Short Communication

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Molecular Identification and Detection of Streptococcus Pneumoniae Serotypes Isolated from Selected Hospitals in Tehran Using Multiplex PCR Method

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ABSTRACT

BACKGROUND AND OBJECTIVE: *Streptococcus pneumoniae* is one of the major pathogens responsible for invasive diseases such as pneumonia and meningitis. Epidemiological studies of these microorganisms are necessary to evaluate the effect of pneumococcal vaccine in any community. Therefore, the present study was conducted for molecular identification and detection of *Streptococcus pneumoniae* serotypes isolated from selected hospitals in Tehran using multiplex PCR method.

METHODS: This cross-sectional study was performed on 32 isolates of *Streptococcus pneumoniae* from clinical specimens of patients admitted to different hospitals in Tehran. The isolates were identified by phenotypic tests and PCR method. Multiplex PCR was used to determine the serotype.

FINDINGS: The number and percentage of *Streptococcus pneumoniae* isolates isolated from cerebrospinal fluid, sputum, blood, bronchoalveolar lavage, eyes, and nasal discharge were 12 (37.6%), 7 (21.8%), 6 (18.7%), 3 (9.5%), 2 (6.2%), and 2 (6.2%) isolates, respectively. In the present study, the identified serotypes were the serotypes 1, 4, 6A/B, 7F, 9V, 11A, 14, 15A, 19A, 19F, and 23F.

CONCLUSION: Based on the results of this study, more than 50% of the serotypes were not among the serotypes present in the vaccines that are commonly used in the community.

KEY WORDS: *Streptococcus pneumoniae, Serogroup, Multiplex Polymerase Chain Reaction, Iran.*

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Introduction

Streptococcus pneumoniae (pneumococcus) bacterium can be colonized asymptomatically in the human nasopharynx, but this pathogen is one of the most important causes of invasive diseases such as pneumonia, septicemia, and meningitis (1, 2). Every year, this bacterium leads to the death of many people, which is estimated to be than 1.6 million, and is more commonly seen in children and the elderly (3). This bacterium is among gram – positive bacteria and has more than 90 serotypes. The most important and major factor of pneumococcal virulence is its polysaccharide capsule, which is the basis for its serotyping (4). By simultaneous comparison of isolates that causes invasive and noninvasive disease, it has been observed that pneumococcal pathogenesis is dependent on the type of capsular serotype. Among all serotypes, fifteen types are the most common serotypes that cause invasive disease in the host in most parts of the world (5, 6). Today, most vaccines against this bacterium are made from polysaccharide capsule. The first polyvalent vaccine, a seven-valent conjugate vaccine (PCV7), was licensed in 2000 (1).

Serotypes in PCV7 include serotypes 4, 23F, 19F, 18C, 14, 9V, and 6B. However, in order to increase the efficacy of the vaccine, 10 – and 13 – Valent Pneumococcal Conjugate Vaccines are currently used; in addition to the aforementioned serotypes, these vaccines include serotypes 3, 1, 5, 6A, 7F, 19A (7). The World Health Organization has recommended the use of polyvalent vaccine against pneumococcus, since it includes the common serotypes (8).

In developed European countries, this vaccine is designed and manufactured based on the serotype circulating in the community. To measure the efficacy of the vaccine and to evaluate the epidemiology of locally circulating serotypes, it is necessary to carry out epidemiologic studies regularly in each region at different times (9, 10). Several studies have shown that the serotypes that cause invasive diseases are highly diverse in different geographic regions. Even this diversity may be different in a geographic area at different times. The variety and diversity of serotypes in the use of vaccines can be of great importance, since the efficacy of each vaccine directly depends on the type of locally circulating serotype (11). In Iran, this vaccine has been imported from abroad and is injected into high-risk groups, but one of the problems is that it is used in the community without conducting a study to show the serotypes. This leads to a reduction and sometimes ineffectiveness of the injected vaccine and leads to the development of dangerous diseases caused by this bacterium (such as meningitis and septicemia). In Iran, few studies have been conducted to determine the frequency of pneumococcal serotypes; there are only six recorded studies that were carried out from 1996 to 2018 (10, 12–16). Considering the importance of the serotypes of this bacterium, the need for such epidemiological studies in different regions and times is necessary in Iran. Therefore, the present study was conducted for molecular identification and detection of *Streptococcus pneumoniae* serotypes isolated from selected hospitals in Tehran using multiplex PCR method.

