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Research Article

Genotypic Profiling of pbp2b, lytA and ply Genes and Multidrug Resistance Patterns in Nasopharyngeal Streptococcus pneumoniae Isolates from Healthy Indian Schoolchildren in Puducherry: A Cross-Sectional Molecular Study

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Abstract

Background: Nasopharyngeal colonisation by Streptococcus pneumoniae is an important reservoir for transmission and antimicrobial-resistant pneumococci. This study characterised antimicrobial susceptibility, multidrug resistance (MDR), and the distribution of lytA, ply and altered pbp2b among pneumococcal isolates recovered from healthy Indian schoolchildren.

Methods: We evaluated 21 nasopharyngeal S. pneumoniae isolates from asymptomatic children aged 6-9 years. Conventional phenotypic testing and PCR detection of lytA and ply supported species identification. We performed oxacillin screening and minimum inhibitory concentration (MIC) testing for penicillin, erythromycin, co-trimoxazole, ceftriaxone, and levofloxacin. Altered pbp2b was examined by PCR. We analysed categorical resistance, MIC50/MIC90, MDR patterns, genotype-phenotype associations, and correlations between oxacillin-zone diameter and MIC.

Results: lytA and ply were detected in all 21 isolates, while altered pbp2b was present in 15 (71.4%). Resistance was most frequent to co-trimoxazole (71.4%) and erythromycin (66.7%), followed by penicillin (33.3%) and ceftriaxone (14.3%); no isolate was resistant to levofloxacin. MDR was recorded in 14 (66.7%) isolates. Altered pbp2b was strongly associated with a smaller oxacillin zone, higher penicillin MIC and MDR (14/15 vs 0/6; $p<0.001$). Oxacillin-zone diameter showed strong inverse correlations with penicillin, erythromycin, co-trimoxazole and ceftriaxone MICs.

Conclusions: The isolates showed a substantial burden of macrolide and co-trimoxazole resistance, frequent MDR, and a close link between altered pbp2b and reduced beta-lactam susceptibility. Continued molecular and phenotypic surveillance of asymptomatic carriage may help define community reservoirs of resistant pneumococci and inform antimicrobial and vaccination strategies.

Keywords: Streptococcus pneumoniae; nasopharyngeal carriage; pbp2b; lytA; ply; antimicrobial resistance; multidrug resistance; schoolchildren; India

1. Introduction

Streptococcus pneumoniae remains one of the most consequential bacterial pathogens in childhood, causing pneumonia, meningitis, bacteraemia and other invasive syndromes across diverse health-care settings. The organism contributes substantially to preventable morbidity and mortality, particularly in children, and its importance extends beyond invasive disease because the same species circulates widely in the upper respiratory tract of otherwise healthy individuals. Global estimates have demonstrated the large historical burden attributable to pneumococcal disease and helped establish pneumococcal prevention as a major child-health priority [1].

The nasopharynx is the principal ecological niche of S. pneumoniae and the starting point for both person-to-person transmission and progression to disease. Colonisation is usually asymptomatic, but it allows pneumococci to persist, compete with other respiratory organisms, exchange genetic material, and acquire determinants that influence antimicrobial susceptibility and virulence. For this reason, carriage isolates offer information that is biologically different from, but complementary to, isolates recovered from sterile clinical sites. The central role of colonisation in pneumococcal pathogenesis and population transmission has been extensively described [2].

Carriage is particularly common during childhood, when repeated acquisition and clearance can occur and when close contact in households, schools and other congregate environments may facilitate dissemination. The composition of the carriage reservoir can therefore influence the strains available for transmission in the community. Carriage studies also provide a practical window into the effects of age, vaccination and antimicrobial selection on circulating pneumococci, and they can detect resistant organisms before these strains necessarily become dominant causes of invasive infection. The epidemiological link between pneumococcal carriage and subsequent disease has consequently become an important component of pneumococcal surveillance and vaccine evaluation [3].

Antimicrobial resistance adds a second layer of concern. Pneumococci can develop reduced susceptibility to beta-lactams through alterations in penicillin-binding proteins and may simultaneously acquire resistance to macrolides, folate-pathway inhibitors and other drug classes. In India, pooled pre-2019 evidence from invasive paediatric isolates showed particularly high non-susceptibility to trimethoprim-sulfamethoxazole, with clinically relevant resistance also observed to erythromycin and penicillin, supporting the need for continued local surveillance rather than reliance on uniform assumptions about susceptibility [4].

Indian carriage data are especially important because invasive disease surveillance alone cannot describe the entire community reservoir. A multicentre cross-sectional study conducted before widespread pneumococcal conjugate vaccine introduction in India documented substantial pneumococcal colonisation and showed antimicrobial resistance among community isolates as well as isolates from children with pneumonia. These findings established a useful pre-vaccine baseline and emphasised

immunisation's potential to alter both carriage serotypes and the distribution of resistant pneumococci [5].

Surveillance across Asian countries has repeatedly identified high frequencies of antimicrobial resistance among pneumococcal isolates, including macrolide resistance and multidrug phenotypes. This regional pattern suggests that local carriage studies should examine not only single-agent resistance but also clustering of resistance across antimicrobial categories, because co-resistance can constrain empirical treatment choices and may reflect successful transmission of resistant lineages [6].

