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Antibiogram profiles and genetic characteristics of *Streptococcus pneumoniae* clinical isolates from Pakistan

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Abstract: **Background**: *Streptococcus pneumoniae* is a major respiratory pathogen responsible for severe infections worldwide, with rising antibiotic resistance posing a significant clinical challenge. **Objectives**: This study aimed to assess the phenotypic and genotypic characteristics of *S. pneumoniae* isolates collected from clinical respiratory samples in different cities of Pakistan between 2022 and 2024. **Methods**: Antibiotic susceptibility was determined using the disc diffusion method and PCR was employed to detect resistance genes and PCV10/13 serotypes. **Results**: High resistance rates were observed against ofloxacin (68.8%), oxacillin (66.9%) and amoxicillin (66.0%), while the lowest resistance was noted to doxycycline (8.4%), cefotaxime (7.5%) and ceftriaxone (3.7%). Genotypic analysis revealed that 68.8% of isolates were positive for the *ermB* gene, 55.6% for *cats* and 50.9% for *tetM*. Additionally, 18.8% of isolates carried the *mefA* gene and 9.4% exhibited resistance potentially linked to the *pbp2b* gene. Serotype analysis showed that 66% of isolates belonged to PCV10/13 vaccine-type serotypes, with serotype 19A being the most prevalent (18.9%), followed by 19F (13.2%) and 14 (9.4%). **Conclusion**: These findings underscore the growing prevalence of antibiotic-resistant *S. pneumoniae* in Pakistan and highlight the urgent need for enhanced antimicrobial stewardship, improved surveillance systems and preventive measures to address the rising threat of resistant strains and shifting serotype distribution.

Keywords: Antibiotic resistance; Macrolide resistance; Resistant genes; Serotyping; *Streptococcus pneumoniae*

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INTRODUCTION

Streptococcus pneumoniae (*S. pneumoniae*) is accountable to cause diverse bacterial infections ranging from mild respiratory tract infection to severe invasive diseases. *S. pneumoniae* has become one of the major public health problems around the globe with about 3.7 million disease cases in children annually. Meanwhile, approximately half of pneumococcal related mortalities occur in Asia and Africa due to pneumococcal diseases (Al-Jumaili *et al.,* 2023). In order to tackle this concern, antibiotic resistance persists as one of the major public health problems worldwide. Continuous increase in drug resistance, leads to the evolution of multidrug resistant (MDR) strains (Ozisik, 2025).

Evolution of antibiotic-resistant pathogens, imposes a significant therapeutic problem outthought the world. According to reports 40% of *S. pneumoniae* strains arise as penicillin-resistant and in these circumstances, it is common to develop resistance against other antibiotics including tetracycline and macrolides (Maraki *et al*., 2024). In underdeveloped countries, antibiotic resistance appears as a major concern, and its growing trend indicates that antibiotic abuse and misuse can be the primary cause of resistance in *S. pneumoniae* globally (Ozisik, 2025).

In Asia, there exists higher concern regarding macrolide and penicillin resistance in *S. pneumoniae* (Oh *et al*., 2021). A study in Iran reported up to 73% erythromycin (macrolide) resistance in *S. pneumoniae* (Behesti *et al*., 2020). Similarly, 76% penicillin resistance was also reported (Tacoli *et al.,* 2025). South Asia is particularly reported as a hotbed region with up to 70% emerging infections cases with antibiotic resistance that pose serious threat to entire region and the world (Bilal *et al.,* 2021).

Despite the introduction of vaccination, Pakistan appears as the fourth most prevalent country around the globe in terms of pneumococcal disease with 14,400 deaths (with an uncertainty range of 9700–17000) (Javaid *et al*., 2024). Being an underdeveloped nation, due to high prevalence of antibiotic resistance, Pakistan experiences severe challenges in both regional and international platforms (Bilal *et al.,* 2021).

Over the last few years, with the continuous rise of antibiotic resistance, MDRs are being reported in Pakistan (Mirha *et al.,* 2024). To control the spread of drug-resistant *S. pneumoniae* strains, vaccines appeared as a potential solution globally as well as in Pakistan (Iqbal *et al*., 2024). With the implementation of pneumococcal conjugate vaccinations (PCVs) since 2000, there seems to be observable declines in pneumococcal infections

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throughout the world (Rivero-Calle *et al*., 2020). In South Asia, Pakistan became the first to include PCV 10 vaccines in its Expanded Program on Immunization (EPI) in 2013 and later introduced PCV13 in 2013 (Iqbal *et al*., 2024).

