



Multiplex PCR Assay for Detection of Pneumococcal Serotypes in Nasopharyngeal Samples of Healthy Children; Tehran, 2009-2010

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript

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ABSTRACT

Background: *Streptococcus pneumoniae* (*S. pneumoniae*), is a major pathogen causing invasive disease, colonizes the nasopharynx constituting a potential source of infection in both children and adults.

Aims: To identify the rate of pneumococcal nasopharyngeal colonization in healthy infants <2 years of age and to define the prevalent serotypes.

Materials and Methods: This cross-sectional study was performed for three months from June to August 2009 on healthy children, aged 6, 12 and 18 months visiting Health centers for routine vaccinations. Trained personnel collected nasopharyngeal samples through

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flexible nasal swabs and sent the specimens for diagnosis of pneumococci; after DNA extraction, microorganisms were serotyped by Multiplex PCR assay.

Results: One thousand two hundred ninety one samples were collected. *S. pneumoniae* was identified in 34%. A total of 761 identified *S. pneumoniae*, belonging to 30 different serotypes were recovered from 441 positive nasopharyngeal specimens. Serotypes 19, 6, 14, 19F, 17, 21, 20, 12F, 11 and three were most common, isolated in frequencies of 8.2, 7.6, 7.2, 7.2, 6.4, 5.3, 5.1, 4.8, 4.5 and 4.3% respectively. There was no significant difference in the diagnosed serotypes from the three age groups. Eleven serotypes (38.5%) are included in the 13-valent pneumococcal conjugate vaccine, (PCV 13). Colonization with more than one strain was seen in 228 samples, (52% of carriers).

Conclusion: Our findings revealed a significant rate of nasopharyngeal colonization with *S. pneumoniae* in young carriers; and draw attention to fact that only 38.5% of the isolated serotypes are included in PCV 13, demonstrating limited coverage of the current vaccine. There is a need to manufacture vaccines with an optimal formulation that would provide effective protection against serotypes prevalent in the community.

Keywords: Streptococcus pneumonia; serotypes; nasopharyngeal colonization; healthy infants.

1. INTRODUCTION

Streptococcus pneumoniae (*S. pneumonia*) has been recognized as a major pathogen causing invasive disease, and serious morbidity in children and adults throughout the world [1,2]. The World Health Organization reports that around 1 million children die each year from invasive pneumococcal disease, including bacteremia, sepsis and meningitis [1-3].

Pneumococcus is a part of the normal resident flora in the nasopharynx. It serves as a potential source of invasive pneumococcal disease (IPD), and a major factor for transmission of infection in the community [4-7]. Colonization of the nasopharynx occurs at a very young age; some authors have reported that >95% of infants may be colonized with more than five different serotypes by two years of age, with a single strain persisting for weeks to be replaced by another strain [8]. In addition, more than one strain may be present at one time in the nasopharynx of young children [8, 9]. Colonization with pneumococci is usually asymptomatic, only 15% of children becoming infected, usually within one month of getting colonized [6]. Although the mechanism that disrupts the balance among the host and the microorganism are not completely defined, certain risk factors have been identified, notably impairment of the immune system and extremes of age, viz. <2years and >65 Years [6]. Genetic similarities have been found between pneumococci carriers in the nasopharynx and in those causing invasive disease, and studies have shown that nasopharyngeal carriage indicate the strains distributed in the populace and may constitute the initial phase progressing to IPD [6,9-11]. Although according to variations in capsular polysaccharide structure, 93 pneumococcal serotypes, belonging to 46 serogroups have been identified, all serotypes do not cause invasive disease [12]. Serotypes causing IPD differ in different geographical locations and also with age; serotype 14, 6B, 19F and 23F being most common in Europe, and serotypes one and five are detected more frequently in children aged >2-5 years [3].

Besides widespread pneumococcal carriage, studies from the United State, South Africa and Europe report a significant prevalence of penicillin resistant pneumococci, with some strains

being resistant to multiple antibiotics [6]. Regional and age-related differences in serotype distribution and the emergence of multi-resistant strains make it imperative to determine the strains circulating in the community so that pneumococcal vaccines that are produced are maximally effective against the prevailing serotypes [10].