At the molecular level, reduced beta-lactam susceptibility in S. pneumoniae is closely linked to structural changes in penicillin-binding proteins rather than beta-lactamase production. Alterations involving PBP2B and related PBPs can reduce affinity for beta-lactam antibiotics and contribute to stepwise increases in MIC. The molecular architecture of beta-lactam resistance has made pbp genes useful targets for studying the connection between genotype and phenotypic susceptibility [7]. Alongside resistance determinants, pneumococcal genes such as lytA, encoding the major autolysin, and ply, encoding pneumolysin, are commonly examined as molecular markers that support species characterisation and describe the virulence-gene profile of isolates.

This study characterised nasopharyngeal S. pneumoniae isolates from healthy Indian schoolchildren. The objectives were to describe antimicrobial susceptibility and MIC distributions for five clinically relevant agents; determine MDR prevalence; define the frequency of lytA, ply, and altered pbp2b; and assess the relationship between altered pbp2b, oxacillin-zone diameter, beta-lactam MIC, and broader co-resistance patterns. By integrating phenotypic and molecular findings at the isolate level, the study aimed to provide a focused view of the resistant pneumococcal reservoir in asymptomatic school-aged children.

This study is a secondary laboratory and statistical analysis focusing on isolate-level phenotypic–genotypic correlations and multidrug resistance patterns across 21 nasopharyngeal Streptococcus pneumoniae isolates previously recovered and identified during a carriage survey conducted in Puducherry [21]. The primary isolation and partial characterization were described previously; the present analysis contributes comprehensive quantitative MIC distributional analyses (MIC50/MIC90), non-parametric correlation matrices between oxacillin zone diameters and multiple antimicrobial classes, and detailed multivariable resistance combination profiling.

2. Materials and Methods

2.1 Study design and isolate population

We conducted a cross-sectional, laboratory-based study using 21 nasopharyngeal Streptococcus pneumoniae isolates from asymptomatic schoolchildren. The children were 6-9 years of age and represented ten coded school clusters. Demographic variables available for analysis included age, sex and pneumococcal conjugate vaccine (PCV) immunisation status. The analytic dataset contained coded isolate and school identifiers without personal names. The study focused on characterisation of recovered pneumococcal isolates and was not designed to estimate population carriage prevalence.

2.2 Nasopharyngeal sampling, transport and primary isolation

We collected nasopharyngeal specimens using sterile flexible swabs and transferred them into skim milk-tryptone-glucose-glycerol transport medium. Specimens were maintained under cold-chain conditions during transport, with longer-term storage at low temperature when required. Laboratory processing followed standard pneumococcal carriage methodology, in which careful nasopharyngeal sampling, appropriate transport medium, controlled storage, and selective culture are essential to preserve pneumococcal viability and minimise methodological variability [8].

After thawing, when applicable, specimens were mixed thoroughly and inoculated onto 5% sheep blood agar supplemented with gentamicin. Plates were incubated at 37 °C in a carbon dioxide-enriched atmosphere for 18-24 hours. Colonies consistent with pneumococci were selected based on small grey alpha-haemolytic morphology and central umbilication. Conventional confirmation included a negative catalase reaction, optochin susceptibility and bile solubility.

2.3 Antimicrobial susceptibility testing

Phenotypic susceptibility testing included oxacillin screening and MIC determination for penicillin, erythromycin, co-trimoxazole, ceftriaxone and levofloxacin. We prepared a 0.5 McFarland inoculum from fresh, pure colonies and inoculated it onto Mueller-Hinton medium supplemented for fastidious pneumococci. We used a 1-microgram oxacillin disk as the screening test and recorded inhibition-zone diameters in millimetres. We performed quantitative MIC testing using a broth microdilution framework appropriate for S. pneumoniae. We used susceptible, intermediate, and resistant categories recorded in the study laboratory dataset for categorical analyses, with susceptibility testing organised within the contemporaneous CLSI M100 framework [9]. In addition to categorical interpretation, we retained raw MIC values for distributional analysis, calculation of MIC50 and MIC90, and genotype-phenotype comparisons.

2.4 DNA extraction and PCR amplification

Fresh colonies were suspended in nuclease-free water and subjected to enzymatic weakening of the Gram-positive cell wall using mutanolysin or lysozyme, followed by thermal lysis. After centrifugation, the DNA-containing supernatant was transferred to a clean tube and stored frozen until amplification. PCR assays targeted lytA, ply and altered pbp2b. The lytA primer pair was CAACCGTACAGAATGAAGCGG and TTATTCGTGCAATACTCGTGCG, yielding an expected amplicon of approximately 273 bp. The ply primer pair was ATTTCTGTAACAGCTACCAACGA and GAATTTTCATAATTTCCATCTTC, yielding an expected amplicon of approximately 348 bp. The pbp2b primer pair was GATCCTAACTTTGATGCAGTCA and TTAATTCCAGCTACTTCTTTTGC, with an expected product of approximately 1500 bp.

Amplification included initial denaturation at 94 degrees C for 5 minutes, followed by 30-35 cycles of denaturation at 94 degrees C for 45 seconds, annealing at approximately 54-56 degrees C for 45 seconds and extension at 72 degrees C for 1 minute, with a final extension at 72 degrees C for 7 minutes. PCR products were resolved on 1.5-2.0% agarose gels and interpreted against a 100-bp molecular-size ladder. Streptococcus pneumoniae ATCC 49619 served as the positive control, and a template-free reaction as the negative control.

