Arch Pediatr Infect Dis. 2020 January; 8(1):e14100. doi: 10.5812/pedinfect.14100.

Published online 2020 January 20. **Research Article**

Relationship Between Prevalence of Pneumococcal Serotypes and Their Neuraminidases in Carriers, Predictive Facts?

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Received 2017 May 27; **Revised** 2019 December 11; **Accepted** 2019 December 23.

Abstract

Streptococcus pneumoniae is one of the leading causes of morbidity and mortality worldwide that mainly colonizes the upper respiratory tract in children and produces invasive infections such as meningitis, bacteremia, pneumonia, and otitis media. *Pneumococcus* species harbor a number of virulent genes that influence disease severity. Neuraminidase is a virulence factor that cleaves sialic acid. Pneumococcal nasopharyngeal carriage is common among healthy individuals and is considered an important transmission source. This topic, however, is less regarded in research from Iran. Therefore, the current study aimed at investigating the prevalence of pneumococcal nasopharyngeal carriage among Iranian children under six years of age. The study also aimed at determining the frequency of pneumococcal serotypes and the neuraminidase virulence genes (*nanA, nanB*, and *nanC*) among carriers. A total of 384 nasopharyngeal samples were collected from children under six years of age referred to healthcare centers of six provinces in Iran, including Tehran, Fars, Ardebil, Lorestan, Khorasan, and Sistan va Baluchistan from 2013 to 2016. Of these, 92 carriers were identified (24.0%; 95% confidence interval [CI]: 20.0, 28.5%). Identified pneumococcal serotypes included 6A/B (14.1%), 14, 3, and 19A (each 12.1%), 4, 19F, 23F (each 5.4%), 11 (4.3%), 18C, 22F, 9V, 19, 12, and 33F (each 2.1%), 5, 12F (each 1.1%) and non-typable (14.1%). Frequency of neuraminidase *nanA, nanB*, and *nanC* genes among 92 carriers was 100% (95% CI: 96.0, 100%), 85.9% (95% CI: 77.3, 91.6), and 62.0% (95% CI: 51.8, 71.2), respectively. The results showed that about 25% of children (n = 92) were pneumococcus carriers. A wide range of serotypes, some of which are known as virulent, were seen in the population. Prevalence of neuraminidase genes was also considerable.

Keywords: *Streptococcus pneumoniae*, Serotyping, Pharyngeal Carrier, Colonization, Polymerase Chain Reaction, Neuraminidase

1. Background

Streptococcus pneumoniae is a human bacterial pathogen considered as one of the main causes of community-acquired pneumonia, meningitis, and bacteremia and is a common cause of mortality worldwide. *S. pneumoniae* can colonize the upper respiratory tract including nasopharynx. The most common serotypes of *S. Pneumoniae* are 19F, 6B, 4, 14, 19A, 3, 23F, and 1. Bacterial attachment to host epithelial cells is required for the development of pneumococcal infections (1-4). Pneumococcus has several virulence factors which contribute to the colonization of the host mucosal surfaces (5). Neuraminidase is a virulence factor that cleaves sialic acid into glycoproteins, glycolipids, and oligosaccharides and contributes to pneumococcal pathogenesis in two ways: (1) decrease of the integrity of mucosa and facilitation of bacterial dissemination into lower layers; (2) facilitation of the attachment of surface receptors to host cells and bacterial colonization.

Two types of neuraminidase are encoded by *nanA* and *nanB* genes. Also, there is a homologous gene named *nanC*. The structural analysis of pneumococcal neuraminidases revealed that 50% of NanB and NanC sequences are similar, but 25% of their sequences are similar with that of NanA (6, 7). NanA is the primary virulence factor for the elimination of sialic acid in three types of neuraminidases. It also removes sialic acid from compounds such as lactoferrin and IgA2 protease (8).

NanA also targets the host glycogen molecules and mediates biofilm formation by pneumococci. *nanA* is upregulated during biofilm development (9). NanB cleaves sialic acid and provides carbon and energy sources for pneumococci growth. NanC function has remained unclear, but it

is essential for invasive infections such as meningitis and hemolytic uremic syndrome (10-12). Since the available vaccines are expensive, serotype-dependent, and their immunogenicity is limited to serotypes included in the vaccine, scientists' endeavors are concentrated on the development of a serotype-independent vaccine.