S. pneumococci diagnostic assays are based primarily on the detection of pneumococcal antigens, DNA, or RNA in blood or body fluids. Although blood culture is the most accurate method to diagnosis pneumococcal disease, is able to detect about 30% of positive specimens [8]. Since many patients with pneumococcal disease have received antibiotics for treatment, any available specimens would contain only nonviable organisms, thus the culture would be negative. Conventional serologic methods give lack uniformity in sensitivity and specificity and are time-consuming because they require reagents in which all serotypes are represented. Therefore, there is a need for a highly sensitive and highly species-specific method of detection that will makes diagnosis of pneumococcal disease less complicated [11,13,14].

In this study our objective was to identify the pneumococcal nasopharyngeal colonization rate in healthy infants <2 years of age by multiplex PCR assay and to define the prevalent serotypes in order to select the type of vaccine which would be effective for prevention of IPD in Iranian children.

2. MATERIALS AND METHODS

2.1 Study Design and Patients

This cross-sectional study was carried out for three months from June to August 2009 on healthy children, aged 6, 12 and 18 months visiting nine Health facility centers affiliated with the Tehran and Shahid Beheshti Universities of Medical sciences for routine vaccinations. The centers were selected through convenience sampling. Children with immunodeficiency, malignant or chronic diseases, (including chronic otitis media), were excluded. We also excluded children with acute respiratory tract infections or patients with consuming antibiotics during the two weeks before sample collection. None of the children had been vaccinated with pneumococcal vaccine.

2.2 Sampling

Flexible nasal swabs was used (Mini-Tip culture rette kits, Becton- Dickinson) to collect nasopharyngeal samples by the assistance of a medical doctor or senior nurse. The swabs were inserted 1-2 cm inside the nasopharynx and keeping for at least 5 seconds. Samples was collected from each patient in a 2ml fluid thioglycolate broth medium, placed on an ice bag and processed in the laboratory within 2h of collection. All specimens were examined just by the serotype specific PCR, since satisfactory reports [12,15].

2.3 DNA extraction and PCR Procedure

DNA was extracted from all specimens by *DNA Extraction Mini Kit (iNtRON, i-genomic CTB, cat.no.17341)*. PCR protocol was set up based on the Pai's report [15]. This protocol consists of seven multiplex PCR sets that each set contained of some serotype specific primer pairs (Table 1). Of the 29 primer pairs designed to target specific serotypes, 18 were completely specific for the targeted serotype and included primer pairs used to detect

serotypes 1, 3, 4, 5, 8, 10A, 14, 15A, 15B/C, 16F, 17F, 19A, 19F, 20, 23F, 31, 34, and 35B. (We consider 15B and 15C as one serotype since they interconvert). The serotype 6B primer set was also cross-reactive with the common serotype 6A *cps* operon sequence. Ten additional primer pairs were not completely serotype specific but were limited to an additional one to three rare serotypes, most within the same serogroup. These included primer pairs for 22F, 12F, 9V, 11A, 7F, 33A, 18C, 38, 35F, and 7C.

Table 1. Primer pairs used in each multiplex PCR according to Pai et al. [14] report

Reaction	Primers	Primer concentration (μM)
<i>Multiplex set 1</i>	19A-f,19A-r	1
	3-f,3-r	1.5
	22-f,22-r	1.5
	6A/B-f(biotin),6A/B-r	0.5
<i>Multiplex set 2</i>	4-f,4-r	1.5
	14-f,14-r	1.0
	12F-f,12F-r	1.5
	9V-f,9V-r	1.5
<i>Multiplex set 3</i>	23F-f, 23F-r	1.5
	7F-f, 7F-r	2.0
	11A-f, 11A-r	1.0
	33F-f, 33F-r	1.0
<i>Multiplex set 4</i>	19F-f, 19F-r	1.5
	16F-f, 16F-r	2.0
	sg18-f, sg18-r	1.25
	35B-f, 35B-r	1.0
<i>Multiplex set5</i>	8-f, 8-r	1.5
	15B/C-f, 15B/C-r	1.5
	38-f, 38-r	1.5
	31-f, 31-r	2.0
<i>Multiplex set 6</i>	1-f, 1-r	1.5
	10A-f, 10-r	1.5
	35F-f, 35F-r	1.5
	34-f, 34-r	1.5
<i>Multiplex set 7</i>	20-f, 20-r	1.5
	7C-f, 7C-r	1.5
	17F-f, 17F-r	1.5
	15A-f, 15A-r	1.5

