The detection of altered penicillin-binding protein 2b, autolysin, and pneumolysin genes in *Streptococcus pneumoniae* colonizing the upper respiratory tract of healthy schoolchildren in Puducherry

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**Abstract**

**Background:** Infections due to *Streptococcus pneumoniae* are responsible for morbidity and mortality in a large proportion of children in developing countries where childhood vaccination has not been widely implemented. *S. pneumoniae* colonizing the upper respiratory tract of children as a normal commensal is a potential pathogen when it crosses the mucosal barrier and enters the bloodstream.

**Aims and Objectives:** The aim of the study was to analyze the carriage rate of pneumococci among healthy school children and in doing so to analyze the presence of penicillin-binding protein 2b (pbp2b) gene which is responsible for penicillin resistance using polymerase chain reaction.

**Methods:** Nasopharyngeal swabs were collected from school children and processed according to standard microbiological procedures. The genes for penicillin resistance were detected using PCR.

**Results:** The carriage rate was 32.2% and about 14 strains had the pbp2b gene.

**Conclusion:** Pneumococcal carriage among children is important as they can cross the mucosal barrier and cause infection. Constant follow up of the carriage rate have to be performed to prevent further spread.

**Keywords:** Autolysin, penicillin-binding protein 2b, pneumolysin, polymerase chain reaction, *Streptococcus pneumoniae*

**INTRODUCTION**

The burden of disease due to *Streptococcus pneumoniae* is high in children across the world. It is known to cause invasive infections in children between the age groups of 5 and 10 years, common infections being otitis media, meningitis, and pneumonia. Pneumococcal disease is preceded by asymptomatic colonization in children. The nasopharynx acts as a reservoir for commensal organisms such as *S. pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis*.

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These organisms can spread to the sinuses, lower respiratory tract, and middle ear via the Eustachian tube and cause bacteremia. According to recent reports, the prevalence of pneumococcal carriage rate in children ranges from 23.5% to 32%. The carrier rate is more in children >24 months of age with evidence to show that pneumococcal carriage starts as early as the neonatal period.[1]

Globally, the burden of pneumococcal disease according to the World Health Organization accounts for 8%–15% of deaths among children aged 1–59 months of age. This wide range in prevalence is of much importance; however, the prevalence among HIV-positive and immunocompromised children is higher than immunocompetent children. In a study done in 2009, in Europe, a high incidence of pneumococcal meningitis was noted, whereas the South Asian countries reported the least number of cases,[2] but the scenario has changed since then.

The detection of penicillin-binding protein 2b (\textit{pbp2b}) gene and pneumolysin (\textit{ply}) and autolysin (\textit{lyt-A}) genes in penicillin-resistant strains of \textit{S. pneumoniae} from throat swabs of children will help to determine the circulation of these potential resistance and virulence genes in the community. The present study was carried out with this aim.

**SUBJECTS AND METHODS**

The study was reviewed and cleared by the Institute Research and Ethics and Committees.

Five hundred apparently healthy schoolchildren between 5 and 10 years of age were included in the study. Children with upper respiratory tract infections were excluded from the study.

Following consent by the parents and from the children and with permission from school authorities, samples were collected from healthy children from schools which were located in Puducherry. Sterile alginate swabs were used for collecting the throat swabs.

**Processing of samples and antibiotic sensitivity testing**

The swabs were then immediately inoculated onto blood agar plates containing 5% sheep blood with 5 μg/ml of Gentamycin and were processed according to the standard procedures. \textit{S. pneumonia} (ATCC 49619) and \textit{Streptococcus viridians} (ATCC 33399) were used as positive and negative controls, respectively.

The zone of inhibition was measured, and the results were interpreted according to the Clinical Laboratory Standards Institute 2013 guidelines.

Further, the isolates were stored in skimmed milk with glycerol at −20°C. For molecular studies, DNA extraction was done using the HiPurA Bacterial Genomic DNA Purification Kit (Himedia, India). DNA thus obtained was stored at −70°C as the first step of molecular analysis to detect the presence of resistant gene \textit{pbp2b} among the penicillin-resistant isolates. A seminested polymerase chain reaction (PCR)-based assay was done for the detection of penicillin-binding protein gene. Amplification was carried out using a thermal cycler. The 50 μl reaction mixture contained 0.5 μg of DNA, 2 mM MgCl₂, 200 mM deoxynucleoside triphosphates (Boehringer Mannheim, Mannheim, Germany), 50 mM KCl, 10 mM Tris-HCl (pH 8), and 1.0 mM each of primers, and 2.5 U of Taq DNA polymerase. The presence of \textit{lytA} gene was detected by the amplification of the 319 bp fragment of the \textit{lytA} gene. For amplification of \textit{lytA} gene, the primers were designed using GeneTool software program by Helini Biomolecules, Chennai 600115 [Table 1]. The PCR products were analyzed by electrophoresis through 2% agarose gel containing ethidium bromide and were visualized with a ultraviolet (UV) transilluminator.

**Table 1: Primers used in the study to detect \textit{pbp2b}, autolysin, and pneumolysin genes**

| Gene detected                      | Primer used                        |
|-----------------------------------|------------------------------------|
| Penicillin binding protein gene (\textit{pbp2b}) | R1 GCCTTTTCTAGGCCAATGCCGATTAC  
R2 GCCTACGATTCATICCCAGAT  
R3 AATTGGCCAATATGGATCTTTTCT   
R4 GTTTAAACTAACAATTAGAATCC  
P5 CTGACCATGATTGCGCTTTCCA   
P6 TTGGCAATAGTTGCTATACATCTG |
|                                    | Each assay contained reactions R1, R3, P5, P6 and primers R2, R4, P5, P6 |
| Autolysin gene                     | Forward primer  
Reverse primer |
| Pneumolysin gene                   | Forward primer  
Reverse primer |
RESULTS

Among the 500 children screened, 161 children (32.2%) were carriers of *S. pneumoniae*, while 67.8% had normal upper respiratory flora. The carriers of *S. pneumoniae* were found to be more in 10 years of age and less in 7-year-old children. Statistical analysis between the carriers and noncarriers with respect to age showed statistically significant ($P = 0.002$) association.

The isolates of *S. pneumoniae* showed a variable susceptibility pattern against commonly used antibiotics [Table 2].

Two genes’ coding for virulence in *S. pneumoniae*, i.e.,: lyt-A and ply, was detected by PCR.

A total of 14 isolates were tested for the presence of lyt-A gene, and all 14 isolates were found to possess the gene and 8 strains tested positive for the presence of ply gene.

DISCUSSION

*S. pneumoniae* have the potential to become pathogenic when they cross the mucosal barrier or gain entry to the bloodstream. In children, the invasive infections caused by the organism are primarily meningitis and septicemia. Despite the availability of potent antibiotics, the mortality rate due to meningitis remains significantly high in India.[3] It is believed that most invasive infections leading to severe pneumococcal disease could be from the patients’ own normal flora. There is a need to routinely monitor the population for carriage and determine the antibiotic susceptibility pattern of this organism, as this might serve as a prerequisite for developing infections in the future.

The nasopharyngeal colonization of *S. pneumoniae* around the world differs with race and socioeconomic status. Among the children who were screened for pneumococcal colonization in the present study, the carriage rate was found to be 32.2%. A study done among children attending a daycare center in Hong Kong showed that the carriage rate of *S. pneumoniae* was 19.4% overall, whereas 28.8% of the carriers belonged to 2–3 years and 32% in children of 4 years.[6] Whereas studies from India have reported the carriage rate to be as high as 86.2% from infants in the first 6 months of life. Nearly half the children were colonized by 2 months of age.[7] This explains that pneumococcal colonization varies from country to country.

Age-wise distribution of the children in the present study showed that the carriage was found to be more in children belonging to 10 years of age (56.4%) compared to children belonging to 7 years of age (21.2). However, the carriage rate between 5 and 9 years of age did not show much difference. Statistical analysis showed that these data were statistically significant ($P = 0.002$) by Chi-square test. This was in contrast to a study done by Kanungo et al. who showed that the prevalence of Pneumococci among children aged <1 year was 62% (755 isolates out of 1218)[8] where the percentage of carriers from the younger age group was 28% and percentage of carriers belonging to 10 years of age was 13%, respectively, from Puducherry. However, there are studies that showed highly variable colonization rate from 24% to 84%, with children being the highest, and the colonization was found to decrease with age.[9]

Increasing antibiotic pressure on the colonizers would reflect on the susceptibility of invasive isolates. There are several studies that show different susceptibility patterns within countries. Reports of variations within race and sex have also been documented.[10] In our study, among the commonly used antibiotics, only 41.6% of the isolates were susceptible to erythromycin; very high rates of erythromycin resistance were observed in Asian countries such as Vietnam (92.1%) and Taiwan (86%). In the present study, 57.8% of the isolates were susceptible to cotrimoxazole and 63.4% were susceptible to cefaclor and penicillin; susceptibility was observed in 62.1%.

The most common antibiotics to show resistance were erythromycin, and trimethoprim-sulfamethoxazole. The isolates amounting to 82.6% were susceptible to chloramphenicol, and 87.6% were susceptible to cefotaxime, and 91.3% of the strains were susceptible to clindamycin, and gatifloxacin susceptibility was seen in 83.9%, and 98.1% were susceptible to moxifloxacin. Meropenem had a susceptibility of 85.1%. The reason could be indiscriminate use of antibiotics for causes not requiring antibiotics, such as viral infections. The other reason could be easy availability of antibiotics as over the counter drugs. Erythromycin is a commonly employed antibiotic for upper respiratory tract infections in most of the primary health centers and hospitals; the

**Table 2: Susceptibility pattern of colonizing pneumococci**

| Antibiotic       | Percentage susceptible |
|------------------|------------------------|
| Erythromycin     | 41.6                   |
| Cotrimoxazole    | 57.8                   |
| Cefaclor         | 63.4                   |
| Penicillin       | 62.1                   |
| Chloramphenicol  | 82.6                   |
| Cefotaxime       | 87.6                   |
| Clindamycin      | 91.3                   |
| Gatifloxacin     | 85.1                   |
| Moxifloxacin     | 98.1                   |

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degree of resistance observed in the present study seems to be of significance.

In the present study, we have tried to correlate the antibiotic resistance pattern between the schools to show a difference between rural and urban populations. When all the four schools were compared, it was seen that there was some difference between susceptibilities to penicillin, cefaclor, and cefotaxime, and this was statistically significant \( P < 0.001 \).

Penicillin resistance is an important marker for detecting multidrug resistance. Until the late 1995, all the strains of pneumococcal were sensitive to penicillin; later on, there was a surge in the resistance pattern observed.\(^8\) Furthermore, one of the mechanisms of penicillin resistance is due to the alteration in the \( pbp2b \) gene, which is the receptor responsible for binding of the drug to the organism. Other mechanisms have emerged at the molecular level. It has also been found that some strains contain altered \( murM \) allele in addition to \( pbp \) genes which can contribute to the mechanism of resistance.\(^9-12\)

To detect the genes for penicillin resistance, an attempt was made to detect the presence of altered \( pbp2b \) gene in these resistant isolates using PCR using specific primers. However, none were found to have an altered gene. Ten strains were positive for the \( pbp2b \) gene [Figure 1]. However, it was found that the proteins were not altered. Perhaps, other mechanisms exist for the resistance to penicillin; the primers used were not specific for altered penicillin-binding protein genes.

Virulence factors coded for by \( ply \) [Figure 1] and \( lytA \) genes could play an important role in the invasiveness of pneumococci. We have tried to detect the presence of these genes among the colonizers to determine their virulence potential. Eleven strains tested were positive for \( lyt-A \) gene and all the isolates were found to possess the gene. Similarly, 14 strains showed that ply genes were present in all strains. On comparing our results with the results obtained by Kanungo et al., out of 24 isolates, \( lytA \) gene was found to be positive in all the strains except one strain. Moreover, 18 strains were positive for ply out of 24 strains.\(^13\)

**CONCLUSION**

Pneumococci continue to be a major colonizer among healthy schoolchildren between 5 and 10 years in schools from different localities within the union territory. Varied environmental factors can influence the carriage rate, as shown in the study. Drug resistance among the colonizers continues to be very high, suggesting the exposure of these children to antibiotic consumption. This highlights the unregulated antibiotic usage in the community. The lack of detection of penicillin-binding protein as a common mechanism for resistance among a few isolates indicates that alternate mechanisms of resistance to beta-lactams may exist in the colonizing \( S. pneumoniae \) among the study population. The presence of genes for ply and \( lyt-A \) among the colonizers indicate that these organisms have the potential of becoming virulent and leading to invasive pneumococcal disease. In the light of these findings, it is necessary to monitor healthy schoolchildren for the carriage of \( S. pneumoniae \) and other potential pathogens such as \( H. influenzae \) and Group A streptococci to prevent invasive infections by these organisms.

**Acknowledgment**

We would like to acknowledge Dr. K. Prasanth Professor, Department of Biochemistry, Pondicherry University, Puducherry, and his students for their expertise and help in the molecular work done in this study.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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