2.5 Outcome definitions

Primary descriptive outcomes were the proportions of isolates classified as susceptible, intermediate or resistant to each tested antimicrobial; MIC50 and MIC90 values; prevalence of lytA, ply and altered pbp2b; and the proportion recorded as MDR. We used MDR terminology to describe acquired resistance across multiple antimicrobial categories, consistent with the broader international framework for standardising multidrug-resistance terminology [10]. Secondary analyses examined associations between altered pbp2b and phenotypic susceptibility, differences between MDR and non-MDR isolates, resistance combinations, demographic distribution of MDR, and correlations between oxacillin-zone diameter and antimicrobial MIC.

2.6 Statistical analysis

We summarised categorical data as counts and percentages. For key resistance proportions and MDR prevalence, we calculated 95% confidence intervals using the Wilson method. Because of the small sample size and the discrete distribution of several MIC values, we summarised continuous variables using the median and interquartile range (IQR); we also reported MIC ranges, MIC50, and

MIC90. We compared continuous variables between groups using the Mann-Whitney U test. We assessed categorical comparisons with Fisher's exact test. Spearman rank correlation was used to quantify the relationship between oxacillin inhibition-zone diameter and MIC values. All tests were two-sided, and $p<0.05$ was considered statistically significant. The analyses were exploratory and no multiplicity adjustment was applied.

2.7 Ethics

The study protocol received IEC approval through the Department of Microbiology, Apollo Institute of Medical Sciences, Chittoor, Andhra Pradesh. Laboratory analyses and statistical evaluation were conducted on coded study isolates.

3. Results

3.1 Study population and molecular profile

We included 21 S. pneumoniae isolates, all from asymptomatic schoolchildren. The median age was 8 years (IQR 7-8; range 6-9 years); 11 children (52.4%) were male and 10 (47.6%) were female. Fourteen children (66.7%) were recorded as PCV-immunised. We detected the species-associated lytA and ply genes in all 21 isolates. Altered pbp2b was present in 15 (71.4%) isolates, and the same number had an oxacillin inhibition-zone diameter below 20 mm. MDR was recorded in 14 isolates (66.7%; 95% CI 45.4-82.8%).

Table 1. Demographic, clinical and genotypic characteristics of the study isolates

Characteristic	Measure	Result
Age, years	Median (IQR)	8 (7-8)
Age 6 years	n (%)	4 (19.0%)
Age 7 years	n (%)	6 (28.6%)
Age 8 years	n (%)	6 (28.6%)
Age 9 years	n (%)	5 (23.8%)
Male	n (%)	11 (52.4%)
Female	n (%)	10 (47.6%)
PCV-immunised	n (%)	14 (66.7%)
Unvaccinated	n (%)	7 (33.3%)
lytA positive	n (%)	21 (100.0%)
ply positive	n (%)	21 (100.0%)
Altered pbp2b present	n (%)	15 (71.4%)
Oxacillin zone <20 mm	n (%)	15 (71.4%)
MDR	n (%)	14 (66.7%)

PCV, pneumococcal conjugate vaccine; MDR, multidrug-resistant; IQR, interquartile range.

3.2 Antimicrobial susceptibility and MIC distribution

Co-trimoxazole showed the highest resistance frequency, affecting 15 of 21 isolates (71.4%), followed by erythromycin in 14 (66.7%). Penicillin resistance was recorded in 7 isolates (33.3%) and ceftriaxone resistance in 3 (14.3%). No levofloxacin-resistant isolate was identified. Intermediate susceptibility was most frequent for penicillin, occurring in 8 isolates (38.1%). Penicillin MICs ranged from 0.015 to 8 micrograms/mL with MIC50 and MIC90 values of 1 and 4 micrograms/mL, respectively. Erythromycin MIC50/MIC90 were 2/16 micrograms/mL, co-trimoxazole 4/8 micrograms/mL, ceftriaxone 0.5/2 micrograms/mL and levofloxacin 1/1 microgram/mL.

Table 2. Antimicrobial susceptibility profile and MIC distribution

Agent	MIC range	MIC50	MIC90	S n (%)	I n (%)	R n (%)	95% CI for R
Penicillin	0.015-8	1	4	6 (28.6%)	8 (38.1%)	7 (33.3%)	17.2-54.6
Erythromycin	0.03-64	2	16	6 (28.6%)	1 (4.8%)	14 (66.7%)	45.4-82.8
Co-trimoxazole	0.25-16	4	8	4 (19.0%)	2 (9.5%)	15 (71.4%)	50.0-86.2
Ceftriaxone	0.06-4	0.5	2	14 (66.7%)	4 (19.0%)	3 (14.3%)	5.0-34.6
Levofloxacin	0.5-2	1	1	19 (90.5%)	2 (9.5%)	0 (0.0%)	0.0-15.5

MIC values are expressed in micrograms/mL. S, susceptible; I, intermediate; R, resistant; CI, confidence interval.

