Microbes and Infectious Diseases 2021; 2(1): 49-59

Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Detection of pneumococcal carriage among under five healthy children with multiple co-colonizing serotypes with impact of pneumococcal conjugate vaccine

Moonmoon Shormin [*1], Samira Afroz [2] , SM Shamsuzzaman [3], Negar Sultana [4]

1- Department of Microbiology, Shaheed Monsur Ali Medical College, Dhaka, Bangladesh.
2- Department of Microbiology, Shahab Uddin Medical College, Dhaka, Bangladesh.
3- Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh.
4- Department of Pathology with Microbiology, Dhaka Dental College, Dhaka, Bangladesh.

ARTICLE INFO

Article history:
Received 2 June 2020
Received in revised form 1 July 2020
Accepted 5 July 2020

Keywords:
Streptococcus pneumoniae
Carriage
PCV
Serotypes
PCR

ABSTRACT

Background: The distribution of serotypes causing invasive pneumococcal diseases (IPD) are diverse, limiting the proportion of IPD cases pneumococcal conjugate vaccine (PCV) can prevent. More studies are needed to estimate the rate of pneumococcal acquisition and serotype replacement following introduction of PCV. **Methods:** The study was conducted in the Department of Microbiology of a tertiary hospital. Nasopharyngeal swabs were collected among 200 under five children from Pediatric outpatient department. *Streptococcus pneumoniae (S. pneumoniae)* were isolated and identified by culture, Gram staining, biochemical test and polymerase chain reaction (PCR). An initial screening of the nasopharyngeal swabs were done by the primer *cpsA* to identify the pneumococci by monoplex PCR. Then primers that are serotype specific were used for serotyping by multiplex PCR with positive and negative control. **Results:** Out of 200 nasopharyngeal (NP) swabs, 67 (33.50%) were positive by culture and 92 (46%) were positive by PCR for *S. pneumoniae*. Out of 200 children, 90 (45%) received PCV and 110 (55%) were not vaccinated. Among vaccinated children, 3 (12%) *S. pneumoniae* were detected in fully vaccinated children and 19 (29.23%) *S. pneumoniae* were detected in partially vaccinated children by culture. In case of PCR, 4 (16%) *S. pneumoniae* were detected in fully vaccinated children and 25 (38.46%) *S. pneumoniae* were detected in partially vaccinated children. Among not vaccinated children, 45 (40.90%) and 63 (57.27%) *S. pneumoniae* were detected by culture and PCR, respectively .The predominant serotypes were *34F, 35B, 6A, 6B, 14, 23F, 3, 19A, 19F, 4, 18C, 7F* as carriage strains. **Conclusion:** Children represent a consistent population of pneumococcal-naïve individuals. So, we found detection rate of *S. pneumoniae* and serotypes in children as carrier was relatively more in unvaccinated children.

Introduction

Streptococcus pneumoniae (*S. pneumonia*) is a major cause of pneumonia, meningitis, and other invasive diseases resulting in high mortality and morbidity among children under the age of five, particularly in lower income countries [1]. *Streptococcus pneumoniae* is a bacterium that colonizes the nasopharynx of human and main source is person to person transmission [2,3]. *Streptococcus pneumoniae* colonization is often asymptomatic but may cause overt infections. It is spread by respiratory droplets and children are the main source of transmission to adults. The pathogenesis of invasive

DOI:10.21608/MID.2020.32348.1021
* *Corresponding author:* Moonmoon Shormin
E-mail address: moonmoon.shormin@gmail.com.

pneumococcal diseases (IPD) begins with nasopharyngeal (NP) colonization that proceeds, often through local infection, to blood stream invasion [4]. Pneumococcal colonization is a dynamic process. One serotype is usually carried at a time, with the first strain often being carried the longest and reacquisition of the same serotype is relatively common [2]. Pneumococcal polysaccharide vaccines and pneumococcal conjugate vaccines (PCV) are two types of vaccines that are used in control of invasive diseases. Use of an effective conjugate vaccine during infancy reduced the rates of invasive pneumococcal infections and antibiotic resistance associated with serotypes [5] . However, infections caused by other serotypes that are not included in different PCV have increased [6].

Although almost all children become colonized with *S. pneumoniae* repeatedly during the first few years of life, a very small fraction of these acquisitions results in invasive disease. Global surveillance demonstrates that a limited number of serotypes cause 80% of IPD, and serotypes vary in their contribution to invasive disease. There are two components to this variation: (i) some serotypes are more commonly carried and thus have more temporal opportunity for invasion, and (ii) some serotypes are more likely to cause invasive disease with each carriage episode, perhaps because of their superior ability to overcome host defenses and penetrate the bloodstream [4]. *Streptococcus pneumoniae* can produce at least 90 capsular serotypes [7], but only a few of these cause most cases of invasive diseases [8]. Since the pneumococcal epidemiology of capsule types varies geographically and temporally, a nationwide serotypes surveillance system is vital to establishing appropriate vaccination strategies [9]. In this study we detect the pneumococcal carriage with multiple serotypes, which is essential for production of the new generation of conjugate vaccines.

Materials and Methods

Data collection

A cross sectional study was done from July 2016 to June 2017. Nasopharyngeal swabs were collected from healthy children aged one month to less than five years who attended the outpatient department of Dhaka Medical College Hospital, Dhaka in Bangladesh for routine immunization, child growth monitoring and nutritional advice. Children whose parents did not give consent and non-co-operative children were excluded from the study. Children with recent history of upper respiratory tract infection, hospitalization and antibiotics usage were also excluded from the study.