Despite the tremendous effectiveness of PCV against pneumococcal infections worldwide, multiple studies have reported a shift from vaccine type serotypes (VTs) to non-vaccine serotypes (NVTs) (Du *et al.,* 2021). In Pakistan, both VTs and NVTs have been reported to be among prevalent serotypes such as 6B, 9V/9A, 19F, 19A and 21 (Nisar *et al.,* 2022). The aim of this study was to investigate antibiotic resistance in *S. pneumoniae* from pneumococcal disease affected patients along with the monitoring of circulating VTs and NVTs. This endeavor aims to address the key deficiency highlighted by a previously published systematic review on antibiotic resistance in Pakistan (Bilal *et al*., 2021) and to respond to the recommendation for continuous monitoring of serotype surveillance in Pakistan (Nisar *et al.,*2022). The potential resistant genes circulating in targeted community will also assist this study.

MATERIALS AND METHODS

Study setting and population

This cross-sectional study was conducted in the microbiology department of the University of Haripur from 2022 to 2024, aiming to evaluate health outcomes in patients diagnosed with pneumococcal-related infections across selected hospitals and clinics in Haripur, Islamabad, Rawalpindi and Mansehra, Pakistan. Permission for sampling was obtained from the respective healthcare facilities. Written informed consent was obtained from all participants or their legal guardians in the case of minors prior to enrollment.

All the participants of the study were outpatients presenting with clinical signs of sinusitis, pneumonia, otitis media or sore throat, based on clinical diagnosis made by licensed physicians, following standard guidelines. Though sore throat is not usually caused by *S. pneumoniae*, it was selected due to overlapping symptoms that suggest the possible involvement of *S. pneumoniae.* Samples were collected by non-invasive sampling technique from nasopharynx, ear discharge and throat, based on clinical presentation by keeping in focus that all samples represent the active infection. To reduce result bias, only one sample was collected per patient to make sure that each isolate represents a unique individual. By following the convenience sampling, to minimize the potential selection bias, diverse patient population with various age groups was included with 261 (58%) females and 189 (42%) males.

Sample collection and S. pneumoniae isolation

A total of 450 swab samples were collected using transport medium, skim milk-tryptone-glucose-glycerol (STGG). During sampling, all the samples were labelled with unique identification code and kept stored at 2-8°C. After reaching the microbiology laboratory, all samples were vortexed for 10-20 seconds and then frozen at -30°C in an upright position until further processing. All handling and storage procedures were carried out according to the previously published consensus protocol (O'Brien *et al.,* 2021). For culturing, blood agar media (Oxoid, UK) supplemented with 5-7% defibrinated sheep blood was used and inoculated plates were incubated for 18-24 hours at 37°C. Initial screening of *S. pneumoniae* was carried out by colony morphology (alpha hemolytic draughtsman colonies), Optochin sensitivity test and Gram staining (Gram positive, diplococci). Molecular level confirmation was done by detecting marker genes through PCR.

Antibiotic susceptibility testing

S. pneumoniae isolates were subjected to antimicrobial susceptibility testing using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid, UK) supplemented with 5% defibrinated sheep blood, following the Clinical and Laboratory Institute (CLSI) 2020 guidelines (CLSI, 2020). A total of 14 antibiotics (Oxoid,UK) belonging to 7 different antimicrobial classes were used: chloramphenicol (C, 30 µg), erythromycin (E, 15 µg), tetracycline (TE, 30 µg), cefotaxime (CTX, 30 µg), amoxicillin (AML, 25 µg), benzylpenicillin (P, 1 µg), doxycycline (DXT, 30 µg), ceftriaxone (CRO, 30 µg), oxacillin (OX, 1 µg), trimethoprim-sulfamethoxazole (SXT, 25 µg), ofloxacin (OFX, 5 µg), azithromycin (AZM, 15 µg), ciprofloxacin (CIP, 5 µg) and cefepime (FEP, 30 µg). Briefly, bacterial suspension was adjusted to a 0.5 McFarland turbidity standard and evenly spread onto the prepared agar plates to create a uniform lawn. Antibiotic discs were placed at least 24 mm apart (center to center) on the inoculated plates. Plates were incubated at 37°C in 5% CO_2 (candle jar) for 20–24 hours by using Isotherm® Forced Convection Lab Incubator (Model: IFA-54-9). After incubation, zone diameters were measured and interpreted according to CLSI 2020 interpretive criteria (CLSI, 2020). Quality control was performed using *S. pneumoniae* D39 to ensure accuracy and reproducibility of results.

Nucleic acid (DNA) extraction and molecular analysis

DNA extraction for molecular characterization was performed using a thermal lysis protocol. Pure bacterial cultures were suspended in 100 µL of nuclease-free water in sterile microcentrifuge tubes. The suspensions were vortexed and subsequently heated at 95 °C for 10 min in a thermal cycler Axygen® MaxyGene II Thermal Cycler (Model THERM-1001). Following heat treatment, the samples were centrifuged at 10,000 rpm for 10 min to pellet cellular debris by using refrigerated microcentrifuge (Hermle Z216-MK). The upper aqueous phase (approximately 50 µL) containing the released DNA was carefully transferred to fresh microcentrifuge tubes and

stored at -20 °C until further use (Likhitha *et al.,* 2022). Species confirmation of *S. pneumoniae* isolates was carried out by targeting conserved genes *lytA* and *SP2020,* as described in previous studies (Travares *et al.,* 2019; Azarsa *et al.,* 2017).

