Molecular Identification and Detection of *Streptococcus pneumoniae* Serotypes Isolated from the Cerebrospinal Fluid of Children with Suspected Meningitis: First Report From Alborz, Iran

Hananeh Tosifi, Omid Safari, Masoud Dadashi, Arman Shafiee, Maryam Beiky, Mahmood Bakhtiyari, Sabahat Haghi, Reza Arjmand, Mohammad Ali Shahbabaie

1Department of Pediatrics, Imam Ali Hospital, Alborz University of Medical Sciences, Alborz, Karaj, Iran
2Department of Microbiology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran
3Department of Psychiatry and Mental Health, Alborz University of Medical Sciences, Karaj, Iran
4Student Research Committee, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran
5Non-communicable Diseases Research Center, Alborz University of Medical Sciences, Karaj, Iran

**Abstract**

**Background:** *Pneumococcus* causes various infections, some of which are life-threatening, including pneumonia, meningitis, otitis media, bacteremia, and sinusitis. Currently, more than 95 serotypes have been identified.

**Objectives:** The purpose of this study was to isolate and determine *Streptococcus pneumoniae* serotypes from the cerebrospinal fluid (CSF) of children with suspected meningitis using the multiplex polymerase chain reaction (PCR) method.

**Materials and Methods:** A total of 864 CSF samples were taken from children with suspected meningitis who were admitted to Imam Ali Hospital of Karaj for one year (January 2019 to January 2020). To collect positive *S. pneumonia* samples, the lytA gene was traced using specific primers and PCR techniques.

**Results:** The results of this study indicated that only 16 patients have a pneumococcal infection (1.85%), of whom 50% have encapsulated pneumococci. Furthermore, serotypes 38 (12.5%), 7F (12.5%), 23F (25%), and 6A/B (50%) were the most common serotypes causing invasive pneumococcal infections in the included samples.

**Conclusion:** Among the techniques for serotyping *S. pneumoniae*, the multiplex PCR technique is known as a fast, easy, and low-cost method that can serotype a large number of samples. The results also showed that the 13-valent pneumococcal conjugate vaccine can cover at least 50% of the strains that cause invasive pneumococcal diseases (IPDs), which are life-threatening, especially in children. Therefore, it is suggested that the healthcare administrators of Iran design and implement a public vaccination program.

**Keywords:** *Streptococcus pneumoniae*, Multiplex PCR, Pneumococcal serotype, Meningitis

**Background**

*Streptococcus pneumoniae*, also identified as pneumococcus, is a pathogen that can asymptptomatically colonize the human nasopharynx and can also cause aggressive diseases such as pneumonia, septicemia, and meningitis. In fact, pneumococcal diseases are responsible for approximately 1.6 million deaths in children under five years of age worldwide.

Community-acquired pneumonia (CAP) is primarily caused by *S. pneumoniae*, accounting for 89% of cases in the United States and 37% globally. Prior to the use of antibiotics, pneumococcus was responsible for 39% of all pneumonia. However, diagnosing pneumococcal pneumonia can be challenging for clinicians as positive blood cultures are only observed in 35-39% of cases caused by this pathogen.

Bacterial meningitis is another intensive and frequent infection caused by *Streptococcus pneumonia*, with a mortality ratio of up to 20% and negative neurological outcomes in 50% of survivors. Despite the development of vaccines and adjuvant therapies, pneumococcal meningitis remains a significant health concern due to challenges such as antibiotic resistance, the emergence of new bacterial serotypes, and limited vaccine effectiveness.

While there has been significant research on the growth of *S. pneumoniae* in the brain environment, our understanding of blood-brain barrier disturbance in pneumococcal meningitis is still incomplete at the molecular level. Moreover, the rapid expansion of...
resistant strains of pneumococcus and the appearance of multidrug-resistant strains have increased worldwide concern about pneumococcal infections. Over the past 30 years, vaccination has been the primary focus of research centers to reduce the mortality and complications related to pneumococcal infections in both children and adults.14,15

Currently, the pneumococcal vaccine is an imported vaccine in Iran and is primarily administered to high-risk groups. However, vaccination is often performed without prior studies to determine the present specific serotypes, which can lead to a decrease in vaccine effectiveness and potentially cause the spread of dangerous diseases such as meningitis and septicaemia in society.

