



Antibiotic Susceptibility and Prevalence of Adhesion Genes in *Streptococcus pneumoniae* Isolates Detected in Carrier Children in Tehran

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Abstract

Background: Pharyngeal carriers are the source and transitional vectors of invasive diseases. Attachment is the first step in pathogenicity. Strains of *Streptococcus* as normal flora can cause diseases in certain circumstances. Adhesion proteins of these bacteria play a fundamental role in the attachment and colonization.

Objectives: In the present study, 5 genes encoding surface proteins namely *phtD*, *pspC*, *phtE*, *lytA*, and *rrgA* were evaluated in *Streptococcus pneumoniae* isolates collected from 4 main care centers and the children's Medical center in Tehran.

Methods: Three hundred and eight nasopharyngeal swab specimens were collected from children under 6 years. The identification of *S. pneumoniae* isolates was performed using biochemical tests confirmed by PCR for the presence of *cpsA* gene. The existence of *phtD*, *phtE*, *pspC*, *lytA*, and *rrgA* genes was studied by PCR amplification assays.

Results: From 308 nasopharyngeal swabs, 102 isolates of *S. pneumoniae* were confirmed by identification tests. Among these isolates, 87 (85.2%), 54 (52.9%), 51 (50%), 43 (42.1%), and 31 (30.3%) were positive for *lytA*, *rrgA*, *phtE*, *pspC*, and *phtD* genes, respectively.

Conclusions: Our study showed that *cpsA* of *S. pneumoniae* is one of the major characteristic genetic markers for diagnostic purposes. Among five adhesion genes, *lytA* was the most frequent one and the strains with a combination of *rrgA* and *lytA* genes were predominant. These findings could be very useful in designing further studies on vaccines against *S. pneumoniae* in our country.

Keywords: Nasopharynx, PCR, *Streptococcus pneumoniae*, Adhesion Genes

1. Background

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterial species. This species is the main reason for mortality, especially in children, patients with chronic diseases, the elderly, and immune-compromised people of all ages worldwide (1). This pathogen causes economically significant diseases like sinusitis, septicemia, otitis, pneumonia, and even meningitis (2). Every year, *S. pneumoniae* takes the life of more than 1000000 children globally. *Streptococcus pneumoniae* usually binds to the epithelial cells of the upper respiratory tract and normally colonizes the nasopharynx and can be found approximately in five up to ten percent and 20 up to 40% of healthy adults and children, respectively (3). The microorganism produces a proliferation of virulence factors, in-

cluding several surface proteins, polysaccharide capsules, and toxin pneumolysin (PLY) (4). However, among these factors, adhesion is essential for pneumococcal colonization and pathogenesis (4).

Among the surface proteins, the pneumococcal surface protein A (PspA) and Pneumococcal surface protein C (PspC) are the best-characterized choline binding proteins (CBPs). The abundance of CBPs in the cell wall is reported as a strain-specific trait (5). Pneumolysin is a released cytoplasmic toxin that can autolyze the cell and it is a very important virulence factor. N-acetylmuramoyl-L-alanine-amidase (LytA) is known as the major hydrolytic enzyme of *S. pneumoniae* (6, 7), which causes the deoxycholate and penicillin-induced cell lysis in the stationary phase (7).

The multi-functional virulence factor of pneumococ-

cus is an adhesion so-called PspC (8) that binds to the polymeric immunoglobulin receptor (9, 10) and regulatory protein factor H of the complement system (11). Among the virulence factors on the cell surface, pili are highly significant (12). The pilus is involved in the virulence and intercedes binding of *S. pneumoniae* to epithelial cells (12). The pilus-encoding gene can code for three main subunits (RrgA, RrgB, and RrgC) and three sortase enzymes. Among the abovementioned sortase proteins, RrgA is a critical virulence factor in murine lung infection model and has a varied prevalence among serotypes of *S. pneumoniae*. The pneumococcal histidine triad protein (Pht) family is a group of surface proteins that do not contain any recognized anchor choline-binding motifs (4).

