Phenotypic and Genotypic Characterization of Some Virulence Factors in Streptococcus pneumoniae Isolated from Patients with LRTI in Najaf Province/Iraq

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Abstract- This study provides genotypic data on 74 isolates of *Streptococcus pneumoniae* recovered from lower respiratory tract infections. In this study, some of them were detected by traditional phenotypic methods while others detected by phenotypic and then genotipically by using monoplex-PCR technique. The results showed that all (100%) isolates were encapsulated isolates. The molecular detection of *cspA* gene by using specific primer for *S. pneumoniae* revealed positive amplification for all isolates (100%). The results showed that overall, 89.2% of investigated isolates contained the *lytA* gene. In spite of, all (100%) of pneumococcus isolates have autolysin phenotypically. The results of this experiment indicated for positive amplification and it has been found that *ply* gene is found in 97.3% of pneumococcal isolates. In this study, the *nanA* and *nanB* genes were found in all *S. pneumoniae* isolates (100%) and (62.2%) respectively.

Index Terms— LRTI, *Streptococcus pneumoniae*, Pneumococci Virulence Factors, *lytA*, *ply*, *nanA*, *nanB*

1 INTRODUCTION

Streptococcus pneumoniae, commonly called the pneumococcus, is responsible for high rates of morbidity and mortality worldwide [1]. It is normally colonizes the human nasopharynx, nose, and throat asymptomatically and such carriage is considered essential for subsequent development of disease in susceptible individuals [2]. This bacterium is globally significant pathogen and causes a wide range of diseases such as pneumonia, meningitis, otitis media, bacteraemia, and other less-frequent infections, e.i. endocarditis and arthritis [3].

One of the prime virulence determinants of this bacterium is the polysaccharide capsule (CPS). This structure is thought to protect the bacterium against harmful environmental conditions and exhibits antiphagocytic properties [4]. The CPS is composed of saccharide repeating units that are polymerized into a polysaccharide chain. Thus far, 90 different capsule serotypes have been identified. The diversity is based on variation in the carbohydrate structure of the oligosaccharide units or the attached side groups [5]. Due to the importance and the role of virulence factors in pathogenicity of *S. pneumoniae,* the present study was designed to detect phenotypicaly and genotypicaly of some virulence factors represented by capsule capsA, autolysin lytA,

pneumolysin ply, neuroaminidase A, B, nanA, nanB.2 Procedure for Paper Submission

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2 MATERIAL and METHODS

Patients and Clinical Specimens: A total of 600 sputum samples were collected from out- and inpatients who suffering from lower respiratory tract infection (LRTI) (pneumonia, COPD) attending to the Chest Unit in Al-Sadder Medical City, Al-Hakeem General Hospital and Clinic Consultive Center for Chest Disease and Al-Zahra'a Hospital for Childbirth and Children in Al-Najaf province during the period from February 2013-Aprile 2014. The patients included both sexes (male and female) and the age range (1-80 years).

Bacterial Isolates and Culture Conditions: Quantitative sputum cultures were made for each specimen according to sputum gram stain for pneumonia infections. Sputum specimens were homogenized with an equal volume of normal saline on a vortex mixer. Blood agar and Chocolate agar were inoculated with 0.1 ml of homogenized specimen and spread on the plates with sterile loope. Plates were incubated in (5-10) % CO_2 candle jar at 37 C° for overnight [6]. The plates were examined thereafter for bacterial growth and positive plates

(α-haemolysis) were then a single pure isolated colony was transferred to trypticase soy agar for the preservation and to submitted the morphological evaluation by Gram staining and carry out other biochemical tests that confirmed the identification of isolates *S. pneumoniae*.

Identification of Bacteria: The identification of *S. pneumoniae* was achieved according to cellular morphology, culture characters and biochemical reactions that discribed in Macfaddin, [7]:-

Optochin Test: A half plate of 5% sheep blood agar was streaked with an inoculum from a pure isolates of the organism to be tested, then an optochin disc was placed in the center of the inoculum and incubated for 24-48 hrs. at 37 C° in a candle jar, then observation of zones of growth inhibition greater than 14 mm surrounding the disc was considered as positive. This test was used to differentiate *S. pneumoniae* (sensitive) from viridans streptococci (resistance) [8].