Given the lack of information in this field, our study aimed to define the frequency of S. pneumoniae serotypes in the cerebrospinal fluid (CSF) of children suspected of having meningitis and referred to Imam Ali (AS) Karaj Medical Education Center using the multiplex polymerase chain reaction (PCR) method.

Materials and Methods

Sample Size and Bacterial Identification in Pediatric Meningitis Study

In this cross-sectional study, we collected a total of 864 samples randomly selected from 2500 children suspected of having meningitis. The study took place at Imam Ali Hospital in Karaj between January 2019 and January 2020. To be included in the study, children had to exhibit fever (defined as rectal temperature > 38.5°C or axillary temperature > 38°C) along with at least one of the following symptoms: Neck stiffness or inflexibility, bulging of the fontanel, convulsions, irritability, or drowsiness. Children without parental consent to participate or those not showing signs of S. pneumonia-induced meningitis were excluded.

All children underwent lumbar punctures performed by a pediatrician. Pneumococcal isolates were cultured on blood agar medium (Merck Co., Darmstadt, Germany) with 5% sheep blood and incubated at 37°C with 5-10% CO2. Identification was based on conventional tests including α hemolysis, bile solubility, and optochin sensitivity. The confirmation of S. pneumoniae was done by identifying the lytA and capsular polysaccharide (CPS) genes using specific primers and multiplex PCR.

The sample size for this study was determined based on a 9% prevalence rate, 95% confidence limits, 5% error rate, and 80% power, using a specific formula. Additionally, S. pneumoniae isolates were further confirmed through the detection of the lytA gene using specific primers and PCR techniques.

DNA Extraction

To extract DNA, bacterial isolates from 24-hour cultures were collected using a sterile loop and dissolved in 250 µL of PBS buffer. DNA isolation followed the manufacturer’s instructions using a kit from Roche Diagnostic Corporation (US). Further, the quantity and concentration of isolated DNA were assessed using nanodrop and electrophoresis on a 1% gel.

Multiplex Polymerase Chain Reaction Serotyping

PCR reactions were performed using 2X Taq Master Mix (premix PCR). Forward and reverse primers (12.5 µL each) were added to microtubes containing the premix. All primers were designed and prepared according to the protocol of the Center for Disease Control and Prevention (CDC). All steps were carried out on ice to prevent unwanted reactions and enzyme deactivation. Initially, samples were screened for the presence of the lytA gene to confirm the presence of pneumococcus in CSF. Subsequently, multiplex PCR was conducted in seven separate reactions on CPS-positive samples to determine capsular serotyping. Reactions were performed individually for serotypes A15 (tm = 54°C) and F22 (tm = 66°C) due to their distinct melting temperatures. The primer information for each reaction is presented in Table 1 (supplementary file). Following multiplex PCR, the products were electrophoresed on a 2% gel.

Statistical Analysis

Data analysis was conducted using SPSS software version 19. Various statistical methods, including student t-test, ANOVA, chi-square, and paired t-tests were employed, and results were reported as mean and standard deviation (SD).

Results

Demographic Information

The current study was conducted on 864 samples collected from children with an age mean ± SD of 3.7 ± 1.2 years. Of these, 54% and 46% were boys and girls, respectively. The demographic information of the children is presented in Table 1.

Table 1. Demographic Information of Included Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients (n = 864)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>3.7 ± 1.2</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>92 ± 13</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>50 ± 9</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>114 ± 28</td>
</tr>
<tr>
<td>Respiratory rate (breath/min)</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>38.4 ± 1.7</td>
</tr>
<tr>
<td>WBC (10³/µL)</td>
<td>10.8 ± 2.6</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>11.3 ± 1.8</td>
</tr>
<tr>
<td>Platelet (10³/µL)</td>
<td>370 ± 102</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>24 ± 16</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate (mm/h)</td>
<td>54 ± 38</td>
</tr>
</tbody>
</table>

Note: WBC: White blood cell.
Among the 864 samples, multiplex PCR identified 16 positive cases of *S. pneumoniae*, accounting for 1.85% of the samples. The presence of the CPS gene was confirmed in 65.5% of the *S. pneumoniae* isolates (n = 10).