Ethical approval

This protocol was approved by Research Review Committee (RRC) of Microbiology Department of Dhaka Medical College, Dhaka in Bangladesh. Informed written consent was obtained from the parents or legal guardians of each child before sample collection.

Sample collection procedure

Samples were collected from nasopharynx of each child by inserting a specially designed swab with a flexible shaft and a small calcium alginate tip [10]. The swabs were gently inserted into the NP area located one-half to two-thirds distance from the nostril to ear lobe, Then the swab was rotated gently and was allowed to remain for 20-30 seconds. The swab was then removed and was placed in Skimmilk-tryptone-glucose-glycerol (STGG) media, labeled and transported to the laboratory [11], then the swabs containing screw-cap bottles were vortexed on high speed for 10-20 seconds and placed at -20°C in upright position for culture and polymerase chain reaction (PCR) [12] .

Culture and isolation [12]

Specimens which were kept at -20°C were hawed at room temperature (25°C) and vortexed for 10-20 seconds. Then specimens were inoculated on blood agar using one loop (10µl) of sample. The plates were streaked into four quadrants and incubated at 37°C for 24 hours with CO_2 atmosphere inside a candle jar Small, smooth and transparent colonies were seen on blood agar plate. Colonies of *S. pneumoniae* were low convex, tiny and they became flattened centrally showing the 'draughtsman form'. A narrow zone of ∝ hemolysis was seen around the colonies. Gram positive diplococci were seen which were ovoid or lanceolate in shape and catalase negative [13]. The isolates with presumptive identification were confirmed by optochin sensitivity test, bile solubility test **(Table 1)** and by PCR **(Figure 1)**.

Procedure of PCR [14]

Samples preserved in STGG medium were brought out from freeze, kept at room temperature to demoisture, then samples were vortexed to make a homogenous suspension. Swab stickswere removed and the vortexed specimens were taken into two micro centrifuge tubes, labeled properly, centrifuged at 10,000 X g for 10 minutes and the supernatant was discarded. The deposit was used as pellet for PCR. The

micro centrifuge tubes containing pellet were kept at -20 °C until DNA extraction.

- ***DNA extraction***

Two hundred micro litter of lytic buffer was mixed with the sample pellets and vortexed until mixed well. Then the tubes were incubated at 60°C for 3 hours. After incubation, tubes were kept in heat block at 100°C for 10 minutes for boiling. Then the tubes were immediately placed on ice for 5 minutes. After that the tubes were centrifuged at 4°C at 14000 X g for 10 minutes. Finally supernatants were taken using micropipette and used as template DNA for PCR. This DNA was kept at -20°C for future use [10] **.**

- ***Serotyping by PCR***

The primer *cpsA* was used for targeted highly conserved gene that exists in all capsular loci thus far characterized [15]. Thirty eight specific primers were used for targeted genes specific for serotypes *1, 2, 3, 4 , 5, 6A, 6B, 7F, 9V, 11A, 12F, 14, 18C, 19F, 19A, 23F, 33F, 34F* and 35B. Of these serotypes *1, 2, 3, 4 , 5, 6A, 6B, 7F, 9V, 11A, 12F, 14, 18C, 19F, 19A, 23F,33F* were vaccine type (VT) and serotypes *34F, 35B* were non vaccine (NVT) type. Vaccine Type serotypes means a serotype included in PCV7 (serotypes *4, 6B, 9V, 14, 18C, 19F*, and *23F*), PCV10 (serotypes *1, 5,* and *7F* added to PCV7), PCV13 (serotypes *3, 6A,* and *19A* added to PCV10), and PPSV23 (serotypes *2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F*, and *33F* added to PCV13, except for 6A). Non-vaccine serotypes are the serotypes which are not covered by PCV7, PCV10, PCV13, and PPSV23. An initial screening of the NP swabs were done by the primer *cpsA* to identify the pneumococci by monoplex PCR **(Table 2a)**. Then primers that are serotype specific were used for serotyping by multiplex PCR with positive and negative controls [10] **(Table 2b)**.

- ***Mixing of master mix and primer with DNA template***

Primers were mixed with Tris-EDTA (TE) buffer according to manufacturer's instruction. For each sample, total 25µl of mixture was prepared by mixing of 12.5 µl of master mix (mixture of dNTP, taq polymerase MgCl2 and PCR buffer), 2µl of forward primer, 2µl of revers primer (Promega Corporation, USA), 2µl of DNA template and 6.5 µl of sterile distilled water in a micro centrifuge tube. For multiplex PCR, total 25 µl of mixture was prepared by mixing of 12.5 µl of master mix , 1µl of each forward primer , 1µl of revers primer , 2µl of DNA template and nuclease free water in a micro centrifuge tube. After a brief vortex, the tubes were centrifuged in a micro centrifuge for few seconds [10].

Amplification in thermal cycler (Eppendorf AG, Master cycler gradient, Hamburg, Germany):

Melting temperature of the primer depends on base pair and calculated using the following formula:

- Up to 20 bp, the melting temperature is (°C) = 4 (G+C) +2 (A+T)-5.
- More than 30 bp, the melting temperature is (°C) = 4(G+C)+2 (A+T)+ 35-2n.

(n= total number of base pair)

Polymerase chain reaction consisted of preheat at 94°C 10 minutes followed by 36 cycles of denaturation at 94 °C for 30 seconds, annealing at 52 °C for 40 seconds, extension at 72 for one minute with final extention at 72 for 10 minutes. Annealing temperature varies with GC contents of primers.

- ***Polymerase chain reaction primer selection***

The selection of PCR primers was based on the published works [15,16] **(Table 2)**.