To investigate the genetic basis of antibiotic resistance in *S. pneumoniae*, a set of CDC recommended and well-characterized resistance genes was targeted based on their known association with phenotypic resistance. These included *pbp2b* (penicillin resistance), *ermB*, *mefA* (macrolide resistance), *tetM* (tetracycline resistance) and *cat* (chloramphenicol resistance). For PCR, primer sequences and protocols were adapted from previous study **(**Velusamy *et al.,* 2020), as recommended by CDC on its website. For each PCR batch, PCR grade water (Solis BioDyne) was used as negative control while *S. pneumoniae* D39 was used as positive control to ensure result specificity and to reduce any possibility of contamination. Each PCR tube in each batch was carefully handled and properly labelled to ensure accuracy of results. All PCR products were analyzed using 2% agarose gel (Invitrogen UltraPure agarose; Owl™ EasyCast™ B1A Mini Gel Electrophoresis Systems) under UV illuminator. Product band sizes were carefully determined by comparing them with the bands of 100bp DNA ladder (Solis BioDyne).

Furthermore, prevalence of VTs and NVTs was assessed by targeting PCV10 and PCV13 serotypes by using primer set outlines in table 1. PCR for serotyping was performed by following the condition described in previous study (Abdoli *et al.,* 2020); initial denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 60 sec 58°C for 60 sec and 72°C for 60 sec with final extension at 72°C for 10 min in a thermal cycler Axygen® MaxyGene II Thermal Cycler (Model THERM-1001).

Statistical analysis

For statistical analysis, IBM SPSS Statistics version 28 was used. Chi square test and Fisher-Freeman-Halton test were utilized for different categorical data. Statistical significance was determined when the p-value was < 0.05. Poisson regression was used to find the association between antibiotic classes in MDR. Regression coefficients (β) were exponentiated to calculate incidence rate ratios (IRRs) with 95% confidence intervals (CI). Meanwhile, Cohen's Kappa coefficient was used to assess association between genotypic and phenotypic resistance. McNemar's test was used to evaluate difference in proportion for paired data.

RESULTS

One hundred and six *S. pneumoniae* isolates were recovered from patients having age ranging from 2 months to 75 years; 46 (43.4%) were children under 18 years old, while 60 (56.6%) were adults with median age of 26 years. The prevalence of *S. pneumoniae* isolates recovered from sinusitis was 7.5% (n=8), throat swab 30.1% (n=32), ear discharge 28.3% (n=30) and most prevalent source was observed to be nasopharynx with 33.9% (n=36) recovered isolates.

Demographic and clinical highlights

The significant associations were observed in multiple clinical and epidemiological parameters. Fisher-Freeman-Halton test showed statistically significant association for sampling method ($p < 0.0001$) and sampling area ($p < 0.0001$) and type of infection ($p < 0.0001$), while no significant relationship was observed for age group ($P = 0.318$). On comparing variables like gender ($\chi^2 = 1.81$, $p = 0.178$), history of self-medication ($\chi^2 = 0.12$, $p = 0.729$), previous treatment ($\chi^2 = 0.18$, $p = 0.671$), vaccination status ($\chi^2 = 0.01$, $p = 0.920$), occupational status ($\chi^2 = 0.92$, $p = 0.631$), educational level ($\chi^2 = 2.12$, $p = 0.145$) and place of residence ($\chi^2 = 0$, $p = 1$), no significant association was observed between the two groups by Chi square test.

Antimicrobial activity of antibiotics

Among the *S. pneumoniae* isolates, 73 (68.8%) were resistant to OFX, while 28 (26.4%) were sensitive. A total of 71 isolates (66.9%) exhibited resistance to OX, 70 (66.0%) to AML and 70 (66.0%) to AZM. Additionally, 54 isolates (50.9%) showed resistance to both E and P, 50 isolates (47.1%) to TE, 23 isolates (21.6%) to SXT, 17 isolates (16.0%) to CIP, 13 isolates (12.2%) to FEP, 12 isolates (11.3%) to C, 9 isolates (8.4%) to DXT, 8 isolates (7.5%) to CTX and 4 isolates (3.7%) exhibited resistance to CRO as illustrated in fig. 1. The measurement of zones of inhibition was conducted in accordance with guidelines and breakthrough points compliance with the Clinical Laboratories and Standards Institute (CLSI, 2020). The resistance patterns and corresponding antibiotic breakpoints are summarized in table 2.

Multidrug resistance patterns

Occurrence of different MDR patterns was identified among 106 isolates. Utmost frequent combination was beta lactam and macrolide (AML+E) with 23.0% (n=45) isolates, followed by P+E, 21.0% (n=41) and P+TE in 16.9% (n=33) isolates. Other distinguished MDR pattern includes resistance to P+E+TE (9.2%, n=18) isolates and AZM+OX+FEP (5.6%, n=11) isolates. Resistance to E+SXT was observed in 5.1% (n=10) isolates while complex resistance pattern was observed was P+AML+DXT+OFX+AZM in 4.1% (n=8) isolates. Further detail of resistance patterns is given in table 3.