The Pht family either alone or in combination with capsular polysaccharides is reported to involve in the protection against *S. pneumoniae* when used as vaccines. The protein contains a protected histidine triad motif (HxxH-HxH), termed Pht (pneumococcal histidine triad), that is conserved and repeated several times and involved in binding of zinc ions (13). The Pht family contains four members *PhtA*, *PhtB*, *PhtD*, and *PhtE* (5). Studies in children and adults showed that *PhtD* and *PhtE* elicit antibodies in response to natural infection are considered important antigens for *S. pneumoniae* in the attachment to nasopharyngeal epithelial cells (14).

2. Objectives

Some of the pneumococcal virulence factors are potential targets for protein-based pneumococcal vaccine production. Thus, this study concerned the presence of the five virulence factors in the *S. pneumoniae* isolates in Tehran province.

3. Methods

3.1. Ethics Statement

This study was approved in the 33rd ethical committee meeting, Pasteur institute of Iran. (2012.Nov.20).

3.2. Sampling

In this cross-sectional study, 308 nasopharyngeal swabs were collected from under 6-year-old, healthy children referred to four main care centers (Torkamani, Shobeir, Hazrate Roghayeh, Ameneh) and the children's Medical center of Tehran from December 2011 to August 2012. We obtained the written informed consent of the nurseries' officials or children's parents before sampling. They declared their willingness for using children's data anonymously for research purposes. Nasopharyngeal

swabs were kept in transport media (Stuart) (Oxoid Limited, Hampshire, England) and sent to the Department of Microbiology in Pasteur Institute of Iran.

3.3. Isolation

Nasopharyngeal swabs were streaked on blood agar (Merck, Germany) and chocolate agar plates (Merck, Germany) and incubated for 24 hours at 37°C in 5% CO₂ atmosphere. The typical *S. pneumoniae* single colonies were morphologically characterized and finally were sub-cultured on 5% sheep blood agar.

3.4. Phenotypic and Molecular Identification Methods

The identification of *S. pneumoniae* isolates was done by biochemical methods, Gram staining, colony morphology (alpha-hemolytic, small, gray, and showing mucoid colonies), optochin susceptibility, and bile solubility. Optochin disks (6 mm; MAST diagnostics, Bostle, Mersey side) were applied to blood agar (with 5% Sheep Blood). After 24 hours incubation at 37°C in 5% CO₂ atmosphere, isolates displaying zones ≥ 14 mm in diameter were considered as *S. pneumoniae*. Molecular tests were used for confirmation of the identified isolates by PCR amplification of *cpsA* gene. *S. pneumoniae* ATCC 6305 was used as the control strain in all the identification tests (15). The PCR amplification was carried out using thermocycler in the following conditions: initial denaturation step at 94°C for 45 seconds and 25 cycles of 94°C for 4 minutes, 54°C for 45 seconds, and 65°C for 2 minutes, followed by a final extension at 72°C for 10 minutes.

3.5. Disk Diffusion Testing

Antibiotic susceptibility testing was performed according to the clinical and laboratory standards institute (CLSI) guidelines and using the Kirby-Bauer method. The ten following antibiotic disks (MAST, UK) were tested for all isolates: gentamicin (10 mcg), cotrimoxazole (25 mcg), oxacillin (1mcg), erythromycin (15 mcg), chloramphenicol (30 mcg), cefotaxime (30 mcg), levofloxacin (5 mcg), vancomycin (30 mcg), amoxicillin (25 mcg), and tetracycline (30 mcg).

3.6. Molecular Analyses

The genomic DNA of the isolates was extracted by a DNA extraction Kit (KiaSpin PCR Template purification Kit, Iran) according to the manufacturer's protocol. Finally, the harvested DNA pellet was re-suspended in Elution Buffer to provide 70 μ l of DNA sample. The presence of *lytA*, *phtD*, *phtE*, *rrgA*, and *pspC* genes was studied in the confirmed *S. pneumoniae* isolates by PCR amplification assays. The primer sequences used in this study are shown in Table 1

(Bioneer, South Korea). To confirm our PCR results, we used reference standard strains for each reaction of PCR. These strains included ATCC 6303 for *pspC* and ATCC 49619 for *lytA*, *phtD*, and *phtE* genes.