Bile Solubility Test: There are two methods for this test, tube method and plate method [7,9].

Inulin Fermentation Method: The isolates were inoculated into tubes of phenol red inulin broth and incubated at 37C° for 24hours. The tubes then examined for the presence of a yellow color indicative of acid formation from the fermentation of inulin and red indicates no inulin fermentation tube [7].

Identification of Bacteria by STREPTO-SYSTEM 9R Kit: STREPTO-SYSTEM 9R for *S. pneumoniae* identification was used according to the recomendation of company product (Liofilchem, England).

Vitek–2 for Identification: GP identification card was used for identification of pneumococci [10].

Phenotypic Detection of Virulence Factor

Detection of Hemolysin Production: α-hemolysin was evaluated on blood agar base supplemented with 5% sheep blood. The plates were incubated for 24 h at 37 ºC under 5% CO_2, when positive samples showed a visible greenish zone of partially hemolysis with blurred edges [11].

Detection of Capsule Production: A loopful of suspected culture was mixed with a loopful of nigrosin stain on a clean and dry slide and allowed to air dry slowly at room temperature. The slide was gently rinsed with water and then stained with methylene blue stain for 2min and allowed to air dry slowly at room temperature. The slide was gently washed with water and examined under oil immersion objective. The nigrosin stain provides a dark background to unstained capsule and methylene blue stain provides blue color to the cells [12].

Extraction and Isolation of DNA: Genomic DNA Extraction Kit (Geneaid) was used for DNA extraction. Concentration of DNA was determined spectrophotometrically by measuring its optical density at 260 nm (Extinction coefficient of dsDNA is 50 µg/ml at 260 nm) the purity of DNA solution is indicated by ratio of OD 260-280 which is in the range of 1.8±0.2 for pure DNA. PCR program that apply in the thermocycler. The PCR products and the ladder marker are resolved by electrophoresis on 1.2% agarose gel [13].

Polymerase Chain Reaction (PCR) Technique

a.Selection of PCR Primers: In this study, monoplex PCR was employed to detect a number of genes that encode virulence factors in *S. pneumoniae* isolates. Monoplex PCR was used to detect cpsA-382, *lytA, ply, nanA* and *nanB* (table 1).

b.PCR Cycling Conditions: PCR mixture was set up in a total volume of 30 µl included 15µL of PCR premix (contains:*Taq* DNA polymerase, $MgCl_2$, dNTPs, KCl, stabilizer tracking dye and tris-HCl), 2µl of each primer and 5µl of extracted DNA have been used. The rest volume was completed 6µl of sterile deionized distilled water, then vortexed. Negative control contained all material except template DNA, so instead that distilled water was added. PCR reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into thermocycler PCR instrument where DNA was amplified as indicating in table 2.

Table 1.
The primers sequences used in PCR for detection of virulence factors

Target Gene	DNA sequence(5'-3')	Product Size bp	References
cpsA-382	F:ACG CAA CTG ACG AGT GTG AC R:GAT CGC GAC ACA CCG AAC TAA T	353	[14]
lytA	F:CAA CCG TAC GAA TGA AGC CGG R:TTA TTC GTG CAA TAC TCG TGC G	308	[15]
ply	F:ATT TCT GTAA CAG CTA CCA ACG A R:GAA TTC CCT GTC TTT TCA AAG TC	329	[16]
nanA	F:ATA GAC GTG CGC AAA ATA CAG AAT CA R:GTC GAA CTC CAA GCC AAT AAC TCC T	550	[17]
nanB	F:ACT ACG AGG TGT TAA TCG TGA AGG R:CCA ATA CCC GCA GGC ATA ACA TC	500	[17]