For this purpose, neuraminidases are studied in some countries, because this molecule has immunogenicity and is preserved in all strains (13). Serotyping of native and prevalent pneumococci in Iran, frequency of neuraminidases in these serotypes, and comparison of the results with those of other studies from other countries are necessary for obtaining more conclusive results for further investigations.

2. Objectives

The current study aimed at investigating the prevalence of pneumococcal nasopharyngeal carriage among unvaccinated children under six years of age in six provinces of Iran. The study also aimed at evaluating the frequency of pneumococcus serotypes and the neuraminidase virulence genes (*nanA, nanB*, and *nanC*) among the identified carriers.

3. Methods

The current cross-sectional study was conducted on 384 nasopharyngeal samples collected by sterile Dacron swabs from children under six years old referred to healthcare centers of six Iranian provinces including Tehran, Fars, Ardebil, Lorestan, Khorasan, and Sistan va Baluchistan from September 2013 to December 2016. The samples were then kept in a combined preserving medium (composed of beef extract 0.4 g, agar 1.2 g, casein 1.0 g, and NaCl 0.3 gin 100 mL double distilled water [BBL Co.]). The samples were sent to the Department of Bacteriology at Pasteur Institute of Iran for isolation and diagnosis. The participants were assigned to the three equal age groups of 2 - 24, 25 - 48, and 49 - 72 months. The nasopharyngeal swabs were streaked onto 5% sheep blood chocolate agar (Quelab Cat. No. 1601a) and then incubated at 37°C in 5% CO_2. The suspected pneumococcal colonies were subcultured onto chocolate agar.

3.1. Identification of Pneumococci

Pneumococci were identified based on the results of culture test and colony morphology, Gram staining, and biochemical tests including catalase, bile solubility and susceptibility of bacteria to optochin disks on chocolate agar plates (Mast diagnostics Cat. No. D42). Growth inhibition zones $\geq$ 14 mm in diameter around the optochin disks were considered as pneumococci. The primary detection of pneumococci was based on the appearance of the green zone of alpha-hemolysis surrounding colonies on chocolate agar, observation of lancet-shaped diplococci in Gram-stained smears and negative results in catalase test (14, 15).

The clearance of turbidity in tubes containing 2-McFarland bacterial suspension and sodium deoxycholate (bile salt) after 15 minutes was considered as pneumococci. The confirmatory method for the detection of pneumococci was based on molecular methods (16-19).

First, pneumococcal DNA was extracted and purified by Qiagen extraction kit (Cat No. 69525; Germany) and then *cpsA* gene was detected by PCR as the key test for confirmation. Specific primers for *cpsA* detection were provided as described in Table 1.

3.2. PCR

The presence of neuraminidase genes in the isolated pneumococci was determined by the detection of *cpsA* gene using PCR. Using the primers provided for the detection of most prevalent serotypes of pneumococci (Table 2), all the confirmed isolates were then serotyped based on multiplex PCR (20).

The multiplex PCR results were confirmed in the current study using standard strains including ATCC 6305, ATCC 6301, ATCC 49619, ATCC 49136, and ATCC 700677. Multiplex PCR was performed in a total volume of 25 μL as the reaction mixture containing 0.5 μL of dNTPs (10 mM), 0.5 μL of each primer (10 pmol), 2.5 μL of PCR buffer (10$\times$), 1.5 μL of $MgCl_2$ (25 mM), and 0.2 μL of Taq DNA polymerase (5U/μL) (Fermentas/thermofischer USA. Cat. No. EP0402). The PCR cycle was run at 94°C for four minutes followed by 30 amplification cycles at 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for two minutes. PCR products were electrophoresed on 1% agarose gel at 100 V for one hour; then, they were stained with ethidium bromide and visualized under a UV transilluminator. The primers used for the detection of the three neuraminidase genes in the current study are shown in Table 3.

