MAEDICA – a Journal of Clinical Medicine
2016; 11(3):203-207

MÆDICA - a Journal of Clinical Medicine

ORIGINAL PAPER

Antimicrobial Resistant Pattern and Capsular Typing of Streptococcus Pneumoniae Isolated from Children in Sistan –Baluchestan

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ABSTRACT

Background: *Streptococcus pneumoniae (pneumococcus) is an important pathogen of respiratory tract in developing countries. It also causes meningitis, otitis media, community-acquired pneumoniae, and bacteremia. Nasopharyngeal carriage is the paramount importance in the transmission of these bacteria. The pathogenesis of disease begins with colonization of the nasopharynx. Due to lack of pneumococcal disease surveillance and epidemiological data on the carriage in Sistan-Baluchestan, this study was done to determine the frequency of common pneumococcal capsular types among non-vaccinated children under 6 years old by Multiplex PCR.*

Materials and method: *In this study 260 nasopharyngeal swabs were taken from non-vaccinated healthy children between 6 months to 6 years old at medical centers in Sistan-Baluchestan during August 2013 to January 2014. These samples were cultured on blood agar. Primary identification of bacterial isolated was determined by biochemical analysis and molecular tests. Capsular typing was performed by Multiplex PCR using primers targeting cps locus that is highly conserved among different capsular types. The master mixes for PCR were grouped them into six multiplex reactions.*

Results: *Out of 260 nasopharyngeal swabs, 42 isolates of Streptococcus pneumoniae were detected and identified. The overall pneumococcal carriage rate was 16.1%. The most frequently isolated capsular types were: 6A/B, 19A, 19F and 23F. These capsular types accounted for 49.9% of all strains detected.*

Conclusion: *We found that the prevalence of pneumococcal carriage among non-vaccinated children under six years old is about 16%. Our study provides much data about carriage rate and pneumococcal capsular types in preschool children, which is necessary for predicting the different valent pneumococcal conjugated vaccines in Iran.*

Keywords: *Streptococcus pneumoniae*, Nasopharynx, Multiplex PCR, Capsular typing.

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Article received on the 30rd of December 2015. Article accepted on the 13rd of September 2016.

BACKGROUND

Streptococcus pneumoniae (*S. pneumoniae*) is an important cause of morbidity and mortality among young children and the elderly people (1). Nearly one million children under age 5 die of pneumococcal diseases every year (2). In recent years treatment of infections with this organism is difficult because of acquiring resistance to the antibiotics (1, 3, 4). Capsule is an important virulence factor in S. pneumoniae isolates causing infectious diseases (sepsis, pneumonia, meningitis, otitis media, sinusitis and bronchitis) (1, 5). As yet, 94 capsular serotypes have been identified in S. pneumoniae but few of them (4, 6B, 9V, 14, 18C, 19F and 23F) have been associated with invasive pneumococcal diseases (IPD) (1). However, predominant serotypes may vary depending on geographic location at different times (4, 6). A suitable vaccine can prevent pneumococcal infections and reduce the mortality rate. Today, many conjugate vaccines are in use for the prevention of infection with this bacterium. A 7-valent and 13- valentant pneumococcal vaccines are already licensed in several countries (1, 7, 8). These vaccines contain trains cause most severe infections in children and about half of infections in adults. However, recent studies show infection with capsular serotypes other than serotypes used in vaccines is growing, PCV13 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) reduced IPD across all age groups when used routinely in children in the USA (9). Here is evidence that routine childhood vaccination reduces the burden of pneumococcal disease in adults and especially high-risk adults, such as those living with HIV/AIDS (9, 10). Hence serotyping is important in following up the vaccination outcome and tracing the emergence of non-vaccine strains. Multiplex PCR is a rapid, simple, and cost-effective molecular method to identify pneumococcal serotypes (10).

Although previous studies showed a high prevalence of pneumococcal disease and high antimicrobial resistance of *S. pneumoniae* strains in Iran, little information is available on the serotypes distribution of *S. pneumoniae* in different parts of country.

