochemical and Vitek, as well as investigation of pneumolysin phenotypically and molecularly through ply gene and sent the PCR products to sequencing by sanger method.

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