Molecular analysis of resistance genes

The observed phenotypic resistance to beta-lactam antibiotics (P 54 (50.9%), OX 71 (66%) and AML 70 (66.9%) was higher than the low *pbp2b* resistance 10 (9.4%) detected by PCR.

Table 1: Primer sequences used for PCR-based detection of *S. pneumoniae* serotypes included in the 13-valent pneumococcal conjugate vaccine (PCV13).

Serotypes	Sequence (5' – 3')	Base-pair size
4	F-ATTCAGAGGCAGCTAGTTCAGG	433
	R-CAGAAGCTACTGTTAGGCTGG	
6B	F-GAAGTAGAAAATCGTGTAAGTGG	211
	R-TCCAACAACTAACCTTATAAGGG	
9V	F-GATCAATGGCAACTATATTTACCC	172
	R-GATTCACTGTCTGACTTTGAACC	
14	F-CCGTCTTTTTGTATGGTGCTATG	84
	R-TGAACAGCCAATCCATCAATCAG	
18C	F-AGTCTTACTAGACGTAATGAACC	220
	R-AAGATAAATTGACTAAGTCCTCCC	
19F	F-TGTTCTTAGTAATGGATATACGGG	523
	R-AAAACTTCACCAGGATCTAATGG	
23F	F-TTCACAAGTGATAGTGAACTTGG	260
	R-TATTAGCTTTATCGGTAAGGTGG	
1	F-TTTGCTAGATGGTGAGTTTGTATC	555
	R-TTTAGAAGCTGCATTGTACTACTC	
5	F-TTATCTATTTTATCGCAGACTCCC	443
	R-CTGCCGATAAAAAGATAGATGCC	
7F	F-TTGACTGCAAGTGTTTCAATGGG	491
	R-AAAGCACAAAATATTGGAACGAGG	
3	F-AGAAATGCTATCCGCGTTGGG	191
	R-TTGTCACGAGATTACGCTCAGG	
6A	F-GAAGTAGAAAATCGTGTAAGTGG	211
	R-TCCAACAACTAACCTTATAAGGG	
19A	F-ATTGGAGTAGCTGAGGTTTTTGG	269
	R-TATCCAATTTAAAACCAGCACGG	