Methods

Bacterial identification: In this cross-sectional study, 32 pneumococcal isolates were collected from Milad, Mofid, Imam Sajjad and Sina Hospitals in Tehran during the years 2016 – 2017. The isolates were obtained from various samples including cerebrospinal fluid (CSF), sputum, blood, bronchoalveolar lavage (BAL), eyes, and nasal discharge. In the following step, isolates cultured on 5% sheep blood agar plate that were phenotypically similar to *Streptococcus pneumoniae* were identified by gram staining, catalase test, bile solubility test, and Optochin sensitivity test. As a qualitative control, the standard *Streptococcus pneumoniae* strain ATCC 49619 was used. For storage, isolates were stored in a STGG medium (Merck, Germany) at a temperature of - 70 °C.

DNA extraction: Bacterial isolates were dissolved in 250 µl phosphate buffered saline (PBS) and DNA was extracted according to the instructions of Roche kit (Germany). The extracted DNA was evaluated using nanodrop and electrophoresis in 1% gel.

PCR: In this study, PCR method was used to confirm the isolates of *Streptococcus pneumoniae*. In the present study, capsular polysaccharide biosynthesis protein *CpsA* was used to identify *Streptococcus pneumoniae* (17). In this study, the HotStarTaq Master Mix Kit (SinaClone, Iran) was used to perform the PCR reaction.

Multiplex PCR serotyping: Multiplex PCR was used to determine the serotype in pneumococcal isolates. Twenty – five primer pairs were used for this purpose (Table 1). Seven Multiplex PCR reactions were performed (Table 2). The primers used in this study were grouped and selected based on the frequency of serotypes that are the cause of invasive diseases as well as the strains found in vaccines (7 –, 10 – and 13 –

Valent Vaccines). After performing Multiplex PCR, the products were electrophoresed in 2% gel.

Statistical analysis: Data were analyzed using SPSS 19, Wilcoxon, and Chi-square tests and $p < 0.05$ was considered significant.

Results

Pneumococcal isolates collected from different specimens included: 12 isolates (37.6%) of CSF, 7 isolates (21.8%) of sputum, 6 isolates (18.7%) blood, 3 isolates (9.5%) of BAL, 2 isolates (6.2%) of the eyes, and 2 isolates (6.2%) of the nasal discharge. 19 (59.4%) patients were male and 13 (40.6%) patients were female, while 22 (69%) patients were children. In this study, twenty–five common serotypes were studied. The identified serotypes were 1, 4, 6A/B, 7F, 9V, 11A, 14, 15A, 19A, 19F, and 23F. The pneumococcal isolates whose serotype was identified were collected from clinical specimens of CSF (6 isolates), blood (3 isolates), sputum (2 isolates), BAL (1 isolate) and nasal discharge (1 isolate).

The most frequent serotype was 6A/B, and this serotype was more isolated from adults. Based on the results of this study, of 32 evaluated pneumococcal isolates, 19 isolates (59%) were not among any of the evaluated 25 serotypes. In other words, these isolates were non-typeable in this study. Chi-square and Wilcoxon tests did not show significant correlation between gender (p=0.26), type of clinical specimen (p=0.38), age (p=0.56) of patients, and the used serotype.