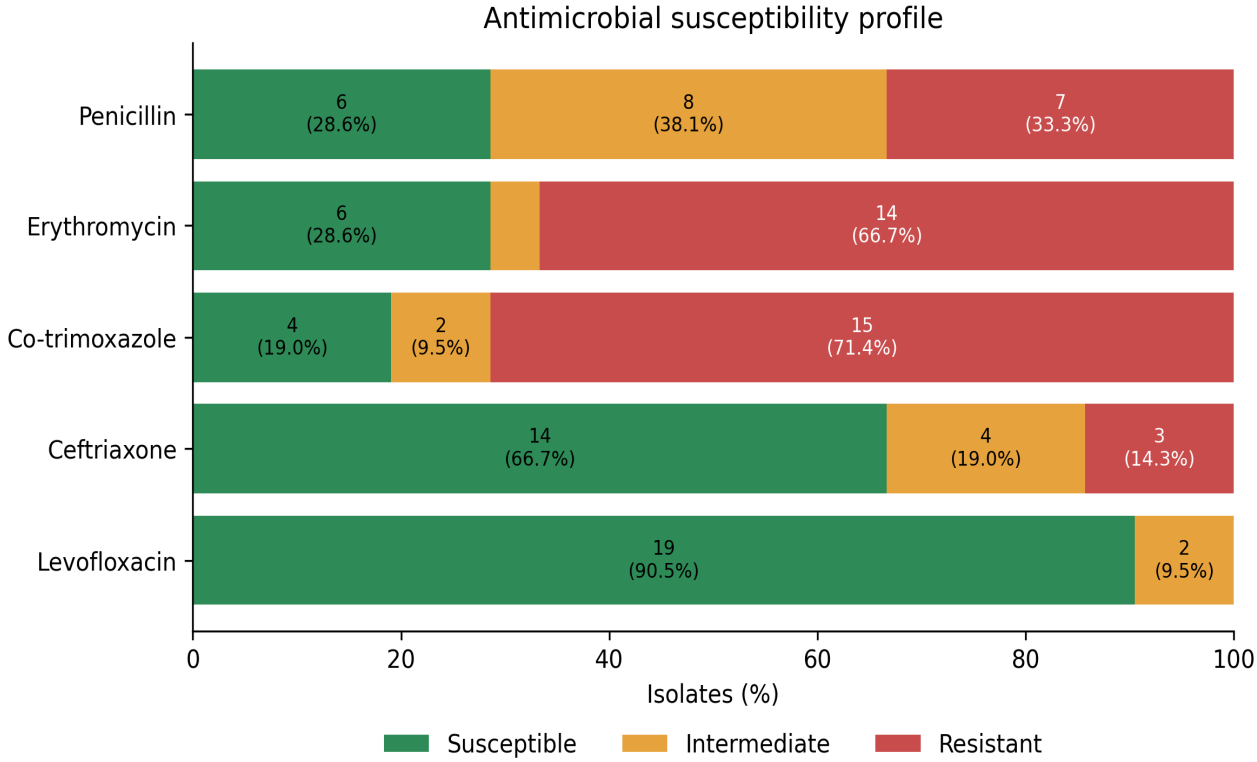


Figure 1. Distribution of susceptible, intermediate and resistant isolates across the five tested antimicrobial agents.

3.3 Association of altered pbp2b with phenotypic resistance

Altered pbp2b was detected in 15 isolates and showed a strong phenotypic relationship with reduced beta-lactam susceptibility. The median oxacillin zone among altered-pbp2b isolates was 15 mm (IQR 10.5-16) compared with 22.5 mm (IQR 22-23.75) among pbp2b-negative isolates ($p<0.001$). Median penicillin MIC was 1 microgram/mL (IQR 1-2) in the altered-pbp2b group and 0.03 microgram/mL (IQR 0.019-0.052) in the negative group ($p<0.001$). Altered-pbp2b isolates also had significantly higher erythromycin, co-trimoxazole and ceftriaxone MICs (all $p<0.001$). MDR was recorded in 14/15 (93.3%) isolates carrying altered pbp2b and in none of the six isolates without the marker (Fisher exact $p<0.001$). Levofloxacin MIC did not differ significantly by pbp2b status ($p=0.155$).

Table 3. Phenotypic comparison according to altered pbp2b status

Variable	pbp2b present (n=15)	pbp2b absent (n=6)	Statistic	p value
Oxacillin zone, mm	15 (10.5-16)	22.5 (22-23.75)	0.0	<0.001
Penicillin MIC, micrograms/mL	1 (1-2)	0.03 (0.019-0.052)	90.0	<0.001
Erythromycin MIC, micrograms/mL	4 (2-12)	0.09 (0.06-0.12)	90.0	<0.001
Co-trimoxazole MIC, micrograms/mL	4 (3-8)	0.5 (0.31-0.88)	90.0	<0.001
Ceftriaxone MIC, micrograms/mL	0.5 (0.5-1)	0.12 (0.12-0.22)	89.0	<0.001
Levofloxacin MIC, micrograms/mL	1 (0.5-1)	0.5 (0.5-0.88)	62.0	0.155
MDR, n/total	14/15 (93.3%)	0/6 (0.0%)	Fisher	<0.001

Continuous data are median (IQR) and were compared using the Mann-Whitney U test. MDR was compared using Fisher exact test.