The aim of this study was to determine the most predominant pneumococcal serotypes among non-vaccinated children under 6 years old in Sistan-Baluchestan, using Multiplex PCR method. ❑

MATHERIAL AND METHODS

1.1-Bacterial isolates:

A total of 260 nasopharyngeal swabs were collected from non-vaccinated healthy children between 6 months to 6 years old at medical centers in Sistan- Baluchestan, Iran from August 2013 to January 2014. Swab samples were transferred into STGG transfer medium (Skimmed milk, Tryptone, Glucose, and Glycerol) and subsequently inoculated into 5% sheep blood agar and incubated overnight at 37°C in 5% CO_2. The isolated colonies were then identified to species level using standard biochemical methods (11). The identity of isolates as *S. pneumoniae* was confirmed by PCR using primers targeting cpsA gene as described previously (12). *S. pneumoniae* ATCC 6305 was used as a control in experiments.

1.2- Antimicrobial Susceptibility testing:

The CLSI protocol for disk diffusion test was used to assess the susceptibility of isolates to Tetracycline (30μg), Gentamicin (10μg), Cotrimoxazole (25μg), Oxacillin (1μg), Amoxicillin (25μg), Vancomycin (30μg), Erythromycin (15μg), Chloramphenicol (30μg), Cefotaxime (30μg) and Levofloxacin (5μg).

1.3- DNA extraction:

Genomic DNAs were extracted using a DNA extraction Kit (Metabion™ international AG, Germany) according to the manufacture's protocol. Finally, the harvested DNA pellet was re-suspended in RNase- Tris-EDTA to provide 70 μl of DNA sample.

1.4. PCR conditions:

The primers were designed based on the sequences available for the capsular types 1, 3, 4, 5, 6A/B, 7F, 9V, 10A, 11, 12, 14, 15, 16, 18C, 19F, 19A, 22, 23F, 33F, and 35B. The primers were used in six different multiplex PCR according to the previously described method (13). Primers have been designed and grouped on major serotypes of invasive and non-invasive isolates exist in different geographical areas (shown in table 1). *S. pneumoniae* type strains including ATCC 6305, ATCC 6301, ATCC

Reaction	Primer pair	Primer sequence (5'-3')
Reaction ≠1	3	F:ATGGTG TGA TTT CTC CTA GAT TGG AAA GTA G R: CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC
	6A/ B	F: AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG R: TTA GCGGAG ATA ATT TAA AAT GAT GAC TA
	19A	F: GTT AGT CCT GTT TTA GAT TTA TTTGGTGAT GT R: GAG CAG TCA ATA AGA TGA GAC GAT AGT TAG
	22	F: GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC R: CAG CAC TTG CGC TGG AAA CAA CAG ACA AC CTC
Reaction ≠2	4	F: CTG TTA CTT GTT CTGGAC TCT CGA TAA TTGG R: GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G
	9V	F: CTT CGT TAG TTA AAA TTC TAA ATT TTT CTA AG R: GTC CCA ATA CCA GTC CTT GCA ACA CAA G
	12	F: GCA ACA AAC GGC GTG AAA GTA GTT G R: CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC
	14	F: CTT GGC GCA GGT GTC AGA ATT CCC TCT AC R: GCC AAA ATA CTG ACA AAG CTA GAA TAT AGCC
Reaction ≠3	7F	F: CCT ACGGGA GGA TAT AAA ATT ATT TTT GAG R: CAA ATA CAC CAC TAT AGG CTG TTG AGA CTA AC
	11	F: GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G R: GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC
	23F	F: GTA ACA GTT GCT GTA GAGGGA ATT GGC TTT TCF R: CAC AAC ACC TAA CAC ACG ATGGCT ATA TGA TTC
	33F	F: GAA GGC AAT CAA TGT GAT TGT GTC GCG 181 338
Reaction ≠4	16	R: CTT CAA AAT GAA GAT TAT AGT ACC CTT CTA C
	18C	F: CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC R: TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC
	19F	F: GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C R: GTA ATA TGT CTT TAGGGC GTT TAT GGC GAT AG
	35B	F: GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG R: CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G
Reaction ≠5	1	F: CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA R: CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C
	10A	F: GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC R: GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C
	15	F: ATT AGT ACA GCT GCT GGA ATA TCT CTT C R: GAT CTA GTG AAC GTA CTA TTC CAA AC
Reaction ≠6	5	F: ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG R: GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG

TABLE 1. Classification of serotypes to molecular capsular typing and the oligonucleotide primers used in this study.