Table 1. Sequencing of primers for serotyping of pneumococcal isolates using Multiplex PCR (10)

Name of primer	Primer sequence 5′ to 3′	bands size range (bp)
1-F	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	280
1-R	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C	
4-F	CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G	430
4-R	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G	
3-F	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G	371
3-R	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G	
5-F	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG	362
5-R	GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG	
6A/B-F	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	250
6A/B-R	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	
7F-F 7F-R	CCT ACG GGA GGA TAT AAA ATT ATT TTT GAG CAA ATA CAC CAC TAT AGG CTG TTG AGA CTA AC	826
7C-F	CTA TCT CAG TCA TCT ATT GTT AAA GTT TAC GAC GGG A	260
7C-R	GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC	
8-F	GAT GCC ATG AAT CAA GCA GTG GCT ATA AAT C	294
8-R	ATC CTC GTG TAT AAT TTC AGG TAT GCC ACC	
9V-F	CTT CGT TAG TTA AAA TTC TAA ATT TTT CTA AG	753
9V-R	GTC CCA ATA CCA GTC CTT GCA ACA CAA G	
10A-F	GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC	628
10A -R	GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C	
11A-F	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	463
11A -R	GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC	
12F-F	GCA ACA AAC GGC GTG AAA GTA GTT G	376
12F -R	CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC	
14-F	CTT GGC GCA GGT GTC AGA ATT CCC TCT AC	208
14-R	GCC AAA ATA CTG ACA AAG CTA GAA TAT AGC C	
15B-F	ATT AGT ACA GCT GCT GGA ATA TCT CTT C	436
15B-R	GAT CTA GTG AAC GTA CTA TTC CAA AC	
16F-F	TTG GAA TTT TTT AAT TAG TGG CTT ACC TA	988
16F-R	CAT CCG CTT ATT AAT TGA AGT AAT CTG AAC C	
17F-F	TTC GTG ATG ATA ATT CCA ATG ATC AAA CAA GAG	693
17F-R	GAT GTA ACA AAT TTG TAG CGA CTA AGG TCT GC	

Name of primer	Primer sequence 5′ to 3′	bands size range (bp)
18C-F	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC	573
18C-R	TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC	
19A-F	GTT AGT CCT GTT TTA GAT TTA TTT GGT GAT GT	478
19A-R	GAG CAG TCA ATA AGA TGA GAC GAT AGT TAG	
19F-F	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	304
19F-R	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG	
20-F	GAG CAA GAG TTT TTC ACC TGA CAG CGA GAA G	514
20-R	CTA AAT TCC TGT AAT TTA GCT AAA ACT CTT ATC	
23F-F	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	384
23F-R	CAC AAC ACC TAA CAC ACG ATG GCT ATA TGA TTC	
31-F	GGA AGT TTT CAA GGA TAT GAT AGT GGT GGTGC	701
31-R	CCG AAT AAT ATA TTC AAT ATA TTC CTA CTC	
34-F	GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC	408
34-R	CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC	
35B-F	GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG	677
35B-R	CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G	
35F-F	GAA CAT AGT CGC TAT TGT ATT TTA TTT AAA GCA A	517
35F-R	GAC TAG GAG CAT TAT TCC TAG AGC GAG TAA ACC	

Table 2. General scheme of the grouping of primers based on Multiplex PCR method

Reaction	Volume of primer (µl)	Primers	Bands size range (bp)	Primer Melting Temperature
1	2	19A	478	62 °C
	2	19F	304	
	2	6A/B	250	
	2	1	280	
	2	*cps*	160	
2	2	5	362	63 °C
	2	14	208	
	2	7F	826	
	2	9V	753	
3	3	F23	384	63 °C
	4	7F	826	
	2	11A	463	
	2	1	280	
	2	*cps*	160	
4	4	16F	988	62 °C
	2.5	18C	573	
	2	35B	677	
	2	12F	376	
5	3	8	294	61 °C
	3	3	371	
	3	15B	496	
	4	31	701	
6	3	1	280	60 °C
	3	10A	628	
	3	35F	517	
	4	34	408	
7	2	20	514	63 °C
	2	7C	260	
	2	17F	693	
	2	44	430	

Discussion

Based on the obtained results, of 32 evaluated pneumococcal isolates, 19 isolates (59%) were not among the strains in 7 –, 10 – and 13 – Valent Vaccines. In other words, these isolates were non-typeable strains. In this study, the prevalence of the most common serotypes in the world has been investigated; the ones that are also present in 7 –, 10 – and 13 – Valent Vaccines. Determination of the serotypes of *Streptococcus pneumoniae* is critical for proper immunization in people at risk and for the development of vaccine (18).

