International Journal of Medical Research Professionals
P-ISSN: 2454-6356; E-ISSN: 2454-6364
DOI: 10.21276/ijmrp

IJMRP

Original Research Article.

Detection of *Streptococcus pneumoniae* Colonization and Serotype Distribution by PCR Directly from Nasopharyngeal Swabs

Shormin M[1*], Afroz S[2], Shamsuzzaman SM[3], Rashed A[4]

[1*]MBBS, M. Phil, Assistant Professor, Dept. of Microbiology, Shaheed Monsur Ali Medical College, Dhaka, Bangladesh.
[2]MBBS, M. Phil, Assistant Professor, Dept. of Microbiology, Shahab Uddin Medical College, Dhaka, Bangladesh.
[3]Professor and Head, Dept. of Microbiology, Dhaka Medical College, Dhaka, Bangladesh.
[4]MBBS, MD, Assistant Professor, Dept. of Microbiology, Mugda Medical College, Dhaka, Bangladesh.

ABSTRACT

Introduction: Detection and monitoring of pneumococcal carriage and distribution of serotypes is important to assess the impact and effectiveness of pneumococcal vaccination programs as being a risk for invasive pneumococcal diseases. This study was conducted to identify *Streptococcus pneumoniae* carrier and serotypes from nasopharyngeal swabs by PCR.

Methods: Nasopharyngeal swabs were collected from 200 healthy children under five from Pediatric OPD from a tertiary Hospital. Detection of serotypes was done by conventional multiplex PCR.

Results: Out of 200 nasopharyngeal swabs, 92 (46%) were positive by PCR for *S. pneumoniae*. Among 92 PCR positive swabs, serotypes were detected in 70 (76.09%) nasopharyngeal swabs by conventional multiplex PCR. The more prevalent serotypes were 34F (17.39%), 35B (13.04%)), 6A and 6B (11.96%), 14(7.61%) and 23F (5.43%). For serotyping Quellung method is the gold standard which need viable isolates. But sometimes culture and isolation will be less sensitive which may due to the fastidious nature of the organism and low load of the organism with co-colonization that can result in false negative results. PCR can detect bacterial DNA even if the numbers of the organism is too low to grow in culture and can also detect from non-viable organism after treatment with antibiotics.

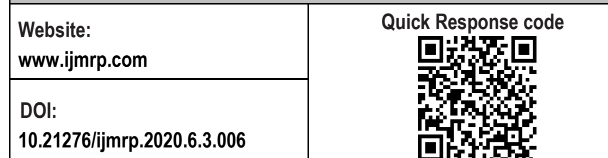
Conclusion: PCR-based serotyping of *S. pneumoniae* from nasopharyngeal swabs will be a valuable tool to assess *S. pneumoniae colonization* and monitor trends in serotype distribution without the requirement for culture and isolation of the organisms.

Key Words: Nasopharyngeal Swabs, Invasive Pneumococcal Diseases, Streptococcal Pneumoniae, Serotypes, Carriage, PCR.

***Correspondence to:**
Dr. Moonmoon Shormin,
Assistant Professor,
Department of Microbiology,
Shaheed Monsur Ali Medical College, Dhaka, Bangladesh.

Article History:
Received: 10-04-2020, **Revised:** 04-05-2020, **Accepted:** 28-05-2020

Access this article online	
Website: www.ijmrp.com	Quick Response code
DOI: 10.21276/ijmrp.2020.6.3.006	



INTRODUCTION

Streptococcus pneumoniae is a bacterium that colonizes the nasopharynx of human and is the most common cause of pneumonia and other invasive pneumococcal diseases (IPD). The capsular polysaccharide, a well-known virulence factor and the serotype determinant of pneumococci, prevents opsonization and phagocytosis of *S. pneumoniae.*[1] *S. pneumoniae* can produce at least 90 capsular serotypes[2], but only a few of these cause most cases of invasive diseases.[3] Identification of these serotypes is essential for production of the new generation of conjugate vaccines and treatment protocols.[4] There are geographical differences in prevalence and distribution of serotypes.[5] Hence, it is necessary to determine the prevalent serotypes of *S. pneumoniae* in different geographical areas. Pneumococcal capsular vaccines protect against disease and nasopharyngeal carriage due to the serotypes included in the vaccine formulations.[6] Nevertheless, surveillance of serotype prevalence patterns is very important since the serotypes responsible for invasive disease can change over time.[7] *S. pneumoniae* is a bacterium that colonizes the nasopharynx of human and main source is person to person transmission. *S. pneumoniae* colonization is often asymptomatic but may cause overt infections. Community-acquired pneumonia (CAP) and infections of normally sterile sites (pleural fluid, cerebrospinal fluid and blood) are the most common infections by *S. pneumoniae* which are collectively called invasive pneumococcal disease (IPD). Pneumococcus is carried in the nasopharynx often with other bacteria, such as *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus*. It is spread by respiratory droplets and

children are the main source of transmission to adults. Universally, carriage rates are highest in young children (40- 60%), compared with older children (12%), adolescents (6 -10%) and adults (3-4%).[8]