The PCR amplifications were performed in 25 μ L reaction mixture containing 0.2 mM of dNTPs, 5 pmol μ L of each primer, 1 \times PCR buffer, 1.5 mM MgCl₂, and 1 unit Taq DNA polymerase (GeNet Bio, Korean). PCR amplification was carried out using PCR in the following conditions: for *lytA* and *pspC* genes, initial denaturation at 94°C for 10 seconds and 25 cycles of 94°C for 2 minutes, 58°C for 15 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes; for *rrgA*, *phtD*, and *phtE* initial, denaturation at 95°C for 30 seconds and 25 cycles of 95°C for 2 minutes, 52°C for 30 seconds, and 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes. PCR products were visually observed in a 1% agarose gel when electrophoresis was performed for 1 hour at 100 V. Agarose gels were stained with ethidium bromide and observed under ultraviolet transilluminator.

3.7. Sequence Analysis

One Amplicon from *S. pneumoniae* with the gene *rrgA* was sequenced by Gen Fanavaran company. Sequences were examined for identity with published sequence data from national center for biotechnology information (NCBI).

4. Results

4.1. Identification of *Streptococcus Pneumoniae* Isolates

From 308 nasopharyngeal swabs, 118 isolates were confirmed as *S. pneumoniae*. Samples were collected from children in three age groups: 0 - 2 years, 2 - 4 years, and 4 - 6 years. 100 (32.5%) of all isolates were from children in the age group of 0 to 2. All pneumococcal strains were sensitive to optochin and bile soluble. The presence of *cpsA* gene was confirmed in 102 *S. pneumoniae* isolates.

4.2. Antibiotic Susceptibility Testing

Among all 102 *cpsA* positive isolates, the resistance pattern to antibiotics was as below: 91% (93 isolates) gentamicin, 68.6% (70 isolates) cotrimoxazole, 59.8% (61 isolates) oxacillin, 23.5% (24 isolates) erythromycin, 22.5% (23 isolates) chloramphenicol, 10.8% (11 isolates) tetracycline, and 1% (1 isolates) amoxicillin. All of 102 isolates were susceptible to cefotaxime, vancomycin, and levofloxacin (Figure 1).

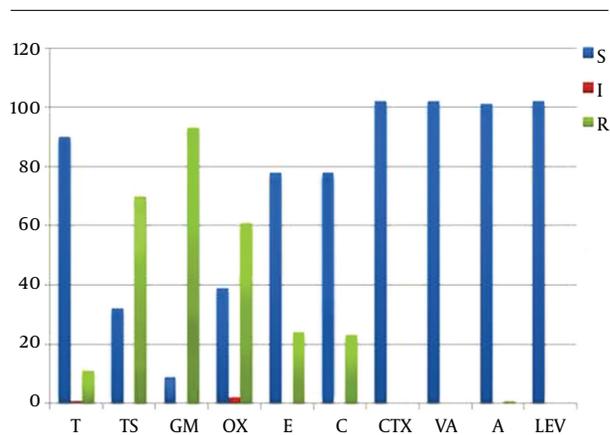


Figure 1. The rate of antibiotic resistance in the study strains. Blue bars, sensitive; red bars, intermediate; green bars, resistant. Abbreviations: T, tetracycline; TS, cotrimoxazole; GM, gentamicin; CTX, cefotaxime; OX, oxacillin; E, erythromycin; C, chloramphenicol; VA, vancomycin; A, amoxicillin; LEV, levofloxacin.

4.3. PCR for *phtD*, *phtE*, *rrgA*, *lytA*, and *pspC* Results

The prevalence of *lytA*, *rrgA*, *phtD*, *phtE*, and *pspC* genes in the isolates of *S. pneumoniae* was determined. Of the 102 isolates of *S. pneumoniae*, 86 (84.3%) were positive for *lytA* gene, having 319 bp bands. The results of this five adhesion genes according to gender, age, and size of bands are shown in Table 2. The *rrgA* gene was detected in 54 (52.9%) of the isolates (a 373 bp band). 51 (50%) isolates harbored *phtE* gene (a 1392 bp band). 43 (42.1%) isolates of *S. pneumoniae* were confirmed by PCR for *pspC* gene. Interestingly, *pspC* gene was characterized in different sizes from 1200 to 2500 bp. The most prevalent variants of *pspC* gene were 1400, 1512, and 2000 bp (Figure 2). 25 out of 43 (58%) isolates had 1512 bp bands, 5 out of 43 (12%) isolates had about 1400 bp bands, 5 out of 43 (12%) had about 2000 bp bands, 4 out of 43 (9%) had about 1200 bp bands, and 4 out of 43 (9%) had about 2500 bp bands. Of the total, 31 (30.3%) isolates harbored the *phtD* gene. All of the isolates had 2454 bp bands.