F: Forward primer; **R:** Reverse primer

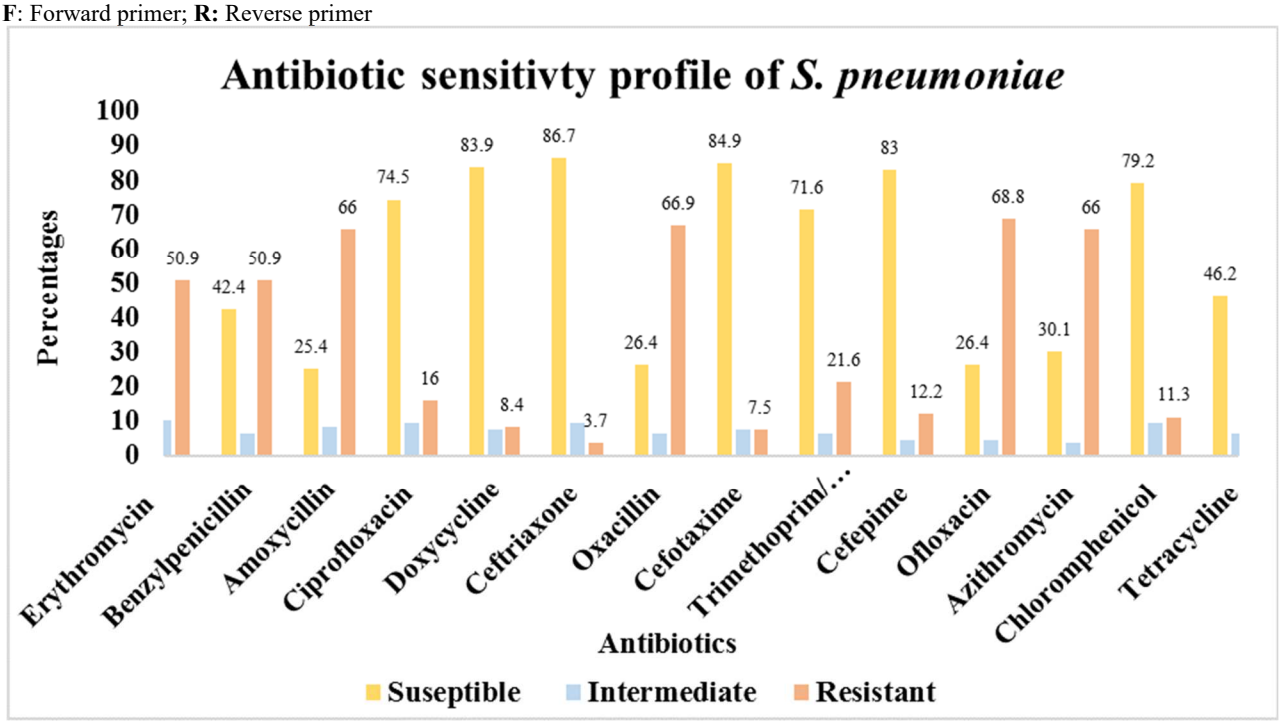

Fig. 1: Antibiotic susceptibility profiles showing resistance, intermediate and sensitivity patterns. The bar graph represents the percentage of isolates classified as resistant**,** intermediate or sensitive to the tested antimicrobial agents.

Table 2: Antibiotic resistance breakpoints for *S. pneumoniae* isolates. The table presents the antibiotic classes, individual antibiotics, corresponding codes, disc concentrations and the breakpoints used to categorize isolates as sensitive (S), intermediate (I) or resistant (R) based on the indicated disc concentration.

Class	Antibiotics	Code	Disc conc.	Break points		
				S	I	R
Macrolides	Erythromycin	E	15µg	≥21	16-20	≤15
	Azithromycin	AZM	15µg	≥18	14-17	≤13
Beta lactam	Amoxycillin	AML	25µg	≥20	-	≤19
Penicillins	Oxacillin	OX	1µg	≥20	-	≤19
	Benzylpenicillin	P	1µg	≥20	-	≤19
Beta Lactam	Cefepime	FEP	30µg	≥22	20-21	≤19
Cephalosporins	Cefotaxime	CTX	30µg	≥31	28-30	≤27
	Ceftriaxone	CRO	30µg	≥22	20-21	≤19
Tetracycline	Tetracycline	TE	30µg	≥28	25-27	≤24
	Doxycycline	DXT	30µg	≥28	25-27	≤24
Fluoroquinoles	Ofloxacin	OFX	5µg	≥16	13-15	≤12
	Ciprofloxacin	CIP	5µg	≥21	17-20	≤16
Sulfonamide	Trimethoprim/Sulfamethoxazole	SXT	25µg	≥19	16-15	≤15
Amphenicol	Chloramphenicol	C	30µg	≥21	-	≤20

E = Erythromycin; AZM = Azithromycin; AML = Amoxycillin; OX = Oxacillin; P = Benzylpenicillin; FEP = Cefepime; CTX = Cefotaxime; CRO = Ceftriaxone; TE = Tetracycline; DXT = Doxycycline; OFX = Ofloxacin; CIP = Ciprofloxacin; SXT = Trimethoprim–Sulfamethoxazole; C = Chloramphenicol. Disc conc. = Disc concentration (µg); S = Susceptible; I = Intermediate; R = Resistant.

Table 3: Multidrug resistance (MDR) patterns identified among *S. pneumoniae* isolates, including antibiotic combinations, classes, number of resistant isolates and Poisson regression analysis of the effect of the number of antibiotic classes on resistance frequency.

A. Distribution of MDR combination pattern				
MDR pattern (combination of antibiotics)	Antibiotic classes	Number of isolates resistant	Percentage of total isolates (%)	
P + E	Beta-lactam + Macrolide	41	21.0%	
P + TE	Beta-lactam + Tetracycline	33	16.9%	
E + SXT	Macrolide + Sulfonamide	10	5.1%	
AML + E	Beta-lactam + Macrolide	45	23.0%	
CTX + E	Cephalosporin + Macrolide	4	2.0%	
P + E + TE	Beta-lactam + Macrolide + Tetracycline	18	9.2%	
P + E + SXT	Beta-lactam + Macrolide + Sulfonamide	9	4.6%	
AZM + OX + FEP	Macrolide + Beta-lactam	11	5.6%	
B. Poisson regression results				
Variables	Coefficient (β)	IRR	95% CI	p-value
Intercepts	4.335	76.34	47.31-123.20	<0.0001
Number of antibiotic classes	-0.546	0.58	0.48 – 0.70	<0.0001

Significant inverse association was observed between number of antibiotic classes by Poisson regression Specifically, 42% reduction in expected number in resistant isolates was observed (incidence rate ratios (IRR) = 0.58**,** 95% confidence intervals (CI): 0.48–0.70; $p < 0.0001$). This indicates that simpler MDR combinations with are more frequently observed compared to complex multi class resistance profiles.

This discrepancy suggests that resistance may not be solely attributed to *pbp2b* absence or mutations. Other *pbp* genes, such as *pbp1a* or *pbp2x*, could potentially contribute to the observed resistance, or other resistance mechanisms may be involved. A high *ermB* prevalence of 73(68.8%) was found. The *mefA* gene was detected in about 20 isolates (18.8%) and the same number of isolates (18.8%) tested positive for both *ermB* + *mefA*. *tetM* and *cat* genes were found in 54 (50.9%) and 59 (55.6%) of the *S. pneumoniae* isolates, respectively. Details of primer sequences, PCR conditions and product size are given in table 4.