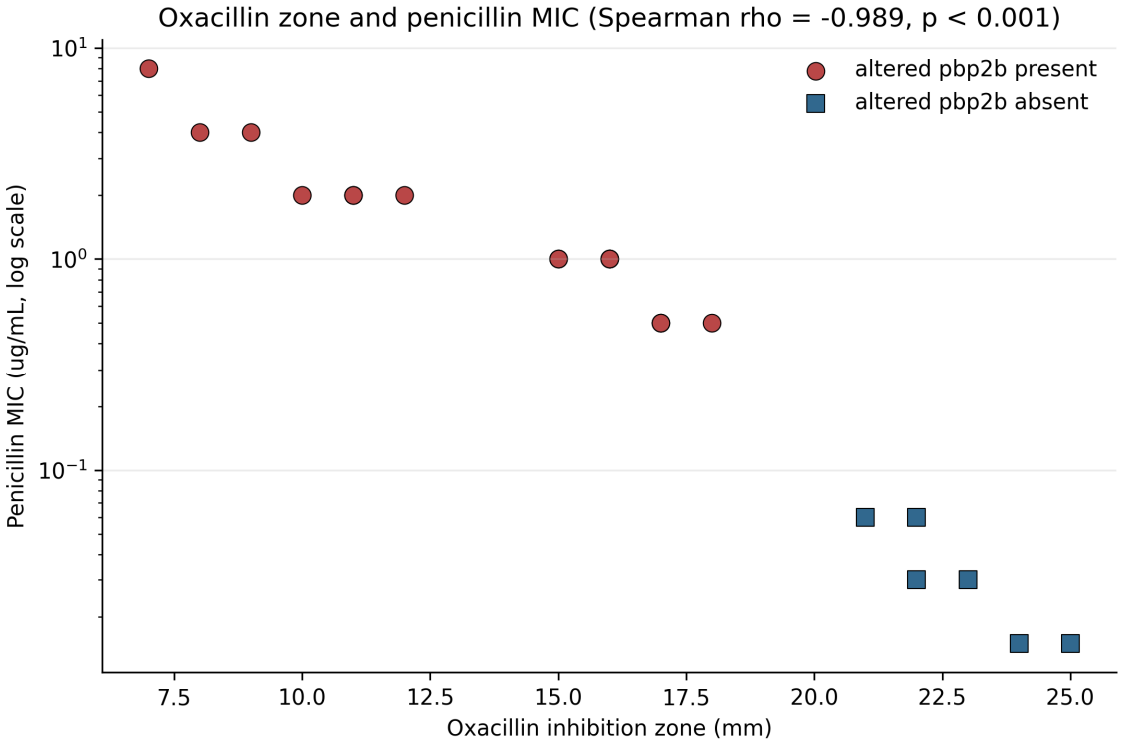


Figure 2. Relationship between oxacillin inhibition-zone diameter and penicillin MIC according to altered pbp2b status.

3.4 Characteristics of multidrug-resistant isolates

The 14 MDR isolates showed a distinct phenotypic profile. Their median oxacillin zone was 13.5 mm (IQR 10.25-16), compared with 22 mm (IQR 21.5-23.5) in non-MDR isolates (p<0.001). Median penicillin, erythromycin, co-trimoxazole and ceftriaxone MICs were all significantly higher in the MDR

group (all p<0.001), while the difference in levofloxacin MIC was not statistically significant (p=0.282). These findings show that the MDR designation was concentrated among isolates with simultaneous elevation of several beta-lactam and non-beta-lactam MICs rather than being driven by levofloxacin resistance.

Table 4. Phenotypic characteristics of MDR and non-MDR isolates

Variable	MDR (n=14)	Non-MDR (n=7)	U statistic	p value
Oxacillin zone, mm	13.5 (10.25-16)	22 (21.5-23.5)	0.0	<0.001
Penicillin MIC, micrograms/mL	1.5 (1-2)	0.03 (0.022-0.06)	97.0	<0.001
Erythromycin MIC, micrograms/mL	6 (2.5-14)	0.12 (0.06-0.18)	98.0	<0.001
Co-trimoxazole MIC, micrograms/mL	4 (4-8)	0.5 (0.38-1)	96.5	<0.001
Ceftriaxone MIC, micrograms/mL	0.75 (0.5-1)	0.12 (0.12-0.25)	93.0	<0.001
Levofloxacin MIC, micrograms/mL	1 (0.5-1)	0.5 (0.5-1)	62.5	0.282

3.5 Resistance combinations

The dominant resistance pattern was combined erythromycin and co-trimoxazole resistance, observed in seven isolates (33.3%). Four isolates (19.0%) were resistant to penicillin, erythromycin and co-trimoxazole, while three (14.3%) were resistant to penicillin, erythromycin, co-trimoxazole and ceftriaxone. One isolate (4.8%) showed co-trimoxazole resistance alone. Six isolates (28.6%) showed no resistance to any of the five antimicrobial agents tested. Thus, macrolide and co-trimoxazole resistance formed the central co-resistance pattern, with progressively broader phenotypes incorporating penicillin and ceftriaxone resistance.

Table 5. Observed antimicrobial resistance combinations

Resistance combination	n	Proportion
Erythromycin + Co-trimoxazole	7	33.3%
No resistant agent	6	28.6%
Penicillin + Erythromycin + Co-trimoxazole	4	19.0%
Penicillin + Erythromycin + Co-trimoxazole + Ceftriaxone	3	14.3%
Co-trimoxazole	1	4.8%

3.6 Demographic distribution of MDR

MDR was observed in 9 of 11 males (81.8%) and 5 of 10 females (50.0%), but this difference was not statistically significant (Fisher exact p=0.183). MDR occurred in 8 of 14 PCV-immunised children (57.1%) and 6 of 7 unvaccinated children (85.7%; p=0.337). Median age was 8 years (IQR 7-8) among children carrying MDR isolates and 7 years (IQR 6-8.5) among those carrying non-MDR isolates (p=0.440). None of the available demographic variables showed a statistically significant association with MDR in this sample.

Table 6. Distribution of MDR according to demographic characteristics

Characteristic	MDR	Non-MDR	p value
Male	9/11 (81.8%)	2/11 (18.2%)	0.183
Female	5/10 (50.0%)	5/10 (50.0%)	
PCV-immunised	8/14 (57.1%)	6/14 (42.9%)	0.337
Unvaccinated	6/7 (85.7%)	1/7 (14.3%)	
Age, years	8 (7-8)	7 (6-8.5)	0.440

We compared binary variables using the Fisher exact test; we compared age using the Mann-Whitney U test.