49619; ATCC 49136 and ATCC 700677 were used as positive control. ❑

RESULTS

Of 260 nasopharyngeal swabs from children under the age of five years, 74 (28.5%) isolates were identified as *Streptococcus pneumoniae* using standard biochemical tests while only 42 isolates (16.1%) were confirmed as *S. pneumoniae* by PCR.

The maximum resistance was observed against oxacillin (100%) followed by cotrimoxazole (93%), tetracycline (61%), erythromycin (56%), gentamicin (46%), amoxicillin (18%) and chloramphenicol (16%). All isolates were susceptible to cefotaxime, vancomycin and levofloxacin.

Serotypes	Number (% frequency)
6A/B	8(19)
19A	5(12)
19F	4(9.5)
23F	4(9.5)
14	2(5)
16	2(5)
1	2(5)
22F	1(2)
18C	1(2)
9V	1(2)
11A	1(2)
Nontypeable	11 (26)
Total	42(100)

TABLE 2. Serotypes distribution of *Streptococcus pneumonia* strains in this study

Multiplex reaction assembly:

The results of the capsular typing are shown in Table 2 and Figure 1. Isolates were assigned into six different capsular types. Of 42 isolates, 11 could not be typed by this method.

The predominant capsular types (40%) were identified in reactions 1 and 4. These reactions showed 19% and 12% of capsular types were 6A/B and 19A respectively. No capsular types were identified in reaction 6. ❑

DISCUSSSION

S. pneumoniae is one of the most common causes of morbidity and mortality in children less than six years and causes life-threatening infections such as meningitis, pneumonia, and febrile bacteremia (14). Serotyping is conventionally based on the Quellung reaction with anticapsular sera, but this is expensive, labor-intensive, and prone to errors (13). PCR-based typing methods including capsular typing are easier, faster, and cost benefit to perform (1). Determination of capsular serotypes is also important for vaccine development since there is difference on the distribution of capsular serotypes causing infection at various areas (15).