- ***Agarose gel electrophoresis***

Polymerase chain reaction products were detected by electrophoresis on 2% agarose gel. Gel was prepared with 1X TBE buffer (Tris Borate EDTA) For 2% agarose gel preparation, 0.25 gram of agarose powder (LE, Analytical grade, Promega, Madison, USA) was mixed with 12.5 ml TBE buffer. After mixing thoroughly it was boiled using microwave oven for few minutes to dissolve the agarose completely. Gel was cooled to 60-70 °C and a comb was placed in gel tray and the agarose gel was poured into the tray and was allowed to stand for 30 minutes for solidification. After solidification of gel, comb was removed from the tray. Six µl of amplicon was mixed with 1µl loading dye on a piece of para film and then the mixture was loaded into the well of agarose gel. Two µl DNA ladder was mixed with 1 µl of loading dye and was loaded into one well of agarose gel. Gel containing amplicons and DNA ladder were then placed on the electrophoresis tank having 1X TBE buffer for 35 minutes at 100 volts. Positive control and negative control were also loaded in separate well.

- ***Staining and de-staining of the gel***

After electrophoresis, the gel was stained with staining buffer for 30 minutes. It was then de-stained with distilled water for 15 minutes.

Data analysis

The results of the study were recorded systematically. Data analysis was done by using 'Microsoft Office Excel 2007' program and according to the objectives of the study. Results were presented in the form of tables and figures. The test of

significance was calculated by using Z test and X^2 test. *P value <0.05* was taken as minimal level of significance and *p value <0.001* was taken as highly significant.

Table 1. Identification of *S.pneumoniae* and its differences with Viridans streptococci.

Characteristic	*S.pneumoniae*	Viridans streptococcus
Colonies	Flattened, draughtsman	Convex
Effect on blood agar	Narrow zone of α haemolysis	Narrow zone of α haemolysis
Optochin sensitivity	+ve	-ve
Bile solubility	+ve	-ve

Table 2a. List of selected *cpsA* primer with annealing temperature and product size.

Name of gene	Oligonucleotide sequence (5'-3')	Annealing temperature°C	Product size
cpsA-f	GGT GTT CTC TAT CCT TGT CAG CTC TGT GTC GCT C	56	657
cpsA-r	GTG TGA ATG GTC GAA TCA ACT CTA TAA ATG CC		
1wzy-f	GGA GAC TAC TAA ATT GTA ATA CTA ACA CAG CG	48	280
1wzy-r	CAA GGA TGA ATA AAG TAA ACA TAT AAT CTC		
3capB-f	TTG TTT TTT GTC TTT ATT CTT ATT CGT TGG	52	818
3capB-r	TAC TGA GAA CCT TCT GCC CAC CTT AGT TGC		
4wzy-f	CTG TTA CTT GTT CTG GAC TCT CGT TAA TTG G	56	430
4wzy-r	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G		
6Bwzy-f	CGA CGT AAC AAA GAA CTA GGT GCT GAA AC	52	220
6Bwzy-r	AAG TAT ATA ACC ACG CTG TAA AAC TCT GAC		
14cpsH-f	GTC TGT TTATTC TAT ATA CAA AGA GGC TCC	51	268
14cpsH-r	GCA TTG CTA CAA TCG CTA TAC TAG ATA TGC		
18CwciY-f	GCA TCT GTA CAG TGT GCT AAT TGG ATT GAA G	52	354
18Cgct-r	CTT TAA CAT CTG ACT TTT TCT GTT CCC AAC		
19FcpsI-f	CAC CTA ATT TTA ATA CTG AGG TTA AGA TTG C	48	408
19FcpsI-r	CAT AGG CTA TCA GAA TTT TAA TAA TAT CTT GC		
19AcpsK-f	GTT AGT CCT GTT TTA GAT TTA TTT GGT GAT GT	51	478
19AcpsK-r	GAG CAG TCA ATA AGA TGA GAC GAT AGT TAG		
23FcpsG-f	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	55	384
23FcpsG-r	CAC AAC ACC TAA CAC ACG ATG GCT ATA TGA TTC		

Table 2b. List of primers selected for serotyping with annealing temperature and product size.

Primer Pair	Oligonucleotide sequence (5'-3')	Annealing temperature°C	Product Size
2-f	GTC ATT GTT ACG ATT AGT TTC GAT AGT TGA GG	51	381
2-r	AAT TCA ATT CCT AAG TCC TCT TCC ATA AAC TC		
6A-f	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	54	250
6A-r	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA		
7F-f	CTA TCT CAG TCA TCT ATT GTT AAA GTT TAC GAC GGG A	54	260
7F-r	GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC		
9V-f	CTT CGT TAG TTA AAA TTC TAA ATT TTT CTA AG	54	753
9V-r	GTC CCA ATA CCA GTC CTT GCA ACA CAA G		
11A-f	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	54	463
11A-r	GAT TAT GAG TGT AAT TTA TTC TTC CAA CTT CTC CC		
12F-f	GCA ACA AAC GGC GTG AAA GTA GTT G	54	376
12F-r	CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC		
33F-f	GAA GGC AAT CAA TGT GAT GAT TGT GTC GCG	54	338
33F-r	CTT CAA AAT GAA GAT TAT AGT ACC CTT CTT CTA C		
34-f	GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC	54	408
34-r	CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC		
35-B-f	GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG	54	677
35-B-r	CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G		

Results

Nasopharyngeal swabs from total 200 under five children were tested **(Table 3)**[in this stusy relations between pneumococci carriage, serotyping, vaccination and age were not seen], among them, 67(33.50%) were positive for *S. pneumoniae* by culture **(Table 4)** and 92 (46%) were positive by PCR using *cpsA* gene specific primer **(Table 5, Figure 1)**. Among 92 PCR positive NP swabs, 19 different serotypes were seen. Among 92 PCR positive NP swabs, 70 (76.09%) were serotypes positive and 22 (23.91%) were serotype negative **(Figure 2)**. Among them more detected serotypes were *34F* (17.39%), *35B* (13.04%), *6A* (11.96%), *6B* (11.96%), *14* (7.61%) *and 23F 4* (5.43%). Less detected serotypes were *3* (3.26%), *19A* (2.17%), *19F* (2.17%), *4* (1.09%), *18C* (1.09%) and *7F* (1.09%) **(Figure 3)**. Out of 200 children, 90 (45%) were received (PCV) and 110 (55%) were not vaccinated. Among vaccinated children, 3 (12%) *S. pneumoniae* were detected in fully vaccinated children (3 doses as recommended in Bangladesh; mentioned in the related table) and 19 (29.23%) *S. pneumoniae* were detected in partially vaccinated children by culture. In case of PCR, 4 (16%) *S. pneumoniae* were detected in fully vaccinated children and 25 (38.46%) *S. pneumoniae* were detected in partially vaccinated children. Among non-vaccinated children, 45 (40.90%) and 63 (57.27%) *S. pneumoniae* were detected by culture and PCR respectively **(Table 6)**. The difference between fully vaccinated and non-vaccinated proportion is statistically significant ($p<0.001$) and the difference between fully vaccinated and partially vaccinated proportion is statistically significant ($p< 0.001$). Among 4 fully vaccinated PCR positive cases, serotypes (mentioned in methodology) were detected among 3 fully-vaccinated children, in the rest one vaccinated child serotypes was not detected as all the serotype specific primers were not used in this study and detected serotypes were *34F* (1) and *35B* (1) and *6B* (1); in the rest one vaccinated child serotypes was not detected as all the serotype specific primers were not used in this study. Among 25 PCR positive

partially-vaccinated children, serotypes were detected in 18 children and detected serotypes were *34F* (7), *35B* (3), *6B* (3), *14*(3), *23*(2) [here mentioned only numerical number which were found in individual type of vaccinated children, percentage(%) of total detected serotypes were mentioned in result pharagraph] **(Figure 4)**. Among 63 PCR positive non-vaccinated children, serotypes were detected in 51 children and the detected serotypes were *3* (3), *4* (1), *6A* (11), *6B* (7), *7F* (1), *14* (4), *18C* (1), *19A* (2), *19F* (2), *23F* (3), *34F* (8), *35B* (8) **(Table 7), (Figure 5)**; [here mentioned only numerical number which were found in individual type of vaccinated children, percentage(%)of total detected serotypes were mentioned in result pharagraph .

Table 3. Results of culture and PCR in different age groups (n=200).

Age groups	Culture positive n (%)	PCR positive n (%)
1month-12 months (n= 40)	13(32.50)	21(52.50)
13months-36 months (n= 65)	29(44.62)	38(58.46)
37months- ≤ 60 months (n= 95)	25(26.32)	33(34.74)
Total	**67**	**92**

N= Total number of samples, n= Total number of positive cases
% were calculated according to the row.

Table 4. Results of culture for *S. pneumoniae* from NP swabs (n=200).

Methods	Nasopharyngeal swabs n (%)
Culture positive	67(33.50)
Culture negative	133 (66.5%)
Total	200 (100%)

Table 5. Results of PCR for *S. pneumoniae* from NP swabs (n=200).

Methods	Nasopharyngeal swabs n (%)
PCR positive	92(46%)
PCR negative	108 (54%)
Total	200 (100%)

Table 6. Distribution of *S. pneumoniae* among study population in relation to vaccination status (n=200).

Vaccination status	Culture positive n (%)	PCR positive n (%)
Fully vaccinated* (n=25)	3 (12)	4 (16)
Partially vaccinated (n=65)**	19(29.23)	25(38.46)
Not vaccinated (n=110)	45(40.90)	63(57.27)
Total	67	92

*Vaccinated with 3 doses of PCV
**Vaccinated with 1 or 2 doses of PCV.

Table 7. Distribution of detected serotypes among vaccinated, partially vaccinated and non-vaccinated children in PCR positive children (n=92).

Vaccination status	Detected serotypes(n)
Fully vaccinated (n=4)	*34F(1), *35B (1), 6B (1)
Partially vaccinated (n=25)	*34F(7),* 35B(3),6B (3), 14 (3), 23F(2)
Not-vaccinated (n=63)	3(3), 4(1), 6A(11), 6B(7), 7F(1), 14(4), 19A(2), 19F(2), 23F(3), *34F(8), *35B(8)

VT=3, 4, 6A, 6B, 7F, 14, 18C, 19A, 19F, 23F, *NVT=34F, 35B
VT-Vaccine Type serotypes: a serotype included in PCV7 (serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F), PCV10 (serotypes 1, 5, and 7F added to PCV7), PCV13 (serotypes 3, 6A, and 19A added to PCV10), and PPSV23 (serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F added to PCV13, except for 6A). NVT-Nonvaccine Type serotypes: serotypes which aret not covered by PCV7, PCV10, PCV13, and PPSV23.

Figure 1. Photograph of amplified *cpsA* gene of *Streptococcus pneumoniae*. Lane 1 shows negative control with DNA of *Staphylococcus aureus* ATCC25923, Lane 2, 3, 5, 6, 7 shows amplified DNA of 657 bp of cpsA gene. Lane 4 shows hundred bp DNA ladder.

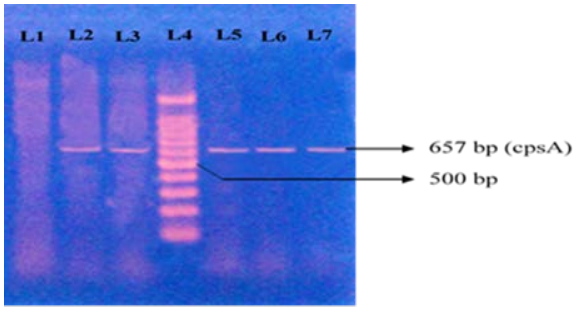


Figure 2. Proportion of detected serotype positive and negative NP by PCR.

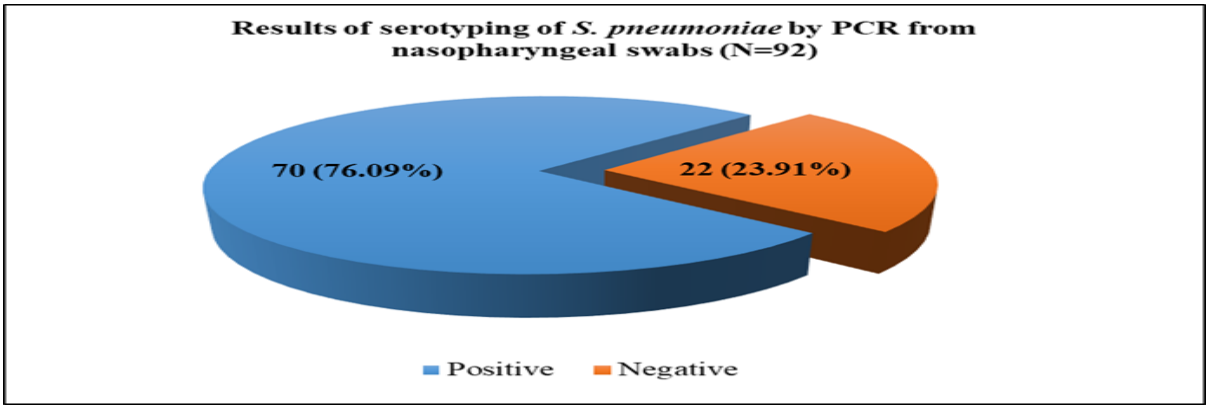


Figure 3. Distribution of serotypes among the study population detected by PCR directly from NP swabs (n=92).

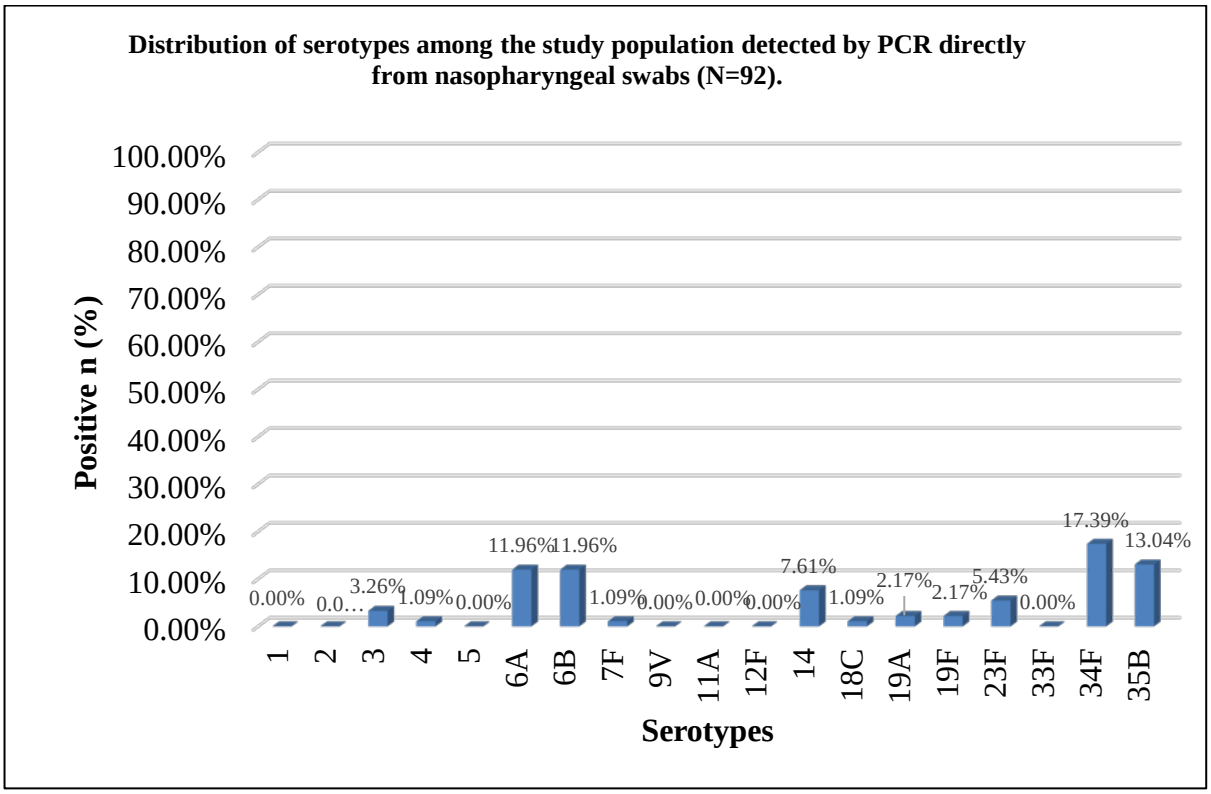


Figure 4. Photograph of gel electrophoresis of detected serotypes. Lane 1: amplified DNA of 268 bp of serotype 14, Lane 2: amplified DNA of 818 bp of serotype 3, Lane 3: amplified DNA of 384 bp of serotype 23F, Lane 4: amplified DNA of 478 bp of serotype 19A, Lane 5: hundred bp DNA ladder, Lane 6: amplified DNA of 408 bp of serotype 19F and Lane 7: amplified DNA of 260 bp of serotype 7F.

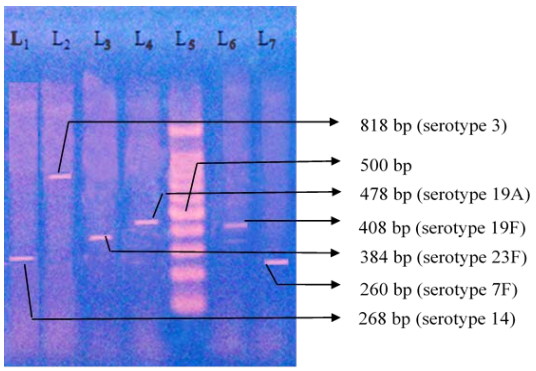
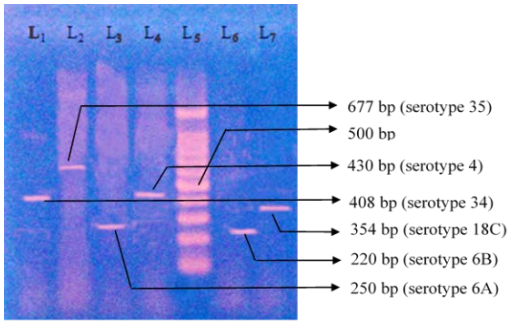


Figure 5. Photograph of gel electrophoresis of of detected serotypes. Lane 1: amplified DNA of 408 bp of serotype 34, Lane 2: amplified DNA of 677 bp of serotype 35, Lane 3: amplified DNA of 250 bp of serotype 6A, Lane 4: amplified DNA of 430 bp of serotype 4, Lane 5: hundred bp DNA ladder, Lane 6:

amplified DNA of 220 bp of serotype 6B, and Lane 7 : amplified DNA of 354 bp of serotype 18C.



Discussion

Streptococcus pneumoniae is a bacterium that colonizes the nasopharynx of human, it's most common cause of pneumonia and other IPD. The capsular polysaccharide, a well-known virulence factor and the serotype determinant of pneumococci that prevents opsonization and phagocytosis of *S. pneumoniae* [17]. Pneumococcal capsular vaccines protect against disease and nasopharyngeal carriage due to the serotypes included in the vaccine formulations [18]. Nevertheless, surveillance of serotype prevalence patterns is very important since the serotypes responsible for invasive disease can change over time [19,20] . The surface capsular polysaccharide of *S. pneumoniae* provokes a type-specific protective immune response and serves as the basis for serotyping of these organisms; currently more than 90 different pneumococcal serotypes have been identified. Serotypes *6, 14, 18, 19,* and *23* are the most prevalent, accounting for between 60 and 80 percent of infections depending upon the area of the world.

Ninety capsular serotypes of *S. pneumoniae* exist, and the prevalence of serotypes differs according to age, region, and time of the surveillance. The 90 serotypes differ in virulence; a minority of serotypes is involved in most of invasive pneumococcal diseases and antimicrobial resistances. Serotypes are classified into VT and (NVT. Vaccine serotype means a serotype included in PCV7 (serotypes *4, 6B, 9V, 14, 18C, 19F,* and 23F), PCV10 (serotypes *1, 5,* and *7F* added to PCV7), PCV13 (serotypes 3, 6A, and 19A added to PCV10), and PPSV23 (serotypes *2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F,* and *33F* added to PCV13, except for *6A).* Nonvaccine serotype is the serotype which is not covered by PCV7, PCV10, PCV13, and PPSV23 [9] .

In the present study, a total of 200 NP swabs were processed for culture and PCR. Out of 200 NP swabs, 67 (33.50%) were culture positive and 92 (46%) were PCR for *S. pneumoniae*. In the present study, PCR base detection of carriage was higher than culture (46% vs 33.50%). Some previous studies [21,22] reported that PCR significantly increased the detection of carriage of *S. pneumoniae* compared to culture. The results of the previous studies and the results of the present study showed that PCR based detection is higher in comparison to culture. *Streptococcus pneumoniae* isolation from NP swab by culture was less sensitive which might be due to the fastidious nature of the organism and low load of the organism with co-colonization that can result in false negative results [23]. Polymerase chain reaction can detect bacterial DNA even if the numbers of organism is too low to grow in culture [24] and can detect from non-viable organisms after treatment with antibiotics [25].

In the present study, among 92 PCR positive NP swabs, 19 different serotypes were seen by PCR. Out of 19, 17 *(1, 2, 3, 4, 5, 6A, 6B, 7F, 9V, 11A, 12F, 14, 18C, 19A, 19F, 23F, 33F)* were vaccine types (VT) and 2 (*34F, 35B)* were non vaccine types (NVT). Among 70 serotype positive NP swabs, 72 different serotypes were detected. More detected serotypes were *34F* (17.39%), *35B* (13.04%), *6A* and *6B* (11.96%), 14 (7.61%), *23F* (5.43%) and less detected serotypes were 3 (3.26%), *19A* and *19B* (2.17%), *4, 7F* and *18C* and were all was 1.09%. Serotypes *1, 2, 5, 9V, 11A, 33F* were not found in the present study. In the present study the detected serotypes were similar to other studies conducted in Bangladesh in different period of times. Two studies [26, 27] detected serotypes (*14, 7F, 6A, 6B, 18C, 19A, 23F)* from invasive cases and from both meningitis (*6, 7, 14, and 18*) and carriage cases (*6, 19, 23, 34, 35, and 14).*

In the present study serotype *1, 2, 5, 9V, 11A, 33F* were not found. The reasons might be , pneumococcal colonization is a dynamic process. One serotype is usually carried at a time, with the first strain often being carried the longest and reacquisition of the same serotype is relatively common [2], besides, serotypes or serogroups carried for a long period would be recovered more frequently from nasal swabs than those carried only transiently, and invasive diseases is more likely to occur soon after the acquisition.and thus, is less well associated with the period of colonization [26]. In the present study, carriage rate was significantly higher in non-vaccinated children than fully-vaccinated children ($p<0.001$) and also significantly higher in partially-vaccinated children than fully-vaccinated children ($p<0.001$).

The findings of the present study might be due to the fact that introduction of PCV reduced the carriage rate in vaccinated children and reduced carriage of VT pneumococci accompanied by an increased NVT [28]. Some previous studies analyzed the impact of pneumococcal vaccination on *S. pneumoniae* carriage rate and serotype distribution [29-31]. They reported that overall carriage rate in residents decreased following vaccination, carriage rates of all age group were lower at first and second post vaccination surveys than at the pre vaccination survey. Some previous studies showed that the prevalence of vaccine type (VT) carriage decreased after PCV vaccination [29,31]. **Isaacman et al.** and **Flasche et al.** reported that reduction in carriage of VT pneumococci due to PCV vaccination is often accompanied by an increase in the carriage of NVT pneumococci and to a lesser extent, an increase in the incidence of IPD caused by NVT pneumococci [32,33].

Limitations

Due to time and resource constraint, all the VT and NVT serotypes could not be detected.

Conclusion

Detection rate of NP carriage of *S. pneumoniae* was relatively more and the predominant serotypes were *34F, 35B, 6A, 6B, 14, 23F, 3, 19A, 19F, 4, 18C, 7F* as carriage strains. These findings highlight the importance of monitoring pneumococcal nasopharyngeal carriage and serotypes distribution for the future pneumococcal conjugate vaccine production.

Recommendation

We recommend that the continuous survey for detecting pneumococcal carriage with different serotypes in children can be useful and improving the ability to diagnose invasive Pneumococcal disease burden and implications for vaccine policy.

Conflict of interest: All the authors declare no conflicts of interest.

Funding

This study was not funded by any profit or nonprofit organization.

Informed consent

Informed written consent was obtained from the parents or legal guardians of each child before sample collection.

References

1-**Normark B, Tuomanen EI.** The pneumococcus: Epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med 2013; 3: e010215.

2-**Gray BM, Converse GM, Dillon HC.** Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. J Infect Dis 1980; 142: 923-933.

3-**Pasinato A, Indolfi G, Marchisio P, Valleriani C, Cortimiglia M, Spanevello V, et al.** Nasopharyngeal carriage in children pneumococcal serotype distribution in 1315 nasopharyngeal swabs from a highly vaccinated cohort of Italian children as detected by RT-PCR. Vaccine 2014; 32: 1375–1381.

4-**Yildirim I, Hanage WP, Lipsitch M, Shea KM, Stevenson A, Huang S, et al.** Serotype specific invasion capacity and persistent reduction in invasive pneumococcal disease. Vaccine 2010; 29: 283-288.

5-**Joloba ML, Windau A, Bajaksouzian S, Appelbaum PC, Hausdorff WP, Jacobs MR.** Pneumococcal conjugate vaccine serotypes of Streptococcus pneumoniae isolates and the antimicrobial susceptibility of such isolates in children with otitis media. Clin Infect Dis 2001; 33:1489–1494.

6-**Linares J, Ardanuy C, Pallares R, Fenoll A.** Changing in antimicrobial resistance, serotypes and genotypes in Streptococcus pneumoniae over a 30-year period. Clin Microbiol Infect J 2010; 16: 402-410.

7-**Henrichsen J.** Six newly recognized types of Streptococcus pneumoniae. J Clin Microbiol 1995; 33: 2759–2762.

8-**Lalitha MK, Thomas K, Kumar RS, Steinhoff MC.** Serotyping of Streptococcus pneumoniae by coagglutination with 12 pooled antisera. J Clin Microbiol 1999; 37: 263–265.

9-**Kim SH, Chung DR, Song JH, Wang H.** Changing Trends in Antimicrobial resistance and serotypes of Streptococcus pneumoniae isolates in Asian countries: an Asian Network for Surveillance of resistant Pathogens (ANSORP) Study. AAC 2012; 56: 1418-1426.

10-**Rubin GR, Rizvi A, Baer A.** Effect of swab composition and use of swabs versus swab-containing skimmilk- Tryptone- Glucose-Glycerol(STGG) on culture or PCR-based detection of Streptococcus pneumoniae in simulated and clinical respiratory specimens in STGG transport medium. J Clin Microbiol 2008; 46: 2635-2640.

11-**Saha S, Modak JK, Naziat H, Al-Emran HM, Chowdury M, Islam M, et al.** Detection of co-colonization with Streptococcus pneumoniae by algorithmic use of conventional and molecular methods. Vaccine 2014; 11: 713-718.

12-**Carvalho Mda G, Pimenta FC, Jackson D, Roundtree A, Ahmad Y, Millar EV, et al.** Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J Clin Microbiol 2010; 48: 1611-1618.

13-**Mawla N, Rahman M, Lutfor AB, Shamsuzzaman SM, Ahamed D, Rashid H, et al.** Emergence of Optochin resistant Streptococcus pneumoniae in Bangladesh. Int J Infect Dis 2008; 12: e261–e262.

14-**Franco MR, Caiaffa-Filho HH, Burattini MN, Rossi F.** Metallo-beta-lactamases among imipenem resistant Pseudomonas aeruginosa in s Brazilian university Hospital. Clinics (Sao Paulo) 2010; 65: 825-829.

15-**Morona JK, Morona R, Paton JC.** Analysis of the 5'portion of the 19A capsule locus identified two classes of cpsC, cpsD and cpsE genes in Streptococcus pneumoniae. J Bacteriol 1999; 181: 3599-3605.

16-**Pai R, Gertz RE, Beall B.** Sequential multiplex PCR approach for determining capsular serotypes of Streptococcus pneumoniae isolates. J Clin Microbiol 2006; 44:124–131.

17-**Watson DA, Musher DM, Verhoef`J.** Pneumococcal virulence factors and host immune responses to them. Eur J Clin Microbiol Infect Dis 1995; 14: 479-490.

18-**Grall N, Hurmic O, Nakib M, Longo M, Poyart C, Varon E, et al.** Epidemiology of Streptococcus pneumoniae in France before introduction of the PCV-13 vaccine. Eur J Clin Microbiol Infect Dis 2011; 30: 1511-1519.

19-**Yasin RM, Zin NM, Hussin A, Nawi SH, Hanapiah SM, Wahab ZA, et al.** Current trend of pneumococcal serotypes distribution and antibiotic susceptibility pattern in Malaysian hospitals. Vaccine 2011; 29: 5688-5693.

20-**Hanquet G, Kissling E, Fenoll A, George R, Lepouue A.** Pneumococcal serotypes in children in 4 European countries. Emerg Infect Dis 2010; 16: 1428-1439.

21-**Brugger SD, Hathaway LJ, Muhlemenn K.** Detection of Streptococcus pneumoniae strain colonization in the nasopharynx. J Clin Microbiol 2009; 47: 1750-1756.

22-**Wyllie AL, Wijmenga AJ, Marlies A, Groot JA.** Molecular surveillance of nasopharyngeal carriage of Streptococcus pneumoniae in children vaccinated with conjugated polysaccharide pneumococcal vaccines. Sci Rep 2016; 6: 238-242 .

23-**Lang ALS, McNeil SA, Hatchette TF, Elsherif M, Martin I, Jason J, et al.** Detection and prediction of Streptococcus pneumoniae serotypes directly from nasopharyngeal swabs using PCR. J Med Microbiol 2015; 64: 836-844.

24-**Rudolph KM, Parkinson AJ, Black CM, Mayer LW.** Evaluation of polymerase chain reaction for diagnosis of pneumococcal

pneumonia. J Clin Microbiol 1993; 31: 2661-2666.

25-**Wheeler J, Murphy OM, Freeman R, Kearns AM, Steward M, Lee MJ.** PCR can add to detection of pneumococcal disease in pneumonic patients receiving antibiotics at admission. J Clin Microbiol 2000; 38: 3907-3911.

26-**Saha SK, Baqui AH, Darmstadt GL, Ruhulamin M, Hanif M, Arifeen SE, et al.** Comparison of .Antibiotic Resistance and Serotype Composition of Carriage and Invasive Pneumococci among Bangladeshi Children: Implications for Treatment Policy and Vaccine Formulation. J Clin Microbiol 2003; 41: 5582-5587.

27-**Saha SK, Naheed A, Arifeen SE, Islam S, Emran HA, Amin R.** Surveillance for invasive Streptococcus pneumoniae diseases among hospitalized children in Bangladesh: Antimicrobial susceptibility and serotype distribution. Clin Infect Dis 2009; 48: 75-81.

28-**Richard AA, DeAntonio R, Hill PC, Roca A, Usuf E, Hoet B, et al.** Carriage of Streptococcus pneumoniae and other respiratory bacterial pathogens in low and lower-middle income countries: A systemic review and meta-analysis. PLoS One 2014; 9: e103293.

29-**Roca A, Hill PC, Tow rend J, Egere U, Antonio M.** Effects of community-wide vaccination with PCV-7 carriage in the Gambia: a cluster-randomized trial. PLoS One 2011; 8: e1001107.

30-**Cheung YB, Zaman SM, Nsekpong ED, Van Beneden CA, Chris A, Adegbola RA, et al.** Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian children who participated in a 9-valent pneumococcal conjugate vaccine trial and in their younger siblings. Pediatr Infect Dis J 2009; 28: 990-995.

31-**Akinsola AK, Ota MO, Enwere GC, Okoko BJ, Zaman SM, Martin O, et al.** Pneumococcal antibody concentrations and carriage of pneumococci more than 3 years after infant immunization with a pneumococcal conjugate vaccine. PLoS One 2012; 7: 1050.

32-**Isaacman DJ, Mclntosh ED, Reinert R.** Burden of invasive pneumococcal disease and serotype distribution among Streptococcus Pneumoniae isolates in young children in Europe: impact of the 7-valent pneumococcal conjugate vaccine and considerations for future conjugate vaccines. Int J Infect Dis 2010; 14: l97-209.

33-**Flasche S, Slack M, Miller E.** Long term trends introduce a potential bias when evaluating the impact of the pneumococcal conjugate vaccination programme in England and Wales. Euro Surveill 2011; 16: 168 -198.

Shormin M, Afroz S, Shamsuzzaman SM, Sultana N. Detection of pneumococcal carriage among under five healthy children with multiple co-colonizing serotypes with impact of pneumococcal conjugate vaccine. Microbes Infect Dis 2021; 2(1): 49-59.