Table 2.
Program used to amplify the genes by PCR

Gene	Stage	Temperature (time)	
cpsA	Initial denaturation	94C° for 5min	
	Denaturation	92C° for 30sec	30cycle
	Annealing	58C° for 30sec	
	Extension	72C° for 40sec	
	Final extension	or 10min	
lytA	Initial denaturation	94C° for 2min	
	Denaturation	94C° for 30sec	30cycle
	Annealing	53C° for 30sec	
	Extension	72C° for 40sec	
	Final extension	72C° for 5min	
ply	Initial denaturation	94C° for 5min	
	Denaturation	94C° for 30sec	30cycle
	Annealing	58C° for 30sec	
	Extension	72C° for 1min	
	Final extension	72C° for 10min	
nanA* and *nanB	Initial denaturation	94C° for 3min	
	Denaturation	94C° for 1min	35cycle
	Annealing	52C° for 1min	
	Extension	72C° for 1.5min	
	Final extension	or 10min	

RESULTS AND DISCUSSION

Virulence Factors of *S. pneumoniae*

It is well known that the pathogenicity of *S. pneumoniae* is associated with many virulence factors. In this study, some of them were detected by traditional phenotypic methods while others detected by phenotypic and then genotipically by using monoplex PCR techniques.

Detection of α-Hemolysine

All *S. pneumoniae* isolates (100%) in this study presented α-hemolysis appearance on sheep blood agar. The α-hemolysin produced by *S. pneumoniae* are considered as one of the important virulence factors that associated with the pathogenesis of these bacteria, α-hemolysin assembles into trans membrane pores in many nucleated cells, and readily lyses sheep or rabbit erythrocytes, while horse or human red cells are less susceptible [18]. α-hemolysin can interact with surface receptors of the host cells, form small heptameric pores, selectively release ions, and/or trigger cell signal transduction pathways, thus inducing apoptosis and/or death in various cell types [19]. *S. pneumoniae* partially digests (lyses) red blood cells, producing around its colonies a greenish-brown discoloration denoted alpha-hemolysis [20]. Since, pneumococci cause a chemical change in the hemoglobin of red cells in blood, resulting in the appearance of a green pigment that forms a ring around the colony. *S. pneumoniae* releases an α-hemolysin that damages red cell membranes, causing colonies to be α-hemolytic [21].

Detection of capsule

Nigrosin stain was used to detection of encapsulated isolates of *S. pneumoniae*. The results showed that all (100%) isolates were encapsulated isolates. The molecular detection of *cspA* gene by using specific primer for *S. pneumoniae* isolates revealed positive amplification for all isolates (100%) as shown in figure (1).

The current *cpsA*-specific primer set differentiated *S. pneumoniae* from *S. pseudopneumoniae*. Thus, this primer set may be useful for discrimination of both species, as it is well known that *S. pseudopneumoniae* strains have no pneumococcal capsules, unlike *S. pneumoniae* [22]. Park *et al.*, [14] reported that the *cpsA*-specific primer sets (*cpsA*-382F and *cpsA*-735R) was highly specific to *S. pneumoniae* 100% with the expected PCR product of 353 bp, and did not amplify any other reference strain (viridence streptococci) and no other reference strain including two *S. pseudopneumoniae* strains was amplified. Therefore, the *cpsA* gene could be used as a new marker for detection of *S. pneumoniae* using a real-time detection assay. Pneumococci individually express one of approximately 90 extracellular and structurally distinct capsular polysaccharides. The chemical composition of the *S. pneumoniae* capsule is generally considered the most important virulence factor [23].

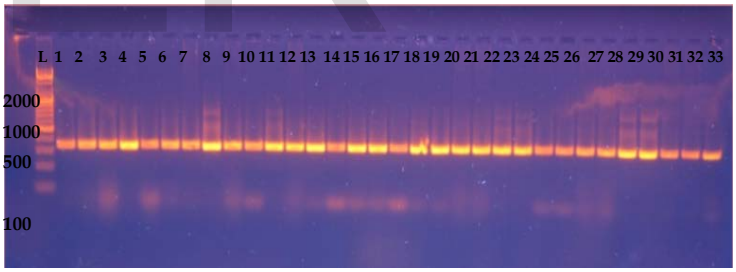

Figure 1. Gel electrophoresis of PCR product of *cpsA* gene primers with product 353 bp. Lane (L), DNA molecular size marker (2000-bp ladder), Lanes (1-32) show positive results with *cpsA* gene.

The genes encoding the enzymes involved in CPS biosynthesis are clustered on the bacterial genome in the capsular polysaccharide synthesis (*cps*) locus. The *cps* locus is typically flanked by the genes *dexB* and *aliA*. At this time, the *cps* loci of 16 different serotypes have been sequenced [24,25]. Nearly all loci have the same genetic organization [26]. The first four genes of the loci are conserved in almost all serotypes, and it has been demonstrated that three of these genes encode enzymes involved in the regulation of capsule production [27,28].

Data from the [2] study indicate that cbpA mRNA is present at elevated levels in the nasopharynx and lungs compared to

the bloodstream. This finding is also consistent with a recent study [29] that demonstrated that CbpA is not required for the entry of pneumococci into the bloodstream from the lungs, nor for survival in the blood. On another note, CbpA has also been shown to interact with factor H and C3 [30] suggesting a dual role for CbpA in colonization (adherence) and in systemic disease. The opaque phenotype is associated with larger amounts of capsular polysaccharide than is the transparent phenotype [31]. In contrast, the transparent phenotype produces increased amounts of teichoic acid, which contains phosphorylcholine [32].

Specific PCR primer was used for the detection of autolysin gene (*lytA*) as shown in figure (2). The results showed that overall, 89.2% of investigate isolates contained the *lytA* gene. In spite of 100% of pneumococcus isolates have autolysin phenotypically.

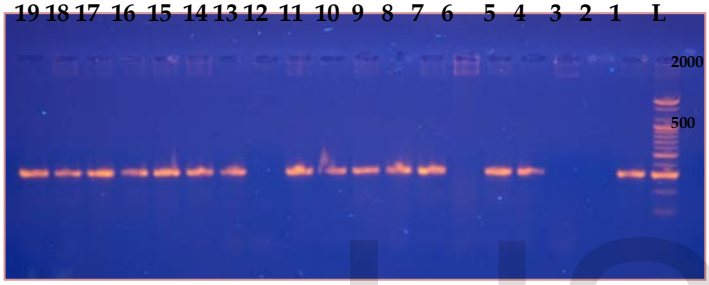

Figure 2. Gel electrophoresis of PCR amplified product of *lytA* gene primers with product 308 bp of *S. pneumoniae* isolates. Lane (L), DNA molecular size marker (2000-bp ladder) ; Lanes (3,6,12) show negative results with *lytA* gene ; Lane (1,2,4,5,7-11,13-19) the isolates were positive results with *lytA* gene.

The *lytA* gene sequence has been suggested as more specific for the diagnosis of pneumococcal disease [33,34].

There is no true gold standard for *S. pneumoniae* identification, but the bile solubility test has been shown to have a high level of accuracy and is frequently used for the identification of *S. pneumoniae* [35]. However, the existence of organisms that appear to be genotypically and phenotypically related to *S. mitis*, but which harbour the *lytA* gene normally associated with pneumococci, has been reported [36]. Moreno *et al*., [37] used PCR to screen the autolysin gene *lytA* and a multiplex PCR, to amplify specific regions of the capsular genes of serogroups 6 and 18 and serotypes 1, 3, 4, 14, 19A, 19F, and 23F.

The analysis of sputum from patients with community acquired pneumonia suggests that the *lytA* primers are the all-powerful primers for detecting *S. pneumoniae*. The *lytA* gene has been frequently used as a reliable target for the identification of *S. pneumoniae* [38], and it has been reported that the *lytA* gene has higher specificity than the gene for *S. pneumoniae* [39].

While some investigators have concluded that amplification of a region of the *lytA* gene can be successfully used to identify *S. pneumoniae* [40,41], others have demonstrated the presence of *lytA* in members of the Smit group [42]. Although it is expected that all virulent isolates of *S. pneumoniae* possess the *lytA* gene, it cannot be presumed that isolates of all other members of the Smit group do not possess the *lytA* gene or may have a facsimile of a *lytA* gene. Therefore, Arbique *et al*., [43] reported that the detection of *lytA* should be used cautiously to identify *S. pneumoniae*.