3.7 Correlation between oxacillin-zone diameter and MIC

Oxacillin inhibition-zone diameter was strongly and inversely correlated with penicillin MIC (Spearman rho=-0.989), erythromycin MIC (rho=-0.991), co-trimoxazole MIC (rho=-0.911) and ceftriaxone MIC (rho=-0.966); all four correlations had p<0.001. Levofloxacin MIC showed a more moderate inverse correlation with oxacillin-zone diameter (rho=-0.555, p=0.009). Overall, isolates with the smallest oxacillin zones tended to have the highest MICs across the major resistance phenotypes in the collection.

Table 7. Spearman correlation between oxacillin inhibition-zone diameter and antimicrobial MIC

MIC endpoint	Spearman rho	p value
Penicillin	-0.989	<0.001
Erythromycin	-0.991	<0.001
Co-trimoxazole	-0.911	<0.001
Ceftriaxone	-0.966	<0.001
Levofloxacin	-0.555	0.009

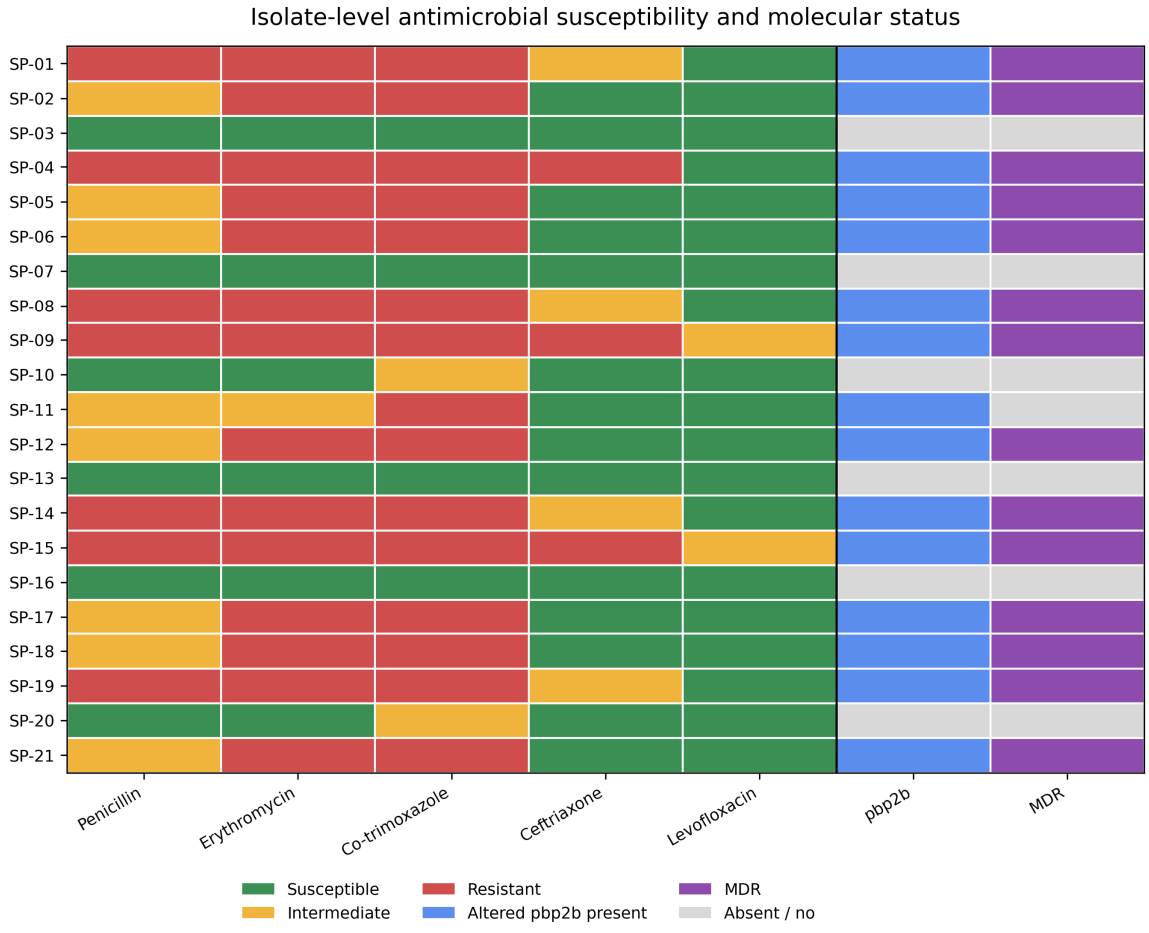


Figure 3. Isolate-level antimicrobial susceptibility pattern with altered pbp2b and MDR status displayed alongside each isolate.

4. Discussion

The present study provides a combined phenotypic and molecular description of nasopharyngeal S. pneumoniae recovered from asymptomatic Indian schoolchildren. Three findings are particularly notable. First, resistance to co-trimoxazole and erythromycin was common, and two-thirds of isolates were recorded as MDR. Second, altered pbp2b was detected in more than 70% of isolates and was closely associated with smaller oxacillin zones, higher penicillin MICs and the MDR phenotype. Third, lytA and ply were detected in every isolate, while levofloxacin resistance was not observed. Together, these findings portray a carriage reservoir in which resistance is concentrated around macrolide, co-trimoxazole and beta-lactam phenotypes rather than fluoroquinolone resistance.