In this study, we considered a total of 29 common serotypes. Within the 10 encapsulated isolates, the recognized serotypes included 23F (2 cases, 20%), 38 (1 case, 10%), 7F (1 case, 10%), and 6A/B (4 cases, 40%). Serotypes 19A, 19F, 1, 5, 7F/A, 9V, 14, 11A, 33F, 18, 35B, 12, 16F, 15B/C, 31, 8, 10A, 35F, 34, 7C, 17F, 20, 4, 15A, and 22F were included in the multiplex PCR analysis but were not identified among the isolates. Detailed results and PCR figures can be found in Supplementary file 1.

**Discussion**

*Streptococcus pneumonia*, along with *Haemophilus influenzae* and *Neisseria meningitidis*, are considered the most common causes of bacterial meningitis; however, according to studies in many developing countries, *S. pneumoniae* is one of the most common causes of bacterial meningitis and noticeably important causes of death in children.¹⁰

According to the recommendations of the World Health Organization (WHO) and the Center for Disease Control and Prevention (CDC), the best strategy to fight against invasive pneumococcal infections that can lead to death or high costs in the field of healthcare is general vaccination of children under two years old. The increase in resistance to different antibiotics, including penicillin and third-generation cephalosporins as well as the high potential of spreading resistance genes to different drugs to sensitive strains clearly shows the necessity of implementing a vaccination program to prevent pneumococcal infections.

In this study, pneumococcal meningitis was evaluated in children with suspected meningitis. It was discovered that *S. pneumonia* was the main cause of meningitis in 16 cases. Further, in 10 patients, the CPS gene, which is related to the pneumococcal capsule, was detected. Other serotypes such as 6A/B, 7F, 23F, and 38 were also identified. Another study reported that 18C, 14, 19A, 6A, and 7F are the most commonly identified serotypes in Tehran.¹⁷ However, in another study in the northeastern region of Iran, the most commonly identified strains were reported to be 23F, 19F, 19A, 1, 14, and 6A/B.¹⁸ The main reason for the disparity of the identified serotypes is likely to be the performance of these studies in different areas and regions.

In our study, the multiplex PCR method was used to identify pneumococcus in children with suspected meningitis. As a standard method for *S. pneumoniae* serotype determination, the quelling reaction is a reference method despite the difficulty, time-consuming, and high cost of this method. One of the alternative methods is the molecular typing method according to the amplification of serotype-specific CPS synthesis genes. Researchers have mostly focused on PCR that identifies multiple serotypes in one augmentation reaction.²⁸ In 2017, a study investigated acute bacterial meningitis in Iran during a systematic review and meta-analysis. In this study, data from 1995 to 2015 were examined in Iran. The outcomes of the current study revealed that the most common cause of bacterial meningitis in Iran is *S. pneumoniae* with a prevalence of 30%, followed by *H. influenzae* and staph coagulase negative. In this study, it was recommended to carry out more studies to investigate the strains of common bacteria that cause bacterial meningitis.²¹

In 2006, Yaro et al²² investigated the epidemiological and molecular characteristics of the most lethal pneumococcal meningitis in Burkina Faso. During 24 months from 2002 to 2005, clinical and laboratory data for cases of acute bacterial meningitis were collected from three districts in Burkina Faso. *S. pneumoniae* was identified by culture, PCR, or antigen detection in CSF samples which were obtained from 1686 people. Pneumococcal genotyping was accomplished on the strains via polynomial repeat dialing and polynomial sequence typing. The results indicated 249 (15%) patients identified with *S. pneumoniae* (annual incidence, 14 cases per 100000), 115 patients (46%) died, and *S. pneumoniae* was noticeably the most common organism, accounting for two-thirds of deaths from bacterial meningitis. Throughout the meningitis epidemic season, an average of 38 cases of *S. pneumoniae* infection were identified each month, compared with broadly 8.7 cases in other months. Of the 48 tested pneumococci, 21 (44%) were classified as serotype 1, and the remaining 27 (56%) were categorized as 15 distinctive serogroups and/or serotypes. Both serotype 1 and other serogroups and/or serotypes were seasonal. The genotypes of serotype 1 isolates were rather opposite during the study period, but they were diverse and similar to the predominant genotypes of Ghana. The results indicated that intervention approaches through the epidemic season in Burkina Faso (and perhaps elsewhere) should develop pneumococcal meningitis in a comprehensive pattern related to meningococcal meningitis. Though a serotype 1 clone was typically isolated, the majority of the cases were affected by other serologies and/or serotypes, and genetic diversity was elevated over a comparatively short period.²⁴ In 2012, one study succeeded in identifying serotypes 1, 3, 4, 5, 6A/B, 7F, 9V, 14, 18C, 19A, 19F, and 23F by one-step multiplex PCR. Considering the 100% sensitivity and specificity of this method, it was suggested that using this method can significantly reduce the costs of detecting different pneumococcal serotypes.⁴