2.4 Modification of Primer Combinations

While the seven mixture reactions (multiplex one to seven) were designed to cover most of the predominant serotypes reported in the United States, reactions one and two were altered based on the prevalence data in other geographic regions (Asia and Africa) to include primers targeting serotypes 1, 5, 6A/B, 7F, 14, 19A, 19F, and 23F. These altered reactions (reaction 1 contained primers for serotypes 19A, 1, 6, and 19F and reaction 2 included serotypes 14, 5, 7F, and 23F) were a combination of serotypes different with that in the original sequential PCR scheme. The universal capsular primers *cpsA*-f and *cpsA*-r were included in all reactions at 0.5 μM each. Primer sequences were ordered to be synthesis based on Pai's work [15].

2.5 PCR Components and Detection Method

The microorganisms were genotyped according to their specific serotype of capsular antigens by Multiplex PCR. Briefly, this comprises a 300nM concentration of each oligonucleotide primer (*Eurofins MWG Operon*); 5.5mM MgCl₂; 200mM (each) deoxynucleoside triphosphates dATP, dCTP, dGTP, and dUTP; and 0.125 U of *Taq*DNA polymerase (from GENET BIO, Prime Taq™ DNA polymerase, lot NO :100914, cat. no. Atype: G-1002, URL: www.genetbio.com). Negative control was considered in all experiments done.

The PCR products were analyzed by gel electrophoresis on 2% BIONEER agarose gels (Cat.No.C-9100-1 Lot.No.1101C) in 1X TBE buffer (890 mMTris, 890mM of boric acid, 40ml of 0.5 M EDTA, pH 8.0) at 100 V for 65 min. Gels were stained with ethidium bromide (0.5g/ml), and gel images were recorded. The sizes of the PCR products were determined by comparison with the molecular size standard (50bp-1Kb linear scale; Low Range DNA ladder or 100bp-3Kb linear scale; Mid Range DNA ladder, Jena Bioscience).

2.6 Specificity and Sensitivity of the Assay

DNA extracted from a variety of non-pneumococcal bacteria (*S.viridans*, *S. pyogenes*, *H. influenza type b* and *K. pneumoniae*) to determine the specificity of the assay. In order to evaluate analytical sensitivity of the assays, serum samples from healthy donors with no recent history of pneumococcal disease were spiked with different amounts of a *S. pneumoniae* suspension to obtain final concentration of 10 to10⁶ CFU/ml, verified by plate counting on 5% sheep blood agar medium. The lowest detection limit of assay was evaluated with a 10-fold dilution of the purified *S. pneumoniae* genomic DNA. The PCR assay was able to detect ≥10 CFU of *S. pneumoniae* per ml. A possible way of overcoming the high rate of apparently false-positive results might have been the use of a lower PCR assay strength. However, this manipulation could have resulted in false-negative results.

2.7 Protocol of Internal Control

An additional primer pair, which has broad specificity for the conserved eubacteria 16S rRNA sequences, was used as amplification control, in these experiments. This primer pair amplifies a 370-bp fragment.

2.8 Statistical Methods

Categorical data were expressed as frequency and percentages. To compare categorical variables in carrier and non-carrier groups, Chi-square analysis (or fisher exact test if appropriate) was performed by SPSS software (version 16). P-value <0.05 was considered as statistically significant.