Pathogenesis of invasive pneumococcal disease (IPD) begins with nasopharyngeal (NP) colonization that proceeds, often through local infection, to blood stream invasion. Although almost all children become colonized with *Streptococcus pneumoniae* repeatedly during the first few years of life, a very small fraction of these acquisitions results in invasive disease. Many studies of the dynamics and ecology of pneumococcal NP carriage, particularly in the inaccurate identification of *S. pneumoniae* masks the exact estimation of diseases and can delay treatment option. This is the common problem in developing countries due to several reasons that include poor infrastructure, insensitive operational procedure and lack of expertise.[9]

Inconsistent methods for phenotypic detection often delay the early identification and confirmation of *S. pneumoniae* and serotype prevalence is not truly estimated in most of the developing countries. Molecular detection and serotype identification of *S. pneumoniae*, by PCR is highly reliable and can also reduce the reliance upon conventional methods. Thus, it can open the possibilities for development of new conjugate pneumococcal vaccines. In Bangladesh, a few studies for identifying *S. pneumoniae* serotypes from nasopharyngeal swabs using PCR have been carried out.So this study has been designed to identify serotypes from nasopharyngeal swabs using PCR.

MATERIALS AND METHODS

Nasopharyngeal swabs (NPS) were collected from healthy children aged one month to less than five years who attended the outpatient department of DMCH (Dhaka Medical College Hospital) for routine immunization, child growth monitoring and nutritional advice. Nasopharyngeal swabs were collected, labeled, and placed immediately in one ml of skim milk-tryptone-glucose-glycerol (STGG) medium and transported to the laboratory.

Procedure of Polymerase Chain Reaction (PCR)

Bacterial Pellet Formation: Samples preserved in STGG medium were brought out from freeze and kept at room temperature to demoisturize and then samples were vortexed to make a homogenous suspension. Swab stick was removed and the specimens vortexed were taken into two properly labeled micro centrifuge tubes, and 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 liter 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, supernatant was taken using micropipette and used as template DNA for PCR. This DNA was kept at -20°C for future use.

Serotyping by PCR: The primer *cpsA* was used for targeted highly conserved gene that exists in all capsular loci as far characterized. Thirty-eight specific primers (table 1) 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 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), or 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. An initial screening of the nasopharyngeal swabs was done by the primer *cpsA* to identify pneumococci by monoplex PCR. Then serotype specific primers were used for typing by multiplex PCR with positive and negative controls (figure 1,2).

Table 1: List of selected primers with annealing temperature and product size

Name of gene	Oligonucleotide sequence (5'-3')	Annealing temperature (℃)	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		
19Fcpsl-f	CAC CTA ATT TTA ATA CTG AGG TTA AGA TTG C	48	408
19Fcpsl-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		

Primer Pair	Oligonucleotide sequence (5'-3')	Annealing temperature (℃)	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		

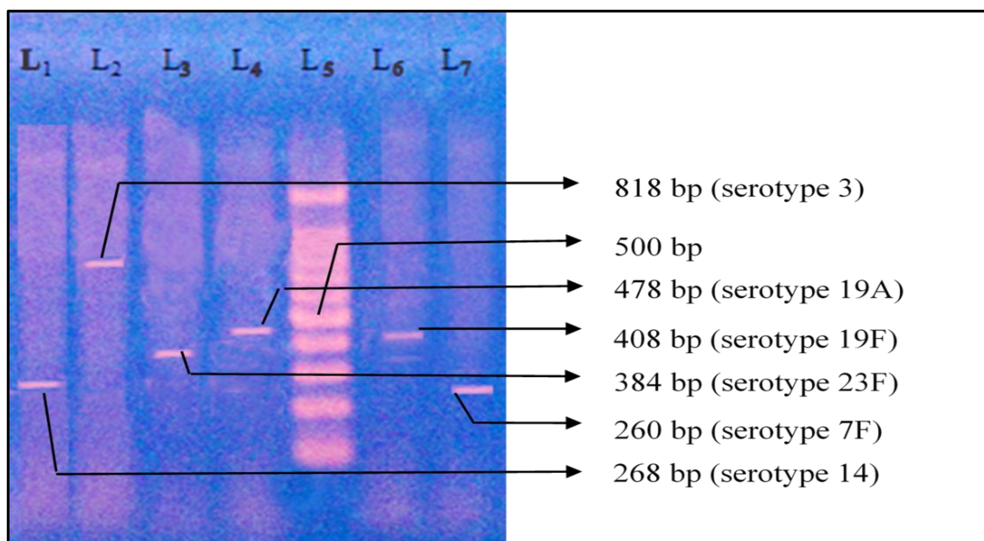


Figure 4: Photograph of gel electrophoresis of detected serotypes

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; **Lane 7:** amplified DNA of 260 bp of serotype 7F.

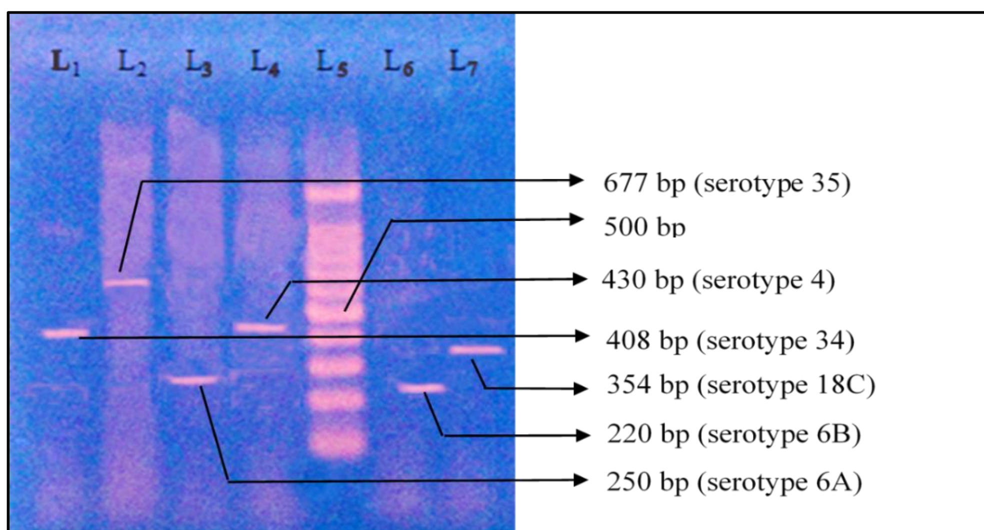


Figure 5: Photograph of gel electrophoresis of detected serotypes

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; **Lane 7:** amplified DNA of 354 bp of serotype 18C.

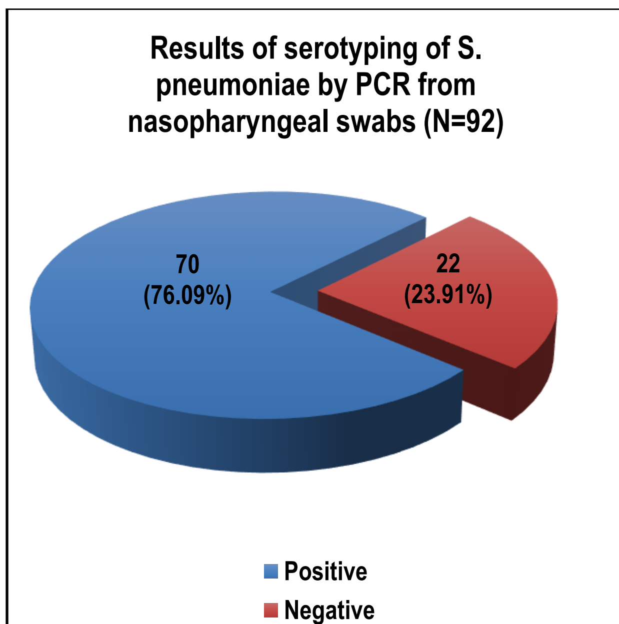


Figure 3: Proportion of detected serotype positive and negative nasopharyngeal swabs by PCR

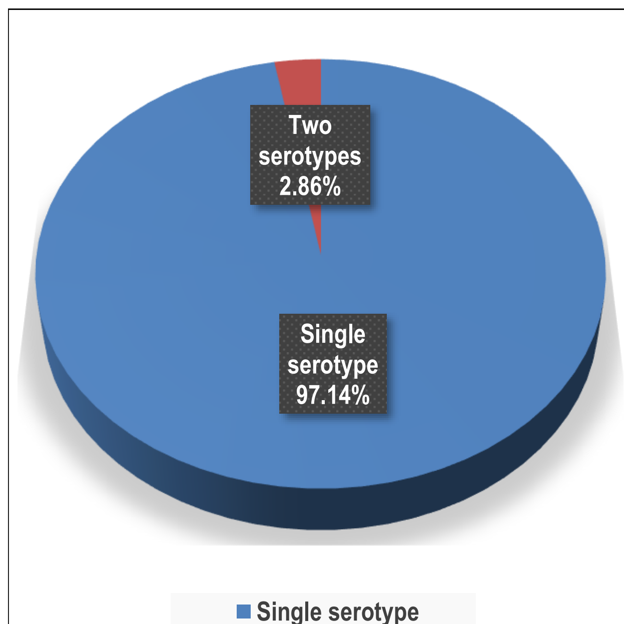


Figure 4: Pattern of serotypes detected in nasopharyngeal swabs by PCR (N=70)

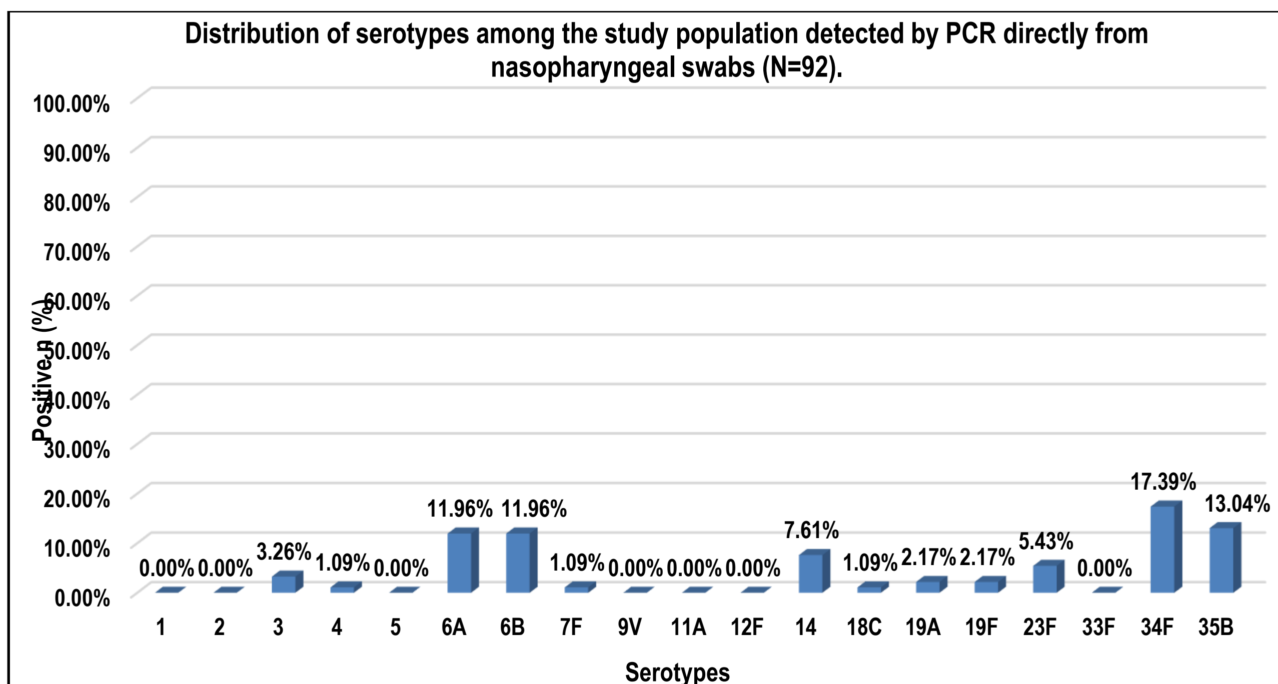


Figure 5 Distribution of serotypes among the study population detected by PCR directly from nasopharyngeal swabs (N=92).

RESULTS

Total 200 under five children were tested, among them, 92 (46%) were positive for *S. pneumoniae* by PCR. Among 92 PCR positive nasopharyngeal swabs for *S. pneumoniae,* 70 (76.09%) were serotype positive and 22 (23.91%) were serotype negative. Figure 3 shows proportion of detected serotype positive and serotype negative nasopharyngeal swabs by PCR.

Among 70 serotype positive nasopharyngeal swabs, 68 (97.14%) had single serotype and 2 (2.86%) had two serotypes (Figure 4).

Among 92 PCR positive nasopharyngeal swabs, 19 different serotypes were seen. 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 5).

DISCUSSION

Streptococcus pneumoniae is a common cause of respiratory infections requiring hospitalization in young children worldwide with increasing rates of antibiotic resistance.[10] Data on serotypes composition of pneumococcal strains from the developing world are scarce.[11] Previously it has been shown that the serotype distribution of invasive *S. pneumoniae* in Bangladesh differs from the distribution in many other parts of the world. The most common serotypes country-wise are as follow: serotype 1 in Nepal; 14 in Bangladesh and India; 19F in Sri Lanka and Pakistan in children of SAARC countries. So, PCV-10 is suitable for countries like India, Nepal, Bangladesh, and Sri Lanka, whereas PCV-13 may be more suitable for Pakistan.[12] Generally, serotype determination of *S. pneumoniae* is performed by culture of the organism followed by serological detection of the capsular type by

Quellung test. This method of serotyping is not widely feasible due to the high cost of antisera, difficulties in interpretation, and requirement of technical expertise, viable pneumococci.[13] The development of PCR-based serotyping has the potential to overcome some of the difficulties associated with the conventional serologic method. In the present study, a total of 200 nasopharyngeal swabs were processed for PCR. Out of 200 nasopharyngeal swabs, In the present study, 92(46%) nasopharyngeal carriage of *S. pneumoniae* was detected by PCR. A study in Switzerland reported that carriage rate was 51.6% by PCR.[14] Another study reported 69% carriage rate in Netherland by PCR.[15] All the results of the previous studies showed higher carriage rate in contrast to the present study. The reasons might be due to different geography, age groups, crowding status, rural area, concomitant respiratory tract illness or HIV infection, nutritional status, co-carriage with other pathogens, seasonal variation and sampling technique. In the present study detected carriage rate was lower than that reported in previous study of Bangladesh.[16] The reasons might be due to the fact that in the previous study data were collected from only lower socio-economic population and from a single community in a government housing area of urban Dhaka. But in the present study data were collected from different socio-economic groups and from the children who attended from different section of society. Moreover, some vaccinated healthy, urban children were enrolled in the present study. Considering culture as gold standard, the sensitivity of PCR to identify *S. pneumoniae* from nasopharyngeal swabs was 100%, specificity was 81.2%, positive predictive value was 72.8%, negative predictive value was 100% and accuracy was 87.5%. Sensitivity of PCR were reported as 100% , 95% and 92%[17-19], which are almost similar to the present study. In the present study, among the serotype positive NP swabs, 68 (97.14%) had one serotype and 2 (2.86%) had two serotypes. No sample was found with more than two serotypes. In the present study serotype distribution pattern is not similar to that reported in previous study of Bangladesh. The differences might be due to sampling technique which described above and as sampling technique was different, detected carriage rate was low than the previous study and multiple serotypes detection rate was low (2.86%) from the low carriage rate (33.50%) in the present study. Besides, a few types of serotype specific primers were used in the present study. 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.[11,20] Detected serotypes (14, 7F, 6A, 6B, 18C, 19A, 23F) from invasive cases and detected serotypes from both meningitis (6, 7, 14, 18) and carriage cases (6, 19, 23, 34, 35, 14). In the present study serotype 1, 2, 5, 9V, 11A, 33F were not found. The reasons might be due to the fact that 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.[11] Serotyping by PCR from NP swabs may be obviated the need for a cultured isolates.[21,22] Several studies[23,24] reported that the high degree of complete concordance (94-100%) between PCR and conventional serotyping methods. The findings of the present study coincide with the findings of previous studies that PCR for detection of pneumococcal serotypes.

LIMITATIONS

Due to time and resource constraint, all the vaccine type and non-vaccine type serotypes could not be detected.

CONCLUSIONS

Quellung method is the gold standard for serotyping of *S. pneumoniae*, which can be performed only on viable isolates, but it is less sensitive. PCR-based serotyping of *S. pneumonia* directly from nasopharyngeal swabs was found to be more sensitive to assess *S. pneumoniae* colonization and monitor trends in serotype distribution without the requirement for culture and isolation of the organisms.

RECOMMENDATIONS

PCR might be included for continuous monitoring and surveillance of serotypes of *S. pneumoniae* for future pneumococcal vaccination program in Bangladesh.

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Source of Support: Nil.

Conflict of Interest: None Declared.

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Cite this article as: Shormin M, Afroz S, Shamsuzzaman SM, Rashed A. Detection of *Streptococcus pneumoniae* Colonization and Serotype Distribution by PCR Directly from Nasopharyngeal Swabs. Int J Med Res Prof. 2020 May; 6(3): 23-28.
DOI:10.21276/ijmrp.2020.6.3.006