The distinct pattern of antibiotic resistance was observed among bacterial isolates and found surpassing results of phenotypic resistance over genotypic resistance for different antibiotics. Around 50.9% phenotypic P resistance was observed, while only 9.4% genotypic resistance exhibit associated with lack of *pbp2b* gene in *S. pneumoniae* isolates. Moreover, significant overlap of phenotypic and genotypic resistance was observed against macrolide antibiotics and associated genes. Meanwhile, a stronger relation was observed between phenotypic TE resistance with 50.9% isolates carrying *tetM* gene.

Table 4: Primer sequences and PCR conditions used for molecular identification of *S. pneumoniae* and detection of antibiotic resistance genes. The table lists gene targets, forward (F) and reverse (R) primer sequences, expected amplicon sizes (in base pairs) and PCR conditions.

Gene	Primer sequence (5′–3′)	Product size (bp)	Annealing temp (°C)	Annealing time (min)	Cycles	References
lytA	F: ACGCAATCTAGCAGATGAAGCA R: TCGTGCGTTTTAATTCCAGCT	75	60	1	45	Azarsa *et al.*, 2017
SP 2020	F: TAAACAGTTTGCCTGTAGTCG R: CCCGGATATCTCTTTCTGGA	155	60	1	45	Tavares *et al.*, 2019
Pbp 2b	F: GGCTGTTTGGACCATATAGGTATT R: ACTCAGGCTTACGGTTCATTC	81	60	1	40	Velusamy *et al.*, 2020
ermB	F: GCAATTGCTTAAGCTGCCA R: ATCTGGAACATCTGTGGTATGG	97	60	1	40	Velusamy *et al.*, 2020
mefA	F: GAGCTACCTGTCTGGATGATTATG R: AAGTGGTGTAACCGCATTGA	93	60	1	40	Velusamy *et al.*, 2020
tetM	F: GCTTATACTATAGCCCTGTTAGTACC R: TGGCTCTAACAATTCTGTTCCA	104	60	1	40	Velusamy *et al.*, 2020
cat	F: GTGACAAGGGTGATAAACTCAAATAC R: TGGCTCTAACTTATCCCAATAACC	87	60	1	40	Velusamy *et al.*, 2020

Table 5: Phenotypic and genotypic antibiotic resistance patterns of bacterial isolates. This table summarizes the antibiotic resistance patterns of bacterial isolates, showing both phenotypic and genotypic resistance profiles.

Antibiotics	Genetic markers	Total phenotypic resistant isolates	Total genotypic resistant isolates	Phenotypically and genotypically resistant isolates	Cohen's Kappa (κ)	McNemar p-value
Benzylpenicillin	*pbp2b*	54 (50.9%)	10 (9.4%)	7 (6.6%)	0.15	0.001
Erythromycin	*mefA*	54 (50.9%)	20 (18.8%)	15 (14.1%)	0.31	<0.0001
	ermB	54 (50.9%)	73 (68.8%)	44 (41.5%)	0.58	<0.0001
	mefA+ermB	54 (50.9%)	20 (18.8%)	15 (14.1%)	0.31	<0.0001
Azithromycin	*mefA*	70 (66.0%)	20 (18.8%)	15 (14.1%)	0.28	<0.0001
	ermB	70 (66.0%)	73 (68.8%)	48 (41.5%)	0.57	<0.0001
	mefA+ermB	70 (66.0%)	20 (18.8%)	15 (14.1%)	0.28	<0.0001
Tetracycline	*tetM*	50 (47.1%)	54 (50.9%)	37 (34.9%)	0.71	<0.0001
Doxycycline	*tetM*	9 (8.4%)	54 (50.9%)	8 (7.5%)	0.12	0.001
Chloramphenicol	*cat*	12 (11.3%)	59 (55.6%)	9 (8.4%)	0.09	0.002

On statistical analysis, slight agreement for *cat* (Cohen's Kappa (κ) = 0.09) and *pbp2b* (κ = 0.15) genes were indicated by Cohen's Kappa analysis. For *mefA* (κ = 0.28–0.31) and *ermB* (κ = 0.57–0.58), fair to moderate agreement was indicated with substantial agreement for *tetM* (κ = 0.71). Meanwhile, κ value represents the level of consistency and reliability of the methods. Higher the value represents more consistent and reliable results. Genotypic and phenotypic result concordance was analyzed by McNemar's test. Results indicates that certain genetic markers such as *ermB, mefA* and *tetM* reliably predict the phenotypic resistance, whereas *cat* and *pbp2b* show poor concordance.