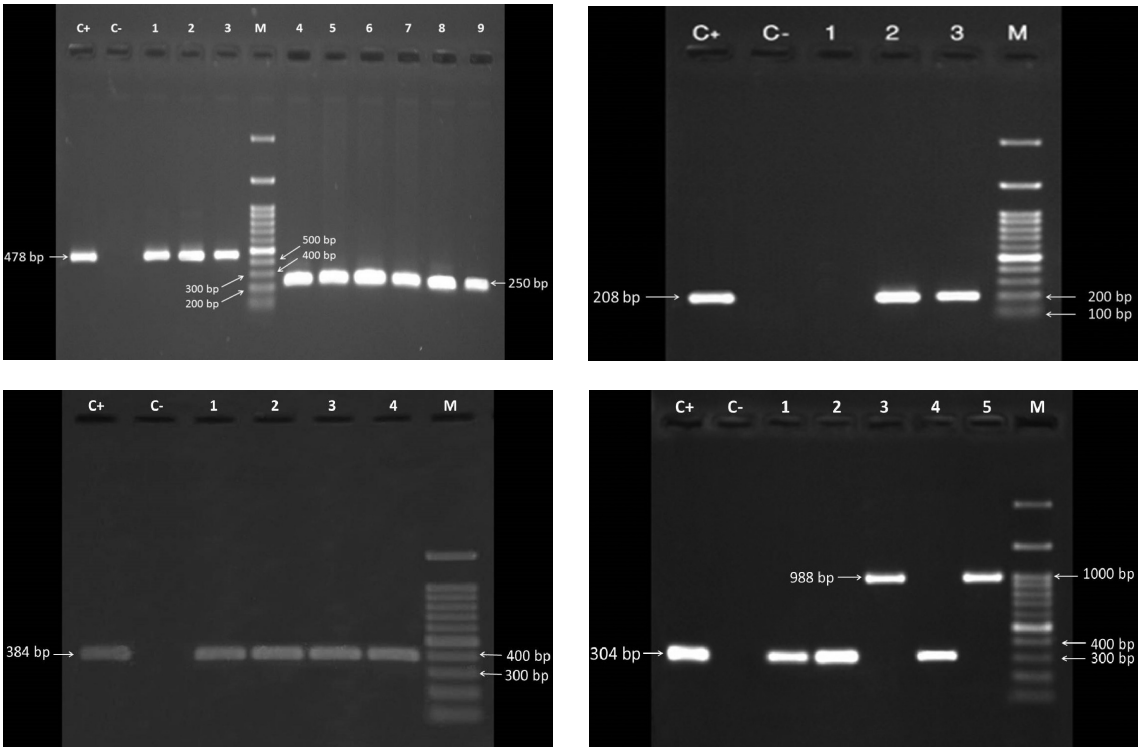

FIGURE 1. Multiplex PCR reactions. Reaction 1 (A); M: size marker, 19A: 478 bp, 6A/B: 250bp. Reaction 2 (B); 14: 280 bp. Reaction 3 (C); 23F: 384 bp. Reaction 4 (D); 19F: 304 bp and 16: 988 bp.

According to this study, cefotaxime was the most effective antibiotics against isolates of *S. pneumoniae* followed by vancomycin and levofloxacin which 100% of the isolates were susceptible to all three mentioned antibiotics completely.

By using multiplex- PCR, 4 isolates couldn't be typed. The remainders were successfully classified into 6 different capsular types. In this survey, similar to other studies from Asia (16, 17), the majority of isolates were typed by three first reactions set in which 6A/B, 19A , 19F and 23F were the predominant capsular types respectively. These results differ somewhat from the results of other studies performed in this field in Iran (11, 18, 19, 21). In previously reported study from Tehran, capsular types 19F and 19A were the most common types and according to the study which is performed by Sanai et al in 2012, the most predominant serotypes were 6, 14, 17, 19, 20, 21, and 23; although in this recent study, serotyping was investigated by quelling test which is less precise than the molecular methods (18, 19, 20). In a study conducted in 2011 in Zahedan, 1,19A,15C,9V,11A,19F were the most predominant capsular types that performed by latex agglutination test (22), while in the present study, the most frequent capsular type was 6 A/B though capsular types 19A and 19F had the next ranks. The differences may be due to the use of different methods for serotyping, different geographical area or may indicate a change in the dominant clone. Given that most of the capsular serotypes obtained in this study are invasive serotypes; their nasopharyngeal colonization in children can be a sign of a lack of proper use of vaccines for prevention in this area. Although all studies that has been done indicate that Serotypes prevalent in most parts of Iran, Similar to serotypes contained in the Pneumococcal Conjugate Vaccines (7 and 13 PCV). According to these results, a few pneumococcal serotypes have been related to the majority of nasopharyngeal carriage and invasive disease (23). It appears that the 7-valent and 13-valent vaccine are suitable for prevention of pneumococcal infections in Iran. However, based on this study PCV13 could cover more serotypes than the others. The vaccine can reduce the risk of life-threatening IPD diseases if properly used, especially in areas prone to infection by this bacterium. In a statistical experiment, among 37868 healthy children carrying *S. pneumoniae* in their nasopharynx who had received at least one dose of conjugate vaccines; invasive pneumococcal disease incidence has decreased to 89% (24). Given

that the highest risk group for infection with this bacterium is children less than 6 years; the groups investigated in this study, lack of regular vaccination program increases the risk of fatal infection and impose high costs to the health system (25, 26). However to determine the actual position of serotypes and evaluating the effectiveness of the vaccine used serotyping should be done on more specimens. ❑

Conflict of interests: none declared.
Financial support: none declared.

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