3. RESULTS AND DISCUSSION

Applied protocols revealed high specificity. The pneumococcal isolates gave a clear band of the expected molecular size in the PCR. The specificity of the PCR assay is demonstrated by its negative results for other organisms including closely related streptococci. One thousand two hundred ninety one infants were subject for this study in 3 different age groups; 429 were 6 months old; 427 at 12 months and 435 at 18 months of age. Six hundreds, (46%) were girls. Only 1.3% went to daycare centers, 17% were exposed to

second hand smoke at home and 17% had received antibiotics during the previous month, (Table 2).

Table 2. Demographic characteristics of children included in the study (n=1291)

Demographic characteristics	Carrier rate	P-Value
Age		0.70
6 mo (n=429)	153 (35.7%)	
12 mo (n=427)	141 (33.0%)	
18 mo (n=435)	147 (33.8%)	
Gender		0.92
Male (n=699)	237 (33.9%)	
Female (n=600)	205 (34.2%)	
Breast-fed Infant		0.52
Exclusive breastfed<3 (n=57)	24 (42.1%)	
Exclusive breastfed 3-6 (n=907)	305 (33.6%)	
Partially breast fed (n=210)	68 (32.4%)	
Formula only (n=87)	32 (36.8%)	
Day-care attendance		0.76
Yes (n=16)	6 (37.5%)	
No (n=1263)	428 (33.9%)	
Presence of school-going sibling		0.81
Yes (n=59)	21 (35.6%)	
No (n=1229)	419 (34.1%)	
Home exposure to secondhand smoke		0.87
Positive (n=226)	76 (33.6%)	
Negative (n=1064)	364 (34.2%)	
Antibiotic consumption during the last month		0.54
Yes (n=218)	78 (35.8%)	
No (n=1073)	361 (33.6%)	

Overall, *S. pneumoniae* was diagnosed by PCR from 34% of participants. Carriage rate was similar in both sexes, 33.9% boys vs.34% girls ($p=0.92$). There was no significant difference in the carrier rate at 6, 12 and 18 months, (35.7%, 33% and33.8%, respectively, $p=0.70$). Similarly, no significant difference was revealed in the frequencies of relevant factors like duration of breast-feeding, home exposure to tobacco smoke, antibiotic consumption or presence of a school-going sibling, among nasopharyngeal carriers of *S. pneumoniae* and non-carriers, ($p>0.05$), (Table 2).

A total of 761 isolates of *S. pneumoniae*, belonging to 30 different genotype specific serotypes were recovered from 441 positive nasopharyngeal specimens, revealing simultaneous colonization of nasopharynx by different serotypes.

Serotypes 19, 6, 14, 19F, 17, 21, 20, 12F, 11 and 3 were the most common serotypes isolated in frequencies of 8.2, 7.6, 7.2, 7.2, 6.4, 5.3, 5.1, 4.8, 4.5 and 4.3% respectively, (Table 3). These ten serotypes comprised almost 60% of all serotypes detected in our samples. There was no significant difference in the serotypes isolated from the three age groups of 6, 12 and 18 months. Colonization with more than one strain was seen in 228 samples, (52% of carriers).

Multiplex PCR technique used in this study is able to detect nasopharyngeal carriers of *S. pneumoniae*, revealed a significant carriage rate of 34% in healthy infants under the age of 2 years, living in Tehran. Previous studies have reported a highly variable carrier rate among 19-93% in young children under two years old who do not attend day care centers [16,17]. A carrier rate of 24% was reported from Turkey and of 21% in healthy Belgian children between three and 36 month [18,19]. Carriage rate of 44% was observed in children six months to 10-year old from Tehran [20]. A high nasopharyngeal carriage rate has been reported from some countries in South Asia and Africa, notably, Indonesia, India and Gambia, (48%, 70 and even 90%, respectively) in notable contrast from some countries like Italy, where carrier rates have been in the order of 8.6% [5]. Differences in age, ethnicity, socio-economic conditions, antibiotic usage in the community, and isolation techniques may account for the widely differing carriage rates observed in different populations [21].