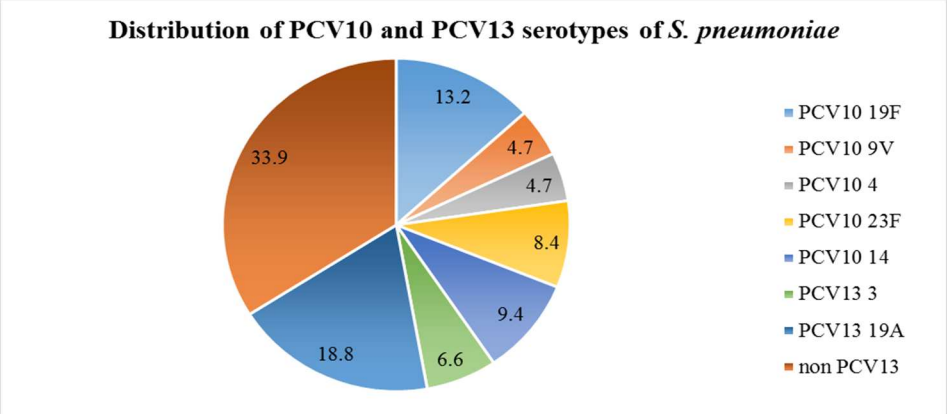

Fig. 2: PCV10 and PCV13 serotypes distribution among *S. pneumoniae* isolates. The pie chart displays the distribution of *S. pneumoniae* serotypes, and each segment represents the proportion of isolates belonging to individual serotype.

These findings underscore the variability in resistance mechanisms, with genetic markers providing valuable insights as illustrated in table 5.

On statistical analysis, slight agreement for *cat* (κ = 0.09) and *pbp2b* (κ = 0.15) genes were indicated by Cohen's Kappa analysis. For *mefA* (κ = 0.28–0.31) and *ermB* (κ = 0.57–0.58), fair to moderate agreement was indicated with substantial agreement for *tetM* (κ = 0.71). Meanwhile, κ value represents the level of consistency and reliability of the methods. Higher the value represents more consistent and reliable results. Genotypic and phenotypic result concordance was analyzed by McNemar's test. Results indicate that certain genetic markers such as *ermB, mefA* and *tetM* reliably predict the phenotypic resistance, whereas *cat* and *pbp2b* show poor concordance.

PCR amplification of PCV 10 and PCV 13 serotypes of S. pneumoniae

PCR amplification was performed on all phenotypically confirmed *S. pneumoniae* isolates using serotype-specific primers targeting PCV10 and PCV13-covered serotypes. Serotyping was performed by using conventional PCR and grade water was used as negative control with *S. pneumoniae* strain D39 as a positive control in all batches. PCR showed 66% (n=70) positive results for PCV10 and PCV13 with 3, 4, 9V, 14, 19F, 19A and 23F serotypes, while 34% (n=36) *S. pneumoniae* isolates do not show any positive results for PCV10 or PCV13 and thus concluded as non PCV13 serotypes. Detailed distribution results of serotypes are illustrated in fig. 2.

Antibiotic sensitivity profiles of serotypes

Diverse phenotypic antibiotic susceptibility trends were observed in *S. pneumoniae* serotypes with notable non-susceptibility patterns among PCV13 and non-PCV13 serotypes. Among 106 serotypes, high resistance was noted in 19A serotype against different antibiotics in particular to E (11.3%), P (8.4%) and AML (12.2%). Multiple antibiotic resistance was also observed in prominent serotypes 23F, 14 and 19F. Considerable resistance to E (23.5%), AML (22.6%), OX (24.5%) and OFX (21.6%) was notable in non PCV13 serotypes. Majority of the serotypes were found to be MDR as shown in table S1.

At genetic level, highest penicillin (*pbp2b)* susceptibility of 90% (n=96) was observed among PCV10, PCV13 and non PCV13 serotypes collectively, highest one was noted in serotype 19A 17.9% (n=19). Utmost genotypic resistance among identified serotypes was observed to be 68.8% (n=73) for *ermB* and *cat* gene with 55.6% (n=59) serotypes as illustrated in table S2.

DISCUSSION

Prevalence and recovery of S. pneumoniae isolates

This study provides comprehensive overview of the epidemiology, phenotypic and genotypic resistance patterns and serotype distribution of 106 *S. pneumoniae* isolates recovered from upper respiratory tract samples. The recovery rate of 23.5%, align with the reported ranges by previous studies from low and middle income countries where prior use of antibiotics, mixed infections and limited diagnostic facilities reduce the culture yield frequently. Role of these factors have been highlighted previously (Belman *et al.,* 2024; Purwanto *et al.,* 2020). Our findings with majority of isolates from patients with suspected pneumonia reinforce the continuing role of *S. pneumoniae* as predominant respiratory pathogen, consistent with regional and local reports (Irfan *et al.,* 2019).

Demographic and clinical associations

Accordance with the result section, statistically significant differences were observed across sampling methods, sampling areas and type of infections. This association indicates that clinical source remains as a major predictor of *S. pneumoniae* recovery. Though, there exists no significant correlations with demographic variables like gender, age, self-medication and vaccination status with *S. pneumoniae* isolation. These outcomes are in agreement with the findings reported in previous epidemiological studies (Nicholson *et al.,* 2024; Mekuria *et al.,* 2022).