S. pneumoniae ATCC49610 was used as positive control in PCR. Polymerase chain reaction in the present study was performed in a total volume of 25 μL with 1X PCR buffer in 2.5 μL, 0.2 mM of dNTPs, 1 U of Taq DNA polymerase, 1.5 mM of $MgCl_2$, 2.5 μL of each primer, and 1μL of DNA template. Thermocycling was performed in Eppendorf Gradient Mastercycler apparatus with the following steps: 98°C for 30 seconds (denaturation), 64°C for 30 seconds (annealing) for *nanA* and 55°C for 30 seconds for *nanB* and *nanC*, 72°C for 60 seconds (extension) and final extension at 72°C for

Table 1. Polymerase Chain Reaction Primers for the Determination of the Main Pneumococcal Serotypes

Serotype	Primers (5' - 3')	Reference
1	F: CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	(20)
	R: CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C	
3	F: ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G	(20)
	R: CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G	
4	F: CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G	(20)
	R: GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G	
5	F: ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG	(20)
	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	(20)
	R: TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	
7F	F: CCT ACG GGA GGA TAT AAA ATT ATT TTT GAG	(20)
	R: CAA ATA CAC CAC TAT AGG CTG TTG AGA CTA AC	
9V	F: CTT CGT TAG TTA AAA TTC TAA ATT TTT CTA AG	(20)
	R: GTC CCA ATA CCA GTC CTT GCA ACA CAA G	
10	F: GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC	(20)
	R: GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C	
11	F: GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	(20)
	R: GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC	
12F	F: GCA ACA AAC GGC GTG AAA GTA GTT G	(20)
	R: CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC	
14	F: CTT GGC GCA GGT GTC AGA ATT CCC TCT AC	(20)
	R: GCC AAA ATA CTG ACA AAG CTA GAA TAT AGC C	
15	F: ATT AGT ACA GCT GCT GGA ATA TCT CTT C	(20)
	R: GAT CTA GTG AAC GTA CTA TTC CAA AC	
16	F: CTG TTC AGA TAG GCC ATT TAC AGC TTT AAA TC	(20)
	R: CAT TCC TTT TGT ATA TAG TGC TAG TTC ATC C	
18C	F: CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC	(20)
	R: TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC	
19F	F: GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	(20)
	R: GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG	
19A	F: GTT AGT CCT GTT TTA GAT TTA TTT GGT GAT GT	(20)
	R: GAG CAG TCA ATA AGA TGA GAC GAT AGT TAG	
22F	F: GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC	(20)
	R: CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC	
23F	F: GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	(20)
	R: CAC AAC ACC TAA CAC ACG ATG GCT ATA TGA TTC	
33F	F: GAA GGC AAT CAA TGT GAT TGT GTC GCG	(20)
	R: CTT CAA AAT GAA GAT TAT AGT ACC CTT CTA C	
35B	F: GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG	(20)
	R: CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G	

Table 2. Polymerase Chain Reaction Primers for the Detection of *cpsA* and Neuraminidase Genes

Genes	Primers	Size of the Gene (bp)	Reference
cpsA	F: GCA GTA CAG CAG TTT GTT GGA CTG ACC	150	(20)
	R: GAA TAT TTT CAT TAT CAG TCC CAG TC		
nanA	F: ATAGACGTGCGCAAAAT-ACAGAATCA	548	(10)
	R: GTCGAACTCCAAGC-CAATAACTCCT		
nanB	F: ACTAGGAGCTGT-TAATCGTGAAGG	492	(10)
	R: CCAATACC-CGCAGGCATAACATC		
nanC	F: TGGGGTAAGTACAAA-CAAGAA	523	(10)
	R: CTAATGGTACTGGCG-CAAAATCA		

five minutes. Polymerase chain reaction product was electrophoresed on 1% agarose gel at 100 V for one hour. The final PCR products were stained with ethidium bromide and then illustrated by UV light. The DNA bands were analyzed by comparison of the visualized bands with a 100-bp ladder.

To confirm the results of PCR for *nanA, nanB*, and *nanC* genes in the current study, two positive PCR products were randomly selected and sequenced with direct Sanger sequencing (Biomatik Co. Canada).

3.3. Statistical Analysis

Data were collected, revised and analyzed by SPSS software (version 16). Frequencies of identified carriers, pneumococcus serotypes, and neuraminidase genes were expressed as number and percentage. The Wilson score interval was used to estimate 95% confidence interval (CI) for the key outcomes (frequencies). Difference in the frequency of pneumococcal nasopharyngeal carriage among six provinces of Iran was determined by the chi-square (χ^2) test. P-value less than 0.05 was considered significant.

4. Results

Of the 384 collected swabs, 119 (31%) and 92 (24%) samples were confirmed as pneumococci by phenotypic and genotypic (detection of *cpsA* gene) assays, respectively (Figure 1). Of the 92 genetically-confirmed pneumococci, the serotype of 79 (86%) isolates was determined using PCR. The most frequent serotype in the studied samples was 6A/B with the frequency of 14.1% (95% CI: 8.5, 22.7%). The least frequent serotypes were 12F and 5, with the frequency of 1.1% each (95% CI: 0.2, 5.9%). The frequency of the identified pneumococcal serotypes in different provinces and overall is presented in Tables 3 and 4, respectively (21).

The presence of neuraminidase genes was determined in the 92 genetically-confirmed isolates (Figure 2). All the confirmed isolates harbored the *nanA* gene, but *nanB* and *nanC* were only detected in 85.9% and 62.0% of the isolates, respectively. Also, 87% of the samples collectively harbored *nanA* and *nanB*, 62% concomitantly carried *nanA* and *nanC*,

Table 3. Frequency of Positive Pneumococcus Serotypes in the Six Provinces of Iran

Serotype	Provinces											
	Tehran (N = 260)		Lorestan (N = 260)		Ardabil (N = 260)		Khorasan (N = 260)		Fars (N = 260)		Sistan and Baluchestan (N= 260)	
	No. (%)	95%CI	No. (%)	95%CI	No. (%)	95%CI	No. (%)	95%CI	No. (%)	95%CI	No. (%)	95%CI
6A/B	5 (1.9)	0.6 - 4	3 (1.2)	0.2 - 4	2 (0.8)	0 - 3	1 (0.4)	0 - 2	2 (0.8)	0 - 3	0 (0.0)	0 - 2
14	5 (1.9)	0.6 - 4	0 (0.0)	0 - 2	2 (0.8)	0 - 3	2 (0.8)	0 - 3	2 (0.8)	0 - 3	0 (0.0)	0 - 2
3	6 (2.3)	0.9 - 5	1 (0.4)	0 - 2	1 (0.4)	0 - 2	0 (0.0)	0 - 2	3 (1.2)	0.2 - 4	0 (0.0)	0 - 2
19A	4 (1.5)	0.4 - 4	4 (1.5)	0.4 - 4	1 (0.4)	0 - 2	1 (0.4)	0 - 2	1 (0.4)	0 - 2	0 (0.0)	0 - 2
4	4 (1.5)	0.4 - 4	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	1 (0.4)	0 - 2
19F	3 (1.2)	0.2 - 4	0 (0.0)	0 - 2	0 (0.0)	0 - 2	1 (0.4)	0 - 2	1 (0.4)	0 - 2	0 (0.0)	0 - 2
23F	2 (0.8)	0 - 3	0 (0.0)	0 - 2	0 (0.0)	0 - 2	1 (0.4)	0 - 2	0 (0.0)	0 - 2	2 (0.8)	0 - 3
11	0 (0.0)	0 - 2	0 (0.0)	0 - 2	1 (0.4)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	2 (0.8)	0 - 3
18C	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	1 (0.4)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2
22F	2 (0.8)	0 - 3	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2
9V	1 (0.4)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	1 (0.4)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2
19	2 (0.8)	0 - 3	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2
12	1 (0.4)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	1 (0.4)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2
33F	1 (0.4)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	1 (0.4)	0 - 2
5	0 (0.0)	0 - 2	0 (0.0)	0 - 2	1 (0.4)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2
12F	1 (0.4)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2	0 (0.0)	0 - 2
Non-typable	6 (2.3)	0.9 - 5	2 (0.8)	0 - 3	1 (0.4)	0 - 2	2 (0.8)	0 - 3	0 (0.0)	0 - 2	2 (0.8)	0 - 3
Total	43 (2.8)	2 - 3.7	10 (0.6)	0.3 - 1.2	9 (0.6)	0.3 - 1.1	11 (0.7)	0.4 - 1.3	9 (0.6)	0.3 - 1.1	8 (0.5)	0.2 - 1.0

Abbreviations: C, central; E, east; N, north; S, south; W, west

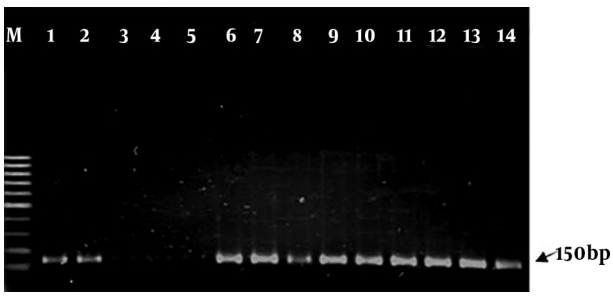

Figure 1. Gel electrophoresis for *cpsA* gene as confirmatory test of Pneumococci isolates. M. indicates the 100 bp. ladder marker, lane 1: Standard strain of *Pneumococcus* for *cpsA*. (ATCC49610). The lanes 2 - 14 represent suspect alpha-hemolytic streptococci that lanes 6 - 14 were detected as Pneumococci because these lanes indicate the *cpsA* and the lanes 3 - 5 removed from our study because they were not detected as Pneumococci.

and 55% harbored *nanB*, *nanC*, and *nanA* genes. Furthermore, 13% and 38% of all isolated strains did not carry *nanB* and *nanC* genes, respectively. Table 4 illustrates detailed data on the frequency of different serotypes.

5. Discussion

Pneumococcal infections are increasing after the emergence of non-vaccine serotypes, capsule switching, and replacement of new serotypes among different communities (22). There are two types of vaccines (i.e., conjugate and polysaccharide) to prevent pneumococcal infections with each covering partial and limited groups of serotypes (23-25). Different serotypes have distinct geographical distributions. Pneumococci express several types of antigens and virulence factors that help them cause infections in humans. One of these antigens is neuraminidase enzyme (Nan) that hydrolyses the neuraminic acids on human cell surfaces. Pneumococci express three variants of neuraminidase. Several pneumococcal proteins and antigens are selected as vaccine candidates including neuraminidases.

The presence of Nan variants, NanA, NanB and NanC, varies among serotypes. Determination of the frequency of Nan variants in native serotypes is important for vaccine studies; thus, the prevalence of different serotypes and nan genes was investigated in the current research. Four

Table 4. Frequency of Pneumococcal Serotypes and Neuraminidases Genes in a Sample of Iranian Children Under Six Years of Age (N = 92[a])

Serotypes	Frequency of Serotypes[a]		Frequency of Neuraminidases Genes[b]					
	No. (%)	95% CI[c]	*nanA*		*nanB*		*nanC*	
			No. (%)	95% CI[c]	No. (%)	95% CI[c]	No. (%)	95% CI[c]
6A/B	13 (14.1)	8.5, 22.7	13 (100)	77.2, 100	12 (92.3)	66.7, 98.6	9 (69.2)	42.4, 87.3
14	11 (12.1)	6.8, 20.2	11 (100)	74.1, 100	9 (81.9)	52.3, 94.9	7 (63.7)	35.4, 84.8
3	11 (12.1)	6.8, 20.2	11 (100)	74.1, 100	8 (72.8)	43.4, 90.3	7 (63.7)	35.4, 84.8
19A	11 (12.1)	6.8, 20.2	11 (100)	74.1, 100	11 (100)	74.1, 100	9 (81.9)	52.3, 94.9
4	5 (5.4)	2.3, 12.1	5 (100)	56.6, 100	4 (80)	37.6, 96.4	4 (80)	37.6, 96.4
19F	5 (5.4)	2.3, 12.1	5 (100)	56.6, 100	3 (60)	23.1, 88.2	3 (60)	23.1, 88.2
23F	5 (5.4)	2.3, 12.1	5 (100)	56.6, 100	4 (80)	37.6, 96.4	1 (20)	36.2, 62.4
11	4 (4.3)	1.7, 10.7	4 (100)	51.0, 100	4 (100)	51.0, 100	1 (25)	4.6, 69.9
18C	2 (2.1)	0.6, 7.6	2 (100)	34.2, 100	2 (100)	34.2, 100	1 (50)	9.5, 90.5
22F	2 (2.1)	0.6, 7.6	2 (100)	34.2, 100	2 (100)	34.2, 100	2 (100)	34.2, 100
9V	2 (2.1)	0.6, 7.6	2 (100)	34.2, 100	2 (100)	34.2, 100	1 (50)	9.5, 90.5
19	2 (2.1)	0.6, 7.6	2 (100)	34.2, 100	1 (50)	9.5, 90.5	1 (50)	9.5, 90.5
12	2 (2.1)	0.6, 7.6	2 (100)	34.2, 100	2 (100)	34.2, 100	0 (0)	0, 65.8
33F	2 (2.1)	0.6, 7.6	2 (100)	34.2, 100	1 (50)	9.5, 90.5	1 (50)	9.5, 90.5
5	1 (1.1)	0.2, 5.9	1 (100)	20.7, 100	1 (100)	20.7, 100	1 (100)	20.7, 100
12F	1 (1.1)	0.2, 5.9	1 (100)	20.7, 100	1 (100)	20.7, 100	1 (100)	20.7, 100
Non serotypes	13 (14.1)	8.5, 22.7	13 (100)	77.2, 100	12 (92.4)	66.7, 98.6	8 (61.5)	35.5, 82.3
Total	92 (5.9)	4.8, 7.2	92 (100)	96.0, 100	79 (85.9)	77.3, 91.6	57 (62.0)	51.8, 71.2

[a]Of 384 swab samples, 92 were genetically confirmed as pneumococci. The numbers presented here show the frequency of each serotype in the sample of 92 pneumococci-positives
[b]Frequency *Neuraminidases* genes are presented for each serotype. The total frequency of each *Neuraminidases* gene among all the 92 samples is also calculated (presented in the 'Total' row of this table).
[c]Confidence intervals are calculated using Wilson Score interval method

serotypes (i.e., 19A, 6, 3, 23F) of S. pneumoniae accounted for 55.7% of all strains isolated from both nasal carriage and clinical samples in Tehran. Serotype 19A was the most frequent one in the two groups of samples (21, 26).

Vaccine serotypes used in conventional vaccines cover only the American serotypes and is ineffective for other geographical serotypes (27). Since the production of conjugated vaccines is expensive, there is a great limitation for the usage of all serotypes in these vaccines. They are produced in limited numbers from polysaccharides (28). To develop an effective vaccine and eliminate remarked conflicts, it is recommended to focus on protein-based extending vaccines as ideal alternatives for conventional vaccines. Thus, the virulence factors of pneumococcus, except the capsule, could be promising vaccine candidates.

As mentioned earlier, neuraminidases are pneumococcal proteins that play key roles in bacterial colonization in the early stages of infection and invasion to organs such as the central nervous system and the incidence of hemolytic uremic syndrome in infected patients (29-31). Thus, determination of the prevalence of neuraminidase genes in Iranian strains is of great importance. The prevalence of neuraminidase genes and its relationship with pneumococcal serotypes in native strains as the main objectives of the current study were determined by PCR.

Serotyping results of the current study were compared with those of other studies performed worldwide in order to explore differences and similarities; for example, Pai et al. (20) perfectly studied isolates of pneumococci by multiplex PCR, and the comparison of the results with those of the current study showed that pneumococcal serotypes were more prevalent in Iran. It can be concluded that the numbers of isolates, *nanA, nanB, nanC*, and serotypes according to the sampling site are comparable and the analysis of the results indicates that *nanA* and *nanB* distribution was not different among distinct serotypes, but results indicated that the presence of *nanC* was higher in invasive serotypes.

The investigation of pneumococcal virulence factors can provide more information as to its pathogenesis and

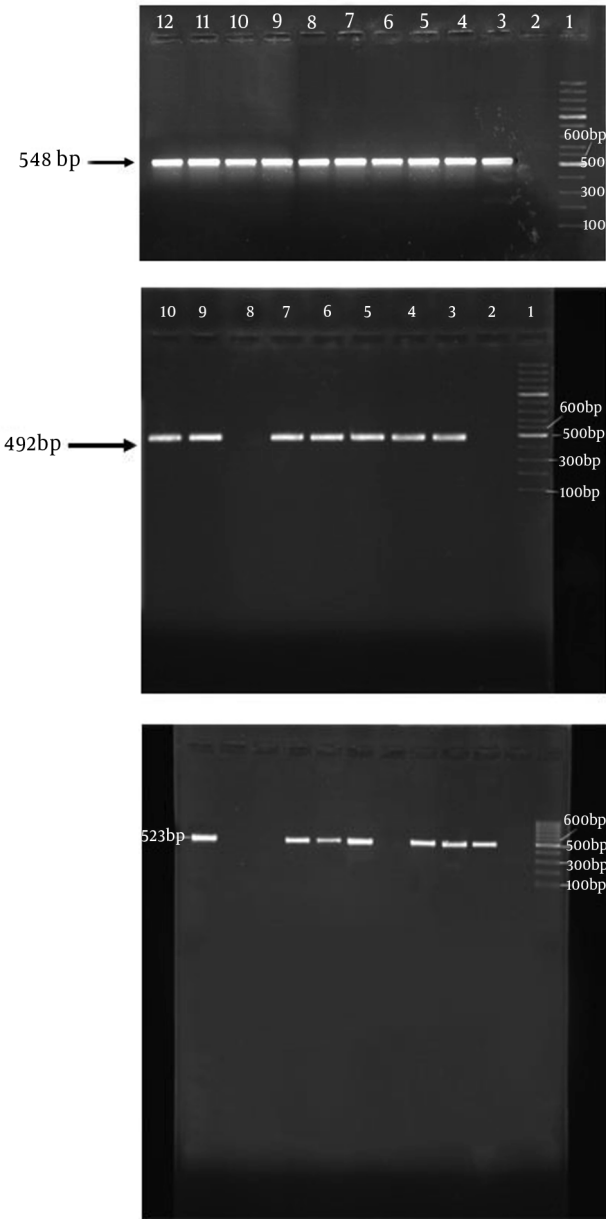

Figure 2. (A) Electerophoresis gel of *nanA* gene PCR. Lane 1: 100-bp ladder, lane 2: negative control, lane 3: positive control, lanes 4 - 12 represent test isolates. (*nanA* gene size: 548 bp). (B) Electerophoresis gel of *nanB* PCR. Lane 1: 100-bp ladder, lane 2: negative control, lane 3: positive control, lanes 4 - 10: represent test samples, lane 8: negative for *nanB*. (*nanB* gene size: 492 bp). (C) Electerophoresis gel of *nanC* PCR. Lane1: 100-bp ladder, lane 2: negative control, lane 3: positive control, lanes 6, 10 and 11 were negative samples.

lead to the development of more appropriate vaccine candidates for the prevention of pneumococcal diseases. Proper vaccines based on neuraminidase proteins (i.e., NanA, NanB and NanC) as pneumococcal virulence factors can result in more immunity, cost-saving and resolutions for some failures in developing pneumococcal vaccines. The prevalence of neuraminidase genes in clinical isolates and expression of these genes can also be evaluated for obtaining more information.

5.1. Conclusion

The analysis of the current study results indicated a correlation between pneumococcal serotypes and the prevalence of *nanA* and *nanB* genes; *nanA* gene was detected in 100%, *nanB* in 85.9%, and *nanC* in 62% of the isolated strains. Although the serotypes 4 and 12F were the most frequent ones in strains isolated from invasive infections, only one strain identified as serotype 12F harbored three neuraminidase genes. The prevalence rate of *nanC* gene in some serotypes found in the current study including 19A, 6A/B, 19F, and 22F was respectively 81.9%, 69.2%, 60%, and 100%. Some other serotypes such as 18C, 5, 12F, 19, and 19V were scarce, but could be the causes of pneumococcal invasive infections. Since a limited number of serotypes were identified in the current study, for certainty about lack of correlation between pneumococcal serotypes and neuraminidase genes, conducting further extensive studies is recommended.

Acknowledgments

Authors hereby acknowledge their gratitude to the study participants and their families, as well as the staff of the healthcare centers for their cooperation with the study.

Footnotes

Authors' Contribution: Fazlollah Mousavi developed the original idea and the protocol, and abstracted. Fatemeh Norolahi analyzed the data, Mohammadali Malekan wrote the manuscript, and is a guarantor. Seyed Davar Siadat, Alireza Janani, Hamid Mousavi and Fatemeh Norolahi contributed to the development of the protocol.

Conflict of Interests: Authors declared no conflict of interest.

Ethical Approval: The study protocol was approved by the Ethics Committee of Pasteur Institute of Iran.

Funding/Support: The work was approved and financially supported by the Research Committee of Pasteur Institute of Iran, Tehran, Iran.

Informed Consent: Informed consent was obtained from parents of the children who participated in the study.

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