The *lytA* gene is usually considered specific for *S. pneumoniae*, but despite the use of the same target gene, the real-time *lytA* PCR described previously by Pillai [44] is not specific for *S. pneumoniae*, whereas the *lytA* PCR described previously by Carvalho *et al* [33]. This discrepancy can be explained by the finding that all *S. pneumoniae* isolates harbored typical *lytA* alleles, whereas *lytA* alleles detected in isolates that belong to other members of the mitis group were always atypical [44].

Detection of pneumolysine

The molecular detection of *ply* gene by using specific PCR primer for *S. pneumoniae* isolates (Figure 3). The results of this experiment indicated for positive amplification and it has been found that *ply* gene is found in 97.3% of pneumococcal isolates.

Figure (3): Gel electrophoresis of PCR amplified product of *ply* gene primers with product 329 bp of *S. pneumoniae* isolates. Lane (L), DNA molecular size marker (2000-bp ladder), All isolates (1-26) were positive results for *ply* gene, except isolate numbered (23) were negative results with *ply* gene.

Pneumolysin is an important virulence factor, contributing to multiple stages of the pathogenic process, and it is also involved in eliciting an immune response from the host. The cytotoxic characteristic of pneumolysin facilitates progression of disease by inhibiting ciliary beating in the human respiratory epithelium, thus helping the migration of bacteria to the lungs and It also acts by disrupting tight junctions between epithelial cells and provide an alternative pathway by which the pneumococcus infiltrates the bloodstream [45]. Additionally, numerous studies have indicated that pneumolysin is important in the development of sepsis, with mutants lacking pneumolysin showing reduced replication and survival in the bloodstream of infected animals [46,47].

PCR amplification from clinical material is indicative of invasive pneumococcal infection. There have been several reports of pneumococcal PCR utilizing amplification of the pneumolysin gene, with a report of PCR for the detection of *S. pneumoniae* DNA in culture-negative samples where meningitis was the diagnosis [38]. Detection of the *ply* gene and/or the expression of the Ply protein has also been used in direct detection assays with clinical samples and to differentiate *S. pneumoniae* from other viridans group streptococci, with various rates of success [48].

Although DNA amplification methods targeting the *ply* gene initially showed promise as a reliable, efficient method for confirmation of pneumococcal isolates. Whatmore *et al.,* [42] have reported on the presence of the *ply* gene in strains of *S. mitis* and *S. oralis*, contraindicating its use in differentiating *S. pneumoniae* from other strains of the Smit group. Similarly, although latex agglutination methods targeting the protein coded for by the *ply* gene showed promise [49], their use, too, is contraindicated by the finding of the *ply* gene in members of the Smit group other than *S. pneumoniae* [43].

Detection of Neuroaminidase genes

In this study, the *nanA* gene was found in all *S. pneumoniae* isolates (100%) as shown in Figure (4). While the results amplification of *nanB* gene primer by PCR revealed that most (62.2%) of *S. pneumoniae* isolates have *nanB* gene, except (37.8%) of isolates as indicated in figure (5).

Two neuraminidases, encoded by *nanA* and *nanB* genes, have been described for *S. pneumoniae*. *NanA* is proposed to aid pathogenesis by revealing carbohydrate receptors for adherence, providing a carbon source for the bacteria and facilitating bacterial adherence by removing terminal sialic acid residues from glycoconjugates. Additionally, *NanA* also has been shown to desialylate lipopolysaccharides of *N. meningitidis* and *H. influenzae* strains [50]. The desialylation of lipopolysaccharide may give pneumococci a competitive advantage over *N. meningitidis* and *H. influenzae*, which reside in the same host niche, by making them more susceptible to complement-mediated clearance. [51]. *NanA* may help promote colonization through desialylation of host proteins that mediate bacterial clearance, such as lactoferrin or immunoglobulin A2 [51].