Phenotypic antibiotic resistance trends

Despite being a first line therapy, high prevalence of beta-lactam antibiotics was observed with 66-67% resistance to AML and OX, while some studies reported the lower rate of resistance up to 20-21% (Zhao *et al.,* 2020). Meanwhile, resistance to P was 50.9% in this study, which is significantly low as documented by other study (Tran *et al.,* 2021).

Macrolide resistance was remarkably high, with AZM (66%) and E (59.9%) resistance. Though, the resistance prevalence was low as compared to reported values to E (98.2%) and AZM (97.6%) in East Asia (Zhao *et al.,* 2020). Resistance to TE (47.1%) and SXT (21.6%) also aligns with the resistance pattern reported in low to middle income countries where broad spectrum antibiotics remains widely in use (Beheshti *et al.,* 2021).

Multidrug resistance patterns

In present study, *S. pneumoniae* isolates were found to be resistant to two or more classes of antibiotics. The most common pattern of MDR was observed in beta lactam and macrolide class of antibiotics. Highest MDR pattern was observed in AML and E (23.0%), followed by P and E (21.0%). However, MDR pattern (99%) to our findings is comparable to that reported in China (89.5%), Shanghai (88.0%) and in Vietnam (Wang *et al.,* 2019). Interestingly, Poisson regression showed significant reverse relation between number of antibiotics and resistance frequency with 42% reduction in resistance with addition of antibiotic class. This trend suggests that MDR pattern is more common in simpler combination rather than complex multi class resistance pattern. These findings are comparable to a documented study from China (Wang *et al.,* 2019).

Genotypic resistance and phenotype – genotype concordance

A noteworthy difference was observed between phenotypic penicillin resistance and pbp2b gene detection. Though 50.9% isolates noted to show high phenotypical resistance to penicillin class antibiotics, only 9.4% isolates were found genotypical resistant due to absence of *pbp2b* gene in this study. These mismatch results highlight the limitation of targeting a particular *pbp* gene, which may account for this discrepancy as mutations in *pbp2x* or *pbp1a* were reported to be associated with elevated beta lactam resistance (Yu *et al.,* 2023; Nakano *et al.,* 2019). Weak agreement in Cohen's kappa and significant McNemar discordance support this interpretation.

However, a strong alignment of phenotypic resistance and macrolide resistant genes *mefA* and *ermB* was observed. The *ermB* (68.8%) was prevalent than *mefA* (18.8%) genes, while both genes (*ermB+mefA)* were observed simultaneously in 18.8% isolates. In contrast, a latest study reported presence of *ermB* (20%), *mefA* (50.4%) and co-occurrence of both genes in 16.7% isolates (Shi *et al.,* 2024). Moreover, *tetM* (50.9%) demonstrated strong concordance with phenotypic TE resistance, while *cat* gene exhibited poor agreement suggesting that there might be involvement of some other mechanism in C resistance.

Serotype distribution and vaccine impact (PCV10/PCV13)

Although the introduction of PCV10 (2012) and PCV13 (2021) vaccine in Pakistan, notable serotype replacement was noted in this study. Sixty-six percent isolates were identified as VTs while the rest of the serotypes were NVTs. The most prevalent serotype was 19A (18.9%), followed by 19F, 14, 23F, 3, 4 and 9V. Comparable serotype pattern has been reported previously in Pakistan and Mozambique (Shahid *et al.,* 2022; Nisar *et al.,* 2018; Sigaúque *et al.,* 2018). Despite PCV10 early implementation, presence of PCV13 and non PCV13 serotypes emphasize the limitation pf PCV 10 coverage and highlight ongoing serotype replacement pressures.

Serotype - specific antibiotic resistance

Across serotypes, resistance varied substantially. NVTs exhibit high resistance to OX (24.5%), E (23.5%), AML (22.6%) and OFX (21.6%). Among VTs, serotype 19A showed highest resistance predominantly to AML (12.2%), E (11.3%), followed by 23F, 19F and 14 serotypes, which reflect the outcome from regional surveillance in South Asia and China (Müller *et al.,* 2022). The serotype resistance highlights the significance of continuous monitoring of vaccine impact and probability to expand vaccine valency in regions with high disease burden.

Broader implication: antibiotic stewardship and regional variations

The observed variations in resistance patterns across regions and studies can be attributed to several factors, including socioeconomic conditions, prescribing practices and antibiotic availability (Sulis *et al.,* 2022). Furthermore, prescribing practices are a key determinant of antibiotic resistance, overuse and misuse of antibiotics being prevalent in certain regions can contribute to antibiotic resistance development (Walsh *et al.,* 2023). In regions where antibiotics are readily available without prescription or regulation, resistance rates tend to be higher (Muteeb *et al.,* 2023). Effective stewardship programs, improved diagnostