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Research Article

**PCR DETECTION OF *STREPTOCOCCUS PNEUMONIAE* DNA
FROM NASOPHARYNX OF THE PATIENTS WITH ACUTE
RESPIRATORY INFECTION**Alieh Lotfi Nejad ^{1*}, Mansour Amin ² and Mitra Salehi ³¹Department of Microbiology, Tehran North Branch, Islamic Azad University, Tehran, Iran²Health Research Institute, Infectious and Tropical Diseases Research Center and Department of Microbiology, School of Medicine, Jundishapur University, Ahvaz, Iran³Department of Microbiology, Tehran North Branch, Islamic Azad University, Tehran, Iran**Abstract:**

Causing pneumonia, sepsis, meningitis, and otitis media often in young children and elderly adults, streptococcus pneumoniae is considered as not only an aggressive pathogen but a normal part of the human respiratory microbiome, as well. To identify S. pneumoniae, some methods which are mostly culture-based are usually used. In general, occasionally being validated by DNA-based methods, culture-based detection methods depend on optochin susceptibility, agglutination, and bile-solubility.

In the current study, a total number of 93 nasopharyngeal samples were collected from patients with acute respiratory infection. Isolates of S. pneumoniae were confirmed by α -hemolysis of colonies appeared, optochin sensitivity, and bile solubility. The presence of ply gene was also discovered by PCR. 7 (7.53%) isolates among 93 nasopharyngeal samples were confirmed as S. pneumoniae by all of phenotypic tests, while the PCR assay revealed that 19 isolates (20.43%) were positive for virulence gene, ply. The present study found out the identification of Streptococcus pneumoniae should be based on phenotypic tests and at least a molecular method such as PCR.

Keywords: PCR, *Streptococcus pneumoniae*, Ply, Nasopharynx**Corresponding Author:****Alieh Lotfi Nejad,**

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INTRODUCTION:

Being able to cause local diseases and acute infections, *streptococcus pneumoniae* (*pneumococcus*) is regarded as a gram positive, lancet-shaped coccus, and a regular inhabitant of the respiratory tract [1]. Moreover, it is one of the most important bacterial causes of inflammatory conditions such as pneumonia, meningitis, bacteremia, sepsis, and acute otitis media [2].

It, as an important pathogen, causes infections in young children particularly during the first two years of life and in elderly patients [1 & 3], by colonizing usually in the mucosal membrane of the nasopharynx and the throat of people in good physical shape [4].

In addition, *streptococcus pneumoniae* being one of the main pathogens which infects human beings universally seems to be the most general reason of community-acquired bacterial pneumonia and otitis media which can lead to severe cases of meningitis and sepsis [5]. It is discovered 1.6 million people die due to pneumococcal diseases each year [6]. The World Health Organization (WHO) asserts *pneumococcus* annually causes more than 700,000 to 1 million deaths among children [7].

Nowadays, more than 90 different serotypes are identified, but specific serotypes usually justify most *S. pneumoniae* nasopharyngeal isolates [5]. However, varying based on the geographic locations, serotypes are temporally distributed [8]. Based on the age, health, and socioeconomic status of the population under study, it is confirmed that the prevalence of *S. pneumoniae* nasopharyngeal colonization ranges from 7-99% [9].

Epidemiological studies of *S. pneumoniae* are important for numerous reasons; for instance, carriage strains can be applied as “indicators for drug resistance, and for pneumococcal serotype distribution and vaccine coverage prediction” [5].

It is worth mentioning that culture-based identification methods are frequently used to both identify *S. pneumoniae* from patients and distinguish it from the less pathogenic viridans streptococci which are often detected in respiratory samples (12. RTehseaerc hd) [10]. To identify *pneumococci*, other than conventional methods like optochin susceptibility, bile solubility, and capsular reaction tests, DNA-based methods have been developed [11].

To detect *S.pneumoniae*, the use of *ply* and *lytA* genes alone or together with each other has been examined [13, 14, & 15].

Predetermining the pneumococcal virulence factor pneumolysin, the *ply* gene is considered as the best target for PCR-based identification of *S. pneumoniae*, which is greatly specific [16].

The present study was an endeavor to determine the frequency of *pneumococcus* bacteria in the

patients with acute respiratory infection by PCR of *ply* gene.

MATERIALS AND METHODS:

Sampling:

A total of 93 samples from nasopharynx of patients (50 females and 43 males) with acute respiratory infection were collected carefully and transferred under cold condition to the laboratory. These samples were obtained from Imam Khomeini Hospital in Ahwaz, Iran.

Cultivation and Identification:

The nasopharynx samples were cultured on blood agar with 5% sheep's blood (Merck KGaA, Darmstadt, Germany). All plates were incubated at 37°C in an atmosphere of 5% CO₂ for 24 hours. By using typical colonial appearance, α hemolysis, and Gram staining, suspected *S. pneumoniae* isolates were re-identified. Confirmatory tests included optochin sensitivity and bile solubility. Finally, the results were confirmed by PCR for *Ply* gene.

In an atmosphere of 5% CO₂, the optochin-susceptibility test was carried out by using a 6.5 mm diameter disc which contains 5 mg optochin. A zone of inhibition of at least 14 mm diameter indicated there was a positive result.

The bile solubility test can be identified as a test to distinguish *Streptococcus pneumoniae* (positive-soluble), which is bile soluble while all the other alpha-hemolytic streptococci are considered bile resistant, from alpha-hemolytic streptococci (negative- insoluble). This test was performed using two tubes containing suspension of a pure culture in 2 ml of 0.85% saline to achieve turbidity in the range of a 0.5-1.0 McFarland standard). Then, 2 drops of 2% sodium desoxycholate were added to test tube and 2 drops of sterile water distilled water were added to the other tube (control tube) and mixed. After that, both tubes were put for 10-15 minutes at 35-37°C. It was also observed for a clearing of turbidity in the tube which contained 2% sodium desoxycholate (If it was negative, it was continued to incubate up to 3 hours and clearing was observed again).

DNA extraction:

Pneumococcal genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany). The virulence gene *ply* was identified by PCR applying the primers which are listed in Table 1.

PCR assays:

Amplification conditions for *ply* gene was as follows: 95°C for 2 min, 30 cycles of 95°C for 30 s, 59.8°C for 45 s, and 72°C for 1.5 min, followed by a final extension at 72°C for 5 min. *S.*

pneumoniae ATCC 3340 was utilized as positive control.

The PCR products and 100 bp DNA ladder were then separated in 1.5% agarose gel with greenview incorporated.

RESULTS:

Phenotypic tests:

Among 93 samples from nasopharynx of patients with acute respiratory, 7 (7.53%) isolates were confirmed as *S. pneumoniae* by all the phenotypic tests (i.e., culturing in blood agar, gram staining, and checking α hemolysis, optochin sensitivity, and

bile solubility). These positive samples contained 5 (71.43%) females and 2 (28.57%) males.

PCR of *ply* gene:

DNAs extracted from the nasopharynx of patients were transferred to the PCR thermocycler. Then, the amplified segments of DNA were revealed by electrophoresis.

The PCR assay unveiled that 19 isolates (20.43%) were positive for virulence gene, *ply*. Figure 1 depicts the results of amplified products. The size of PCR products were 329 bp. Out of 19 patients, 10 patients (52.63%) were female and 9 (47.37%) were male.

Table 1: Primers utilized in the PCR assay

Primer	Sequence (5-3)	Amplicon size (bp)
Ply-F	TGC AGA GCG TCC TTT GGT CTAT	329
Ply-R	CTC TTA CTC GTG GTT TCC AAC TTG A	

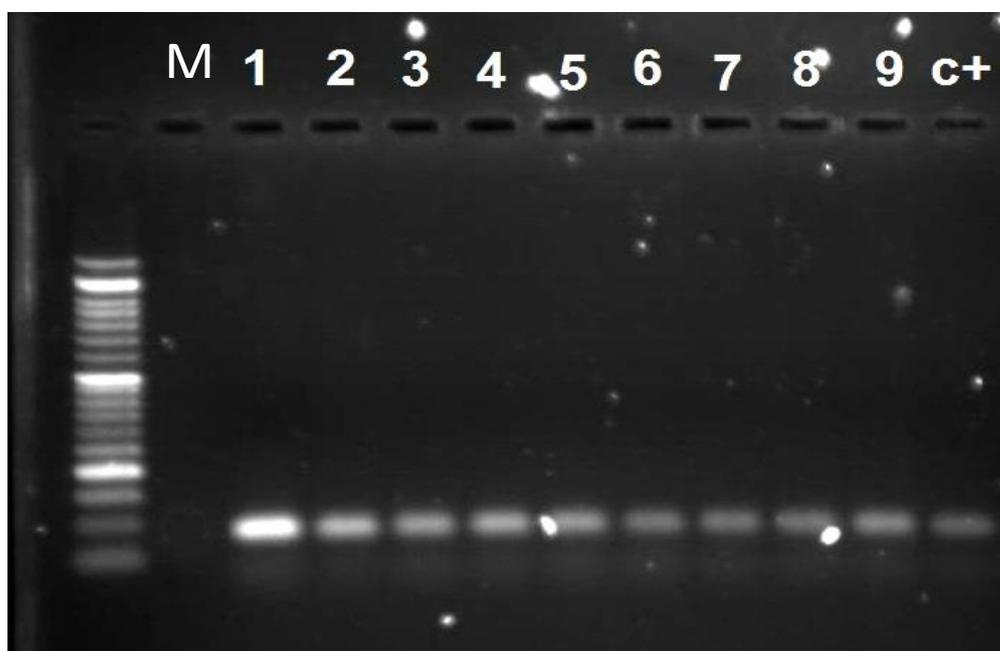


Fig1: Result of amplified DNA fragment. M: molecular weight marker, lanes 1-9: positive samples of patients, C+: positive control (*S. pneumoniae* ATCC 3340).

DISCUSSION:

As mentioned earlier, *streptococcus pneumoniae* gives rise to numerous infections all over the world; thus, not only does it cause invasive diseases with significant mortality but also it causes moderately benign and so common mucosal infections like acute otitis media [17]. Moreover, frequently with no adverse effects to the host, it is usually in the nasopharynx of human beings. However, it can increase and result in upper or lower respiratory tract infections. It has been found that the majority of children carry *S. pneumoniae* in their nasopharynx throughout the first years of their life [18]. Approximately half of children in developed countries such as the United States are colonized with *S. pneumoniae* no less than one time by age one [19]. All over the world, *streptococcus pneumoniae* is responsible for approximately 1.6 million deaths per year [20].

Nasopharyngeal colonization with *Streptococcus pneumoniae* is different in various parts of the world; it is probably due to both differences in the populations under study such as their age, race, and socioeconomic status and differences in sampling and related separation techniques [21]. It is estimated that 25 cases of *S. pneumoniae* occur per hundred thousand cases of young adults and 280 cases occur per hundred thousand cases of elderly people annually. The uncertainty is due to problems in microbiological diagnosis of *S. pneumoniae* because *pneumococcus* isolation from sputum raises a possible diagnosis because there is the possibility of rejection, and the certain diagnosis is subject to *pneumococcus* isolation from sterile fluids and blood [22].

In Iran, the primary problem with *pneumococcus* is the false diagnosis of bacteria. In a routine procedure, the final diagnosis is usually based on sensitivity to optochin that can lead to errors in diagnosis. On the other hand, since *pneumococcus* is a normal nasopharynx flora, unnecessary treatments can lead to antibiotic resistance [23, 24]. Over the past 10 years, researchers have considered the advantage of molecular diagnosis based on PCR and have used this method to diagnose only one bacterium because it enjoys higher speed and accuracy than the existing methods like culturing [25]. Based on existing studies, the *lytA* and *ply* genes are the most important genes to detect and identify *streptococcus pneumoniae*. On the other hand, recent studies have shown that *ply* is a more appropriate gene due to the isolation of higher number of *Streptococcus* strains [26, 27]. The increased correct diagnosis of the organisms can reduce indiscriminate use of antibiotics, the cost of treatment, and drug resistance.

In the present study, the combination of applied microbiological techniques such as gram stain,

optochin test, bile solubility and advanced molecular methods such as PCR of the *Ply* gene was used to identify *S. pneumoniae* from nasopharyngeal samples that collected from Imam Khomeini Hospital in Ahwaz. According to the results from 93 patients under study, 19 patients (20.65%) were positive by PCR methods and among them 9 subjects were male (47.37%) and 10 subjects were female (52.63%). In addition, based on the results of phenotypic tests, 7 positive isolates (7.61%) were identified in 2 males (28.57%) and 5 females (71.43%).

In 2005, by using primers prepared from *ply*, *ctrA*, and *bex* genes, the single-stage PCR multiplex method was designed for simultaneous diagnosis of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* and 425 samples from 336 patients were examined and 66 *Streptococcus pneumoniae* isolates were detected, while the sensitivity and diagnosis of this method was 93% [28]. In 2006 in Rio de Janeiro, Brazil, a test was conducted by Matos et al. to diagnose *pneumococcus* bacterium in patients with meningitis by culturing, gram staining, agglutination tests and *Ply* gene PCR methods on 106 patients and it was found that the PCR of this gene was the best method for the diagnosis of *pneumococcus* since this technique was positive in 104 (96%) patients [29].

In another study conducted in Iran in 2006, of 1161 children under 6-years old [572 (49.3%) males and 589 (50.7%) females] with an average age of 4.2 years, 102 (8.78%) samples were diagnosed as *Streptococcus pneumoniae* by phenotypic methods [30]; thus, the phenotypic results of the mentioned study is consistent with those of the current study. To identify *streptococcus pneumoniae* from the sputum of some healthy children in Finland, Saukkoriipi et al. (2004) used the *ply* gene because of the specificity in *Pneumococcal* genus of this gene. After examining 400 sputum collected, it became clear that 40% of the samples were positive by culturing and 69% were positive by the PCR method [31]. In a study carried out by Abdeldaim et al. in Switzerland, 92 plasma samples were collected from the patients with acute respiratory infection and 91 plasma samples from healthy people as controls and all were analyzed by *lytA*, *ply*, and *spn* genes. Among the samples, 8 (9%), 11 (12%), and 19 (21%) were reported as positive by *lytA*, *spn*, and *ply* genes, respectively. Furthermore, among the control samples, 8 subjects (9%) were reported positive by *ply* gene [32].

In the current study, out of 93 samples collected from the patients, 19 samples (20.43%) were positive for *Streptococcus pneumoniae*. The difference of the present results with the findings of earlier studies may be due to the use of antibiotic

prior sampling. Moreover, some other factors such as different geographic regions or human errors could affect the final result.

CONCLUSION:

The results of this study in identifying the *Streptococcus pneumoniae* isolates from samples collected from Ahvaz showed that the PCR of *ply* gene was more reliable for identifying *Streptococcus pneumoniae* because the use of antibiotics causing false-negative culture results would be largely eliminated.

Besides, the findings of the study revealed that the identification of *Streptococcus pneumoniae* should be based on phenotypic tests and at least a molecular method such as PCR.

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