LeMessurier *et al.* [2] revialed that the expression of *nanA* was significantly elevated in the nasopharynx of infected mice compared to the other niches examined. And these results provide further support for an important role for *NanA* in colonization of the nasopharynx by pneumococci. Influenza virus encodes the neuraminidase *NA*, which is similar to *S. pneumoniae NanA* in substrate specificity [52] *NanA* cleaves N-acetylneuraminic acid (sialic acid) residues on red blood cells, platelets and endothelial cells leading to the exposure of the Thomsen–Friedenreich antigen (TA) and allowing normally circulating anti-T antigen antibodies to react with the exposed TA on cells [53].

The results of the current study were compatible with Pettigrew *et al*., [17] results who found that *nanA* is present in all strains of *S. pneumoniae* isolates, while *nanB* and *nanC* are present in 96% and 51% of isolates, respectively. The distribution of *nanC* varied among the strain collections from different tissue sources and suggested that the presence of *nanC* may be important for tissue-specific virulence. Studies that both incorporate MLST and take into account additional virulence determinants will provide a greater understanding of the pneumococcal virulence potential.

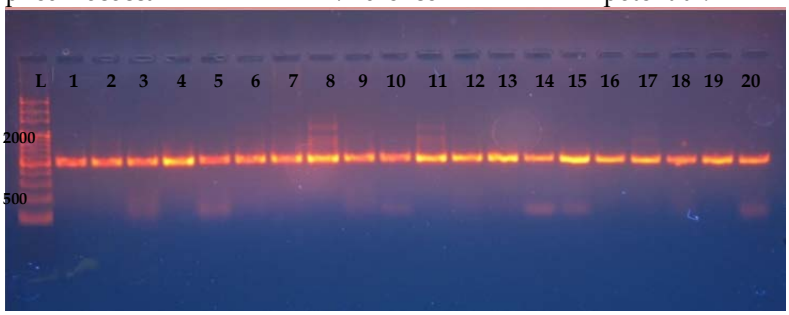

Figure (4): Gel electrophoresis of PCR product of *nanA* gene primers with product 550 bp. Lane (L), DNA molecular size marker (2000-bp ladder), Lanes (1-20) show positive results with *nanA* gene.

Figure (5): Gel electrophoresis of PCR product of *nanB* gene primers with product 500 bp. Lane (L), DNA molecular size marker (2000-bp ladder), Lanes (11, 12, 17) show negative results with *nanB* gene.

Previously less is known about the 78 kDa NanB protein, which has low sequence identity (24%) with NanA. Recent investigations suggest that NanB plays an important role during pneumococcal infection of the respiratory tract and sepsis as well as playing a role in bacterial nutrition. Gut *et al.*, [54] reported that the first structure of a neuraminidase from *S. pneumoniae*, the crystal structure of NanB in complex with its reaction product 2, 7-anhydro-Neu5Ac., and showed that NanB differs in its substrate specificity from the other pneumococcal neuraminidase NanA. Gut *et al*., [54] also, confirmed this finding and establish that free Neu5Ac (the reaction product of pneumococcal NanA) can act as a substrate for NanB. In addition, our biochemical assays clearly demonstrate the strict specificity of NanB towards a2-3 glycosidic substrate linkages

and highlight the differences in substrate specificity between NanA and NanB.

Interestingly, hybridization analysis indicated that these two neuraminidase genes are different and that individual pneumococcal isolates contain both genes [55]. Berry *et al*., [56] demonstrated that *nanB*, a gene encoding a second *S. pneumoniae* neuraminidase, is located on the pneumococcal chromosome approximately 4.5 kb downstream of *nanA*. *nanB* appears to be part of a large operon consisting of at least six ORFs. Janapatla *et al*., [57] isolated and characterized a second neuraminidase gene (designated *nanB*), which is located close to *nanA* on the pneumococcal chromosome (approximately 4.5kb downstream). *nanB* was located on an operon separate from that of *nanA*, which includes at least five other open reading frames. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis suggested that *NanB* has a molecular size of approximately 65 kDa.

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