In a study from Finland where nasopharyngeal specimens were taken at 4-age intervals among 2, 6, 12, 18 and 24 months, pneumococcal carriage rates increased from 9% at 2months to 43% at 24 months. However, in our study, no significant differences in carrier rates were observed in different age groups [21]. One possible explanation for this phenomena observed in Finland is that a large proportion of the children at 24 months of age attend day-care centers which are well-known as transmission sources for pneumococci and more than 98% of our children were at home [21]. A large study performed in 11 Asian countries on 4963 <5 year-old healthy children, recovered 1105 isolates of *S. pneumoniae* from anterior nasal swabs, revealing a carriage rate of 22.3%; highest figures were from India,(43.2%) and lowest from Singapore, (9%) [22].

It is frequently reported colonization with pneumococci occurs earlier than the industrialized nations in developing countries; figures reveal about 50% colonization of < 2 month old infants in Bangladesh and India, in contrast with 12% of < 3 month olds in Finland [8]. In Ozdemir's study a carriage rate of 9.7% was seen in young infants <2months of age [17]. Day care attendance has been quoted as a risk factor for pneumococcal carriage [10,23]; only 1.3% of children in our study attended at daycare centers, that was too few to be analyzed. We also found no significant difference in the colonization rate between infants who were exposed to school-going siblings and babies without such exposure. This is in contrast with Ozdemir's study from Turkey where presence of a school-going child in the household was associated with a significant increase in the carriage rate in infants staying at home [17].

Studies on the prevailing serotypes are effective in planning strategies about vaccines; Lee et al. [20] has reported in the Asian region found 6, 23, 19 and 14 to be the most common strains in the nasopharynx, making up about 60% of the total isolates, responsible for invasive disease, antibiotic resistance and prolonged carriage. Three of these isolates viz. 19, 6, and 14, comprised the most frequent serotypes in our study as well. Serotypes 6, 19, 23, 15, 9, 10 and 14 were reported to be most prevalent in a study from Nepal [7]. In 3-13 month-old healthy Fijian infants most frequent serotypes found in the nasopharynx were 6A, 23F, 19F and 6B; the 7-valent pneumococcal conjugate vaccine, (PCV7) would be effective against 30% of the serotypes circulating in the Fijian community [24]. Serotypes 6B, 6A, 11, 19F and 23 F were isolated from Finnish infants as well, and serotypes 3, 19F, 23F, 19A, 6B, and 14 in decreasing order in the Italian study, (6.6%) [5,21]. Serotypes 6, 14 and 19 and 23 have been between the most frequently isolated strains in most studies; all these strains cause invasive disease and are covered by the heptavalent vaccine, PCV7. Our figures for the prevailing serotypes are largely similar except for serotype 23, which was

detected in 19 samples only i.e. about 2.5% of total isolates and 4.3% of carriers; we do not know the reason for this discrepancy.