4.4. Sequence Analysis

The Amplicon represented expected sequences with 99% identity with the published data from NCBI (GenBank: EF560633.1). This was used as a positive control for the *rrgA* gene in PCR reactions.

5. Discussion

Colonization of *S. pneumoniae* in the respiratory tract can cause bacteremia and finally induce systemic infections such as septic arthritis, meningitis, and pneumonia,

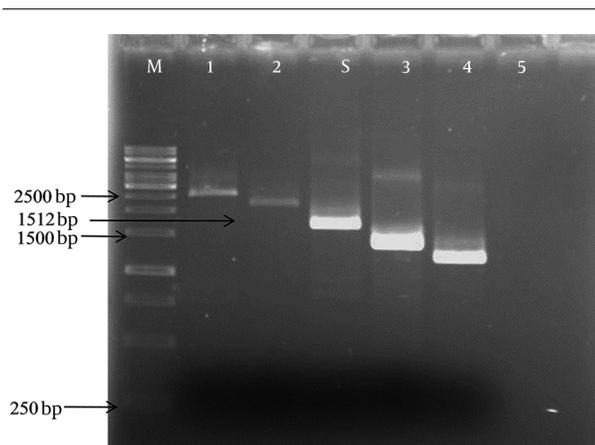
Table 1. Oligonucleotides Used as Primers to Amplify Particular Sequences of *S. Pneumoniae*

Primer Pair	Primer Sequence (5' - 3')	Size of Amplicon, bp	References No.
<i>lytA</i> -sense	CAA CCG TAC AGA ATG AAG CGG	319	(16)
<i>lytA</i> -antisense	TTA TTC GTG CAA TAC TCG TGC G'		
<i>pspC</i> - sense	AAGATGAAGATCGCCTACGAACAC	2265	(17)
<i>pspC</i> - antisense	AATGAGAAACGAATCCTTAGCAATG		
<i>rrgA</i> - sense	CACTTTTATACGCTTTTGCTA	373	(18)
<i>rrgA</i> - antisense	TAATACGACTACTATAGGTGCCATCCGTAITGTTTTTC		
<i>phtD</i> - sense	GCATGCTCCTATGAACCTGGTCGTC	2454	(14)
<i>phtD</i> - antisense	AAGCTTTTACTATATAGGAGCCGGTTGACT		
<i>phtE</i> - sense	GCATGCGCCCTATGCACTAAACCAGCA	1392	(14)
<i>phtE</i> - antisense	CTGCAGCTAAATGTTTTTGGCCACCT		

Table 2. Results of Examining Specimens for Five Adhesion Genes by PCR^a

Gene	No (%)	Female	Male	Size, bp	Age		
					0 - 2	2 - 4	4 - 6
<i>lytA</i>	86 (84.3)	41 (47.6)	45 (52.4)	319	53 (60.3)	18 (20.4)	17 (19.3)
<i>rrgA</i>	54 (52.9)	25 (46.3)	29 (53.7)	373	30 (55.5)	14 (26)	10 (18.5)
<i>phtE</i>	51 (50)	21 (41.1)	30 (58.9)	1392	32 (63)	8 (15.5)	11 (21.5)
<i>pspC</i>	43 (42.1)	20 (46.5)	23 (53.5)	1512	24 (55.8)	8 (18.6)	11 (25.6)
<i>phtD</i>	31 (30.3)	13 (42)	18 (58)	2454	21 (68)	5 (16)	5 (16)

^aValues are expressed as No. (%).

**Figure 2.** The gel agarose electrophoresis of PCR product of *pspC* gene; from left to right: lane M, 1000 bp ladder; lane S, control strain (*S. pneumoniae* ATCC 6303); lanes 1,2,3,4 positive strains for *pspC* gene of *S. pneumoniae* and lane N, negative control.

especially in children under 6 years of age (8). Non-pili adhesion is also important in enabling binding to target cells and colonization of *S. pneumoniae*. These adhesion genes can cause the expansion of direct interaction and invasion of bacteria to epithelial surfaces. In addition, these genes

play a role in survival and colonization of *S. pneumoniae* in the host. *Streptococcus pneumoniae* strains are identified by phenotypic detection and confirmed with PCR amplification of *cpsA* gene. To differentiate *S. pneumoniae* from the closely viridans group streptococci as well as from other pneumococcus-like streptococci such as *S. pseudopneumoniae*, this PCR amplification assay seems to be critical.