The high frequency of co-trimoxazole resistance is consistent with older Indian carriage data. In a large study of North Indian schoolchildren aged 5-10 years, Jain and colleagues found very high co-trimoxazole resistance among nasopharyngeal pneumococci, while penicillin resistance was comparatively uncommon. Their work showed that healthy school-aged children can harbour drug-resistant pneumococci even without clinical disease and highlighted the community reservoir as an informative surveillance target [11]. The current study similarly found co-trimoxazole to be the most frequently resistant category, although penicillin resistance was higher in the present isolate set.

A second Indian study among healthy children younger than five years reported co-trimoxazole resistance in 91% of isolates, penicillin resistance in 17%, erythromycin resistance in 9% and MDR in 19%. Those values differ quantitatively from the present findings, especially for erythromycin and MDR, but the studies converge in showing that resistant pneumococci can be recovered from healthy children and that co-trimoxazole resistance is a prominent component of the carriage phenotype [12]. Differences between studies may reflect geography, age structure, antimicrobial exposure, circulating clones, vaccine-era effects, laboratory interpretive criteria and the limited number of isolates available in the present analysis.

Comparison with invasive-disease surveillance provides a further perspective. A multicentre Indian surveillance study in children younger than five years reported co-trimoxazole resistance in 66% of invasive pneumococcal isolates, erythromycin resistance in 37%, penicillin non-susceptibility in 8% and MDR in 9%. The current carriage isolates therefore show a similar order of prominence for co-trimoxazole and macrolide resistance but a substantially higher recorded MDR frequency [13]. Carriage and invasive collections should not be expected to produce identical resistance distributions because they represent different ecological and clinical populations. Nevertheless, resistant carriage strains are epidemiologically important because colonisation precedes transmission and may supply strains capable of subsequent invasive disease.

The magnitude of erythromycin resistance in the current study also fits within the wider Asian resistance landscape. ANSORP surveillance from 2008-2009 documented erythromycin resistance above 70% and MDR in nearly 60% of pneumococcal isolates across participating Asian countries. Although that surveillance predominantly involved clinical isolates, its resistance frequencies demonstrate that the high macrolide/MDR burden observed here is biologically plausible in a regional context [14]. The present isolate set therefore appears to represent a local expression of a broader Asian pattern rather than an isolated phenomenon.

Macrolide resistance among pneumococci in Asia has long been recognised as both common and genetically heterogeneous. Earlier ANSORP work characterised high macrolide resistance across

multiple Asian settings and showed the importance of acquired macrolide-resistance mechanisms in circulating pneumococci [15]. In the current study, erythromycin resistance was rarely isolated from the wider resistance phenotype: it most often co-occurred with co-trimoxazole resistance and, in a subset, with penicillin and ceftriaxone resistance. This clustering is relevant because co-selection can preserve a resistant lineage even when exposure to one particular drug class changes.

The relationship between altered pbp2b and beta-lactam phenotype was one of the strongest findings. Isolates carrying altered pbp2b had markedly smaller oxacillin inhibition zones and substantially higher penicillin MICs, and nearly all were classified as MDR. Experimental work has established PBP2B, together with PBP2X and other altered PBPs, as a primary determinant of reduced beta-lactam affinity in S. pneumoniae, with progressive PBP alterations contributing to increasing resistance [16]. The current genotype-phenotype association is therefore mechanistically coherent. At the same time, pbp2b should not be viewed as an isolated determinant of every resistance outcome: the association with elevated erythromycin and co-trimoxazole MICs most likely reflects co-carriage of resistance determinants within the same resistant isolates rather than a direct mechanistic action of pbp2b on those drug classes.

All isolates were positive for lytA and ply. Molecular confirmation is useful in pneumococcal work because closely related viridans-group streptococci can complicate identification by phenotypic tests alone. Studies evaluating molecular detection have shown that lytA-targeted PCR can enhance pneumococcal detection and can be incorporated alongside culture-based approaches, while multiplex molecular methods can improve the characterisation of carriage specimens [17]. In this study, complete lytA and ply positivity, together with conventional phenotypic identification, supported a consistent pneumococcal identity across the isolate collection. The uniform presence of ply also indicates that the pneumolysin locus was conserved in this small carriage sample, although gene presence alone does not quantify expression or toxin activity.

The oxacillin-screening results also deserve attention. Oxacillin-zone diameter was almost perfectly inversely correlated with penicillin MIC and strongly inversely correlated with erythromycin, co-trimoxazole and ceftriaxone MICs. This relationship with penicillin is expected because oxacillin screening flags pneumococci with reduced beta-lactam susceptibility. Interpret correlations with non-beta-lactam agents as markers of co-resistance: isolates with the most reduced oxacillin zones were often the same isolates that carried broader resistance phenotypes. This provides a useful isolate-level picture of resistance clustering but does not imply that oxacillin zone predicts the molecular mechanism of macrolide or co-trimoxazole resistance.

Vaccination adds an important population-level dimension. In the present sample, MDR was numerically less frequent among PCV-immunised than unvaccinated children, but the difference was not statistically significant. With only 21 isolates, this analysis has insufficient precision to infer a protective effect against MDR carriage. Nevertheless, the biological rationale for vaccine effects on resistant pneumococci is well established. After introduction of pneumococcal conjugate vaccination in the United States, invasive disease caused by vaccine serotypes fell sharply and disease due to penicillin-nonsusceptible strains also declined, demonstrating that vaccination can reduce circulation and disease caused by resistant vaccine-type pneumococci [18].