Table 3. The most common serotypes isolated in this study

	Serotypes	Age groups			Total n (%)	P-Value
		6 months n (%)	12 months n (%)	18 months n (%)		
1	19	22 (5.1)	17 (3.9)	24 (5.5)	63 (8.2)	0.56
2	6	22 (5.1)	18 (4.2)	18 (4.1)	58 (7.6)	0.74
3	14*	16 (3.7)	19 (4.4)	20 (4.6)	55 (7.2)	0.79
4	19F*	19 (4.4)	16 (3.7)	20 (4.6)	55 (7.2)	0.81
5	17	18 (4.1)	15 (3.5)	16 (3.6)	49 (6.4)	0.87
6	21	14 (3.2)	11 (2.5)	16 (3.6)	41 (5.3)	0.65
7	20	15 (3.4)	14 (3.2)	10 (2.3)	39 (5.1)	0.55
8	12F	13 (3.0)	12 (2.8)	12 (2.7)	37 (4.8)	0.97
9	11	16 (3.7)	8 (1.8)	10 (2.3)	34 (4.4)	0.21
10	3*	11 (2.5)	11 (2.5)	11 (2.5)	33 (4.3)	1.00
11	4*	10 (2.3)	9 (2.1)	6 (1.3)	25 (3.2)	0.57
12	22	7 (1.6)	8 (1.8)	7 (1.6)	22 (2.8)	0.95
13	6A*	6 (1.4)	5 (1.1)	11 (2.5)	22 (2.8)	0.25
14	1*	6 (1.4)	6(1.4)	8 (1.8)	20 (2.6)	0.83
15	23	7 (1.6)	7(1.6)	5 (1.1)	19 (2.4)	0.79
16	16	5 (1.1)	3(0.7)	10 (2.3)	18 (2.3)	0.12
17	6B*	5 (1.1)	5(1.1)	8 (1.8)	18 (2.3)	0.62
18	5*	3 (0.7)	5(1.1)	9 (2.0)	17 (2.2)	0.20
19	19A*	6 (1.4)	5(1.1)	6 (1.3)	17 (2.2)	0.95
20	22A	4 (0.9)	5 (1.1)	8 (1.8)	17 (2.2)	0.48
21	15B	8 (1.8)	3 (0.7)	6 (1.3)	17 (2.2)	0.33
22	18C*	7 (1.6)	4 (0.9)	6 (1.3)	17 (2.2)	0.67
23	9V*	4 (0.9)	2 (0.4)	8 (1.8)	14 (1.8)	0.16
24	10	4 (0.9)	5 (1.1)	4 (0.9)	13 (1.7)	0.89
25	23C	5 (1.1)	3 (0.7)	5 (1.1)	13 (1.7)	0.83
26	9	2 (0.4)	3 (0.7)	5 (1.1)	10 (1.3)	0.58
27	18	1 (0.2)	5 (1.1)	2 (0.4)	8 (1.0)	0.19
28	23B	3 (0.7)	3 (0.7)	2 (0.4)	8 (1.0)	0.83
29	15	1 (0.2)	0 (0.0)	0 (0.0)	1 (0.1)	0.66
30	4B	1 (0.2)	0 (0.0)	0 (0.0)	1 (0.1)	0.66

*11 serotypes covered by 13-valent vaccine

We were able to detect co-colonization of nasopharynx with multiple serotypes in >50% of carriers in our study by using Multiplex PCR with 30 types of primers, which is higher than most studies. Young age patients of our study, geographical variation, or the use of multiplex PCR could have contributed to this discrepancy.

Multiple pneumococcal carriage has been observed in both adults and children in other studies as well, but because of variations in isolation techniques, which are time-consuming and expensive, widely different rates have been reported by various researchers [2,8,20-26]. Eleven serotypes identified in our study, constituting about 38.5% of diagnosed samples, are included in the 13-valent pneumococcal conjugate vaccine, (PCV 13).

4. LIMITATION

As a limitation, it would be better to consider a confirmatory diagnostic test for all specimens by any diagnostic method before testing them for genotyping. In addition we determined the carrier rate in healthy children; it may be argued that the pneumococcal serotypes isolated from children with active infection would represent the strains that actually cause IPD and that intensely invasive strains may rapidly cause acute infection without colonizing the nasopharynx for any length of time. As almost all children referred to two large children's hospitals in Tehran and Shiraz with suspected IPD had already received antibiotics, isolating pneumococci from these patients was not a feasible option. Furthermore, studies have found no significant difference in pneumococcal serotypes isolated from nasopharyngeal specimens among healthy children and patients presenting with pneumococcal disease [7,21,23]. Also evidently there has been a significant reduction in the rate of IPD concurrent with the decline in nasopharyngeal colonization by vaccine related serotypes in countries carrying out universal immunization with pneumococcal vaccines effective against the common serotypes isolated from the nasopharynx [1].

Our findings reveal a high percentage of nasopharyngeal co-colonization with *S. pneumoniae* in young carriers; and indicate that the current PCV13 would only give coverage to <40% of the virulent pneumococcal serotypes circulating in children most susceptible to invasive disease. These results emphasize the need to conduct further large-scale national studies, and more importantly, to manufacture vaccines with an optimal formulation, that would be effective in our society.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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