Among the techniques for determining pneumococcal capsular types, the multiplex PCR technique is known to be a fast, easy, and low-cost method that has the possibility
of determining the types of a large number of samples. Therefore, it seems necessary to conduct comprehensive and complete studies throughout different cities of Iran in order to design a suitable and efficient vaccine. These results were also confirmed in other studies.\textsuperscript{3, 23}

According to the serotypes observed in this study, it is possible to reduce the burden of this disease in this area by using the PCV-7 vaccine, which covers half of the found serotypes. One study that explored the comparison of the incidence of invasive pneumococcal disease (IPD) between two vaccines, PCV-7 and PCV-13, reported that the incidence of IPD was reduced in children vaccinated with PCV-7, while there was no similar effect in PCV-13. In addition, herd immunity after PCV-7 was more significant than after PCV-13.\textsuperscript{24}

**Limitations**

One limitation of this study was the inability to compare the typing patterns of the isolated strains in Alborz with known serotypes from other cities in Iran. To address this limitation, it is recommended that future research expands its scope to include a wider extensive and diverse statistical population as well as a larger sample size. This would allow for the identification of common serotypes across various medical centers in different cities throughout Iran. Additionally, it is suggested that future studies incorporate a control strain in their PCR analyses to enhance the validity and certainty of their findings.

**Conclusion**

The findings of the present study have highlighted that serotypes 38, 7F, 23F, and 6A/B are the most prevalent serotypes responsible for causing IPD in children suspected of sepsis and bacterial meningitis who were referred to Imam Ali (AS) Karaj Hospital in 2018. To date, Iran has not established a comprehensive and widespread immunization program targeting individuals at risk of pneumococcal infections, particularly children. Pneumococcal vaccines are still considered optional and often come with a high cost. Unfortunately, this lack of awareness and accessibility has left many members of society uninformed about the critical importance of vaccination for achieving population immunity.

Furthermore, based on the results of this study and a few others conducted in Iran, it has been shown that the 13-valent pneumococcal conjugate vaccine has the potential to cover at least 50% of the strains responsible for causing IPDs, which can be life-threatening, especially in children. Therefore, implementing a comprehensive immunization program targeting children under the age of two and other high-risk individuals could potentially prevent at least 50% of IPD cases. Given this, it is strongly recommended that healthcare centers and professionals in the country work together to design and execute a nationwide vaccination program.

**Authors’ Contribution**

**Conceptualization:** Reza Arjmand.

**Data curation:** Hananeh Tosifi.

**Formal analysis:** Mahmood Bakhtiyari.

**Methodology:** Mahmood Bakhtiyari.

**Project administration:** Reza Arjmand.

**Resources:** Sabahat Haghi, Mohammad Ali Shabhbabaei.

**Validation:** Masoud Dadashi.

**Writing—original draft:** Arman Shafiee, Maryam Beiky, Reza Arjmand.

**Writing—review & editing:** Arman Shafiee, Maryam Beiky, Reza Arjmand.

**Competing Interests**

The authors declare no conflict of interest.

**Ethical Approval**

The study was approved by the Regional Ethics Committee at Alborz University of Medical Sciences, Karaj, Iran. (IR.ABZUMS.REC.1398.150).

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**Supplementary Files**

Supplementary file 1 contains Table S1 and Figures S1-S7.

**References**