Many virulence genes contribute to the colonization of *S. pneumoniae*; however, our study only demonstrated this for *pspC*, *phtD*, *phtE*, *rrgA*, and *lytA* genes. Our study showed that *cpsA* of *S. pneumoniae* is one of the major characteristic genetic markers for diagnostic purposes. The most important finding of this study was the high level of penicillin resistance in strains isolated from the nasopharynx of children. In this study, cefotaxime, vancomycin, and amoxicillin were the most effective drugs against *S. pneumoniae* infection. However, cotrimoxazole and gentamicin were the less effective drugs against the study *S. pneumoniae* isolates. It should be mentioned that the number of penicillin-resistant isolates is considerable. The frequency of the adhesion genes was 86 (84.3%), 54 (52.9%), 51 (50%), 43 (42.1%), and 31 (30.3%) for *lytA*, *rrgA*, *phtE*, *pspC*, and *phtD*, respectively.

Among the five adhesion genes, *lytA* was the most frequent one. 87 (85.2%) children were positive for *lytA* gene. Most of the *S. pneumoniae* isolates used *lytA* gene for binding to epithelial cells of the nasopharyngeal region. Autolysin, which was found in all strains, appears to be a suitable target virulence factor and apparently highly conserved for inclusion in a potential vaccine (19). The *LytA* protein of *Pneumococcus* is regarded as the main autolysin factor of *S. pneumoniae*, which has a significant function in the pathogenesis of *S. pneumoniae*. Actually, the pathogenesis starts by releasing this pneumolysin. Thus, this autolysin has a fundamental role in the lysis after exposure to certain antibiotics. In addition, it has been reported that *lytA* gene has higher specificity than *ply* gene for the identification of *S. pneumoniae* (16).

Our study showed that *lytA* allocated the highest percentage and the frequency of *lytA* gene in healthy children of Tehran (Iran) was close to that of children in Mashhad (Iran) (20). On the other hand, *rrgA* was more frequent compared to in Mashhad (20). Of the total, 45 children had both *rrgA* and *lytA* that illustrated the important role of these factors in colonization (21). The frequency of the *phtE* gene in nasopharyngeal samples of care centers of Tehran was less than that in other countries (22) and 32 (62.7%) cases were positive for *phtE* gene belonging to healthy children less than 2 years of age. Our results showed the *pspC* gene prevalence was more than that in Mashhad (21). In this study, we observed the genetic diversity of *pspC* gene in isolates from children under 6 years of age. The bands of 25 of 43 strains were the same as standard reference strain. The PCR product size of 43 strains was about 1200 bp to 2500 bp. These results were consistent with a study conducted in the UK (17) that showed 78 out of 102 *S. pneumoniae* strains had PCR products ranging from 1.5 to 2.2 Kb in size (17).

In our study, *phtD* had the least frequency. The frequency of the *phtD* gene in healthy children in Tehran was less than that in other countries (22) and 21 (67.7%) cases that were positive for *phtD* gene belonged to healthy children under 2 years of age. These differences may be related to the geographical differences or different types of specimens. We found no correlation between the gender of children and the presence of adhesion genes; in contrast, we suggested a probable significant correlation between the age of children and adhesion genes. Of the 102 isolates, 97 (95%) from positive cases had at least one of these adhesion genes and only 5 (5%) samples harbored none of these five adhesion genes. It is likely that strains without these genes may use other adhesion factors. Similar studies can determine the dominant virulence factors of native strains. Such studies can be very useful in designing vaccines against *S. pneumoniae*.

6. Conclusions

Our study showed that *cpsA* of *S. pneumoniae* is one of the major characteristic genetic markers for diagnostic purposes. Among five adhesion genes, *lytA* was the most frequent one and strains having a combination of *rrgA* and *lytA* genes were predominant. These findings could be very useful in designing further studies on vaccines against *S. pneumoniae* in our country.

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Footnotes

Financial Disclosure: Upon completion of the study, all authors had no conflict of interest.

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