Subsequent surveillance specifically examining drug-resistant pneumococci showed major reductions in invasive disease caused by penicillin-nonsusceptible and multidrug-nonsusceptible strains after PCV introduction,

while also identifying increases in some non-vaccine serotypes [19]. This dual effect is relevant to carriage studies: vaccination may decrease resistant vaccine-type strains yet reshape the nasopharyngeal niche in ways that allow non-vaccine serotypes to expand. Therefore, vaccine status should ideally be analysed together with serotype and molecular lineage information, which were not available in the present dataset.

Carriage surveillance in the PCV era has further shown that resistant pneumococci do not disappear uniformly after vaccination. Studies of healthy children have documented shifts in serotype distribution and antimicrobial susceptibility after PCV introduction, reinforcing the importance of repeated local measurement rather than assuming that vaccine-era susceptibility profiles are static [20]. The current observation that MDR was present in both vaccinated and unvaccinated subgroups is consistent with the idea that resistant carriage can persist despite immunisation, particularly when the resistance phenotype is carried by serotypes or lineages not fully targeted by the vaccine used.

The absence of levofloxacin resistance is reassuring within this small collection. Nineteen isolates were susceptible, and two were intermediate, and levofloxacin MIC did not differ significantly between pbp2b-positive and -negative groups or between MDR and non-MDR isolates. This contrasts with the pronounced separation seen for penicillin, erythromycin, co-trimoxazole and ceftriaxone. The pattern suggests that fluoroquinolone resistance was not yet embedded in the dominant co-resistance profiles represented by these isolates. Because fluoroquinolone resistance in pneumococci can emerge through target-site mutations under selective pressure, continued surveillance remains appropriate even when baseline resistance is absent.

The resistance-combination analysis adds practical detail beyond single-agent percentages. Seven isolates carried combined erythromycin and co-trimoxazole resistance without additional tested resistance, four combined these two agents with penicillin resistance, and three had the broadest phenotype incorporating ceftriaxone resistance. This stepwise pattern suggests an underlying resistant subset in which accumulation or co-selection of determinants progressively broadens the phenotype. The fact that six isolates remained susceptible to all five tested agents also indicates substantial heterogeneity within a small collection and argues against treating the carriage reservoir as microbiologically uniform.

Several strengths merit emphasis. The study integrates conventional identification, quantitative MIC measurements, oxacillin screening and targeted molecular detection in the same isolates. It also retains isolate-level information rather than reporting only aggregate resistance percentages, allowing direct examination of genotype-phenotype coupling and co-resistance patterns. Using MIC50/MIC90 values provides a distributional view of susceptibility that categorical resistance alone does not capture. Finally, the focus on asymptomatic schoolchildren addresses a community reservoir that is often underrepresented compared with hospital-based invasive disease surveillance.

The study also has limitations. The sample comprises only 21 isolates, which limits precision and statistical power, particularly for demographic comparisons and vaccine-status analyses. The isolates represent a focused school-based collection and should not be interpreted as a population prevalence estimate for all children in Andhra Pradesh or India. Serotyping, multilocus sequence typing and detailed resistance-gene profiling for macrolide, folate-pathway and fluoroquinolone determinants were not available, preventing assignment of the observed co-resistance patterns to specific serotypes or clones. The analytic dataset also did not include information on recent antibiotic exposure, household crowding, or other recognised carriage determinants. Cross-sectional sampling cannot

determine the duration of colonisation or the direction of transmission between children. These limitations support cautious interpretation of effect sizes while preserving the descriptive value of the observed molecular and susceptibility patterns.

Overall, the study identifies a compact but clearly structured resistance phenotype among pneumococci carried by healthy schoolchildren. Macrolide and co-trimoxazole resistance dominate, beta-lactam non-susceptibility is concentrated among altered-pbp2b isolates, and broader MDR is tightly linked to the same low-oxacillin-zone subset. The results support continued community-based pneumococcal surveillance that combines susceptibility testing with molecular characterisation and, where feasible, serotyping or genomic analysis. Such surveillance can complement invasive-disease monitoring by identifying resistant lineages within the asymptomatic reservoir before changes become apparent in clinical disease.

5. Conclusion

Nasopharyngeal S. pneumoniae isolates from healthy schoolchildren showed substantial antimicrobial resistance, with co-trimoxazole and erythromycin resistance predominating and MDR recorded in approximately two-thirds of isolates. Altered pbp2b was frequent and strongly associated with smaller oxacillin zones, increased penicillin MIC and the multidrug-resistant phenotype. Universal detection of lytA and ply supported the molecular profile of the pneumococcal collection, while the absence of levofloxacin resistance indicated preservation of fluoroquinolone activity in this sample. The findings emphasise the value of linking molecular markers with quantitative susceptibility data when evaluating pneumococcal carriage and support continued surveillance of community reservoirs alongside vaccination and antimicrobial-stewardship programmes.

Declarations

Ethics approval: The study protocol received IEC approval through the Department of Microbiology, Apollo Institute of Medical Sciences, Chittoor, Andhra Pradesh.

Prior publication and secondary analysis declaration:

The 21 *Streptococcus pneumoniae* isolates evaluated in this study were originally isolated and partially characterized as part of a previous carriage survey published in the *Journal of Current Research in Scientific Medicine* [21]. The present work does not report new patient recruitment or primary carriage prevalence. Instead, it represents an extended secondary laboratory and biostatistical analysis reporting quantitative MIC distributional profiles (MIC50 / MIC90), non-parametric correlation matrices evaluating oxacillin disk diffusion zone diameters across multiple antimicrobial classes, and granular isolate-level multidrug resistance clustering.

Data availability: The analysed data are contained within the study dataset and the tables presented in this manuscript.

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