In a study by Habibian et al. in Tehran, the highest prevalence of serotypes was related to serotypes 14, 3, 23F, 19F, 19A, 6A, 9V, and 6B, respectively (19). Comparing the results of this study and the study present, the prevalence of serotypes 6A, 9V, 14, 19F and 23F was similar. In the study of Mousavi et al. in Tehran, the most frequent serotypes were 23F, 3, 6, 19A among the studied serotypes (20). In another study in Tehran conducted by Houri et al., the frequency of serotypes was 23F, 19F, 19A and 9V (10); comparing the results of these two studies with our study showed that the prevalence of serotypes 6A, 9V, 19A, 19V and 23F are the same.

The similarity in the prevalence of serotypes is due to the fact that the location of the study is similar. Another similarity between these two studies and our research is serotyping method, which is the Multiplex PCR method, but the difference in the prevalence of other serotypes is due to the difference in the time of the study. Finding such differences confirms the need for consistent follow-up for serotypes.

Except for the two mentioned studies, no similar study was found that evaluated pneumococcal serotypes in clinical specimens and therefore more studies should be conducted to detect pneumococcal serotypes in clinical isolates. In a study in Turkey, the most common serotypes were 19F, 6B, 23F, and 18C (21). Comparison of these studies shows that three common serotypes in Turkey are also present in Iran. However, 18C serotype was not observed in our study. In a study conducted in Saudi Arabia, the highest frequency was related to strains 4, 3, 19F, 9V, 19A and 14 (22). The prevalence of pneumococcal serotypes in Saudi Arabia is very similar to our study. The possible reason for this is because of the pilgrimage of Iranians to the city of Mecca, and when the carriers of bacteria return to Iran, they spread the strains in the community. In Pakistan, the highest frequency of serotypes was reported for 18, 14 and 19F (23). There are fewer similar serotypes compared to our study, which may confirm the theory that the travel of Iranian people to neighboring countries or vice versa has a major role in the diffusion of different strains of pneumococcus in Iran. One of the significant findings in this study was the isolation of serotypes 1, 4, 7F and 15A. According to evaluations in other studies, these serotypes have rarely been reported in Iran (21).

One of the reasons for the differences in the results of the studies may be due to the fact that these studies were conducted at different points in time. The second reason for difference in the results is due to the number of the studied samples, since the sample size was different in each of the studies. Among the limitations of this study was small number of pneumococcal isolates due to the slow-growing bacterium. Based on the results of this study, it should be noted that more than half of the pneumococcal isolates were not part of the serotypes in vaccines used for immunization of children in Iran. For matching different studies in Iran, it is suggested that similar sample size be used by different studies so that we can make a reasonable comparison based on the obtained results.

Considering the importance of *Streptococcus pneumoniae* in causing diseases with high mortality, such as septicemia and meningitis, and also the correlation between the serotype and the type of complication that leads to it (24, 25), it is necessary to evaluate the prevalence of serotypes of this bacterium in Iran. Then, vaccination should be done in people at risk, such as children and the elderly. In Iran, like other countries of the world, the supply and administration of polyvalent pneumococcal vaccines should be based on the type of circulating serotype, in which case the immunization of individuals will be fully done. Because of the limited information on the prevalence of pneumococcal serotypes in Iran, it is suggested that the prevalence of pneumococcal serotypes be tracked in all regions of the country for several consecutive years and a suitable polyvalent pneumococcal vaccine be introduced and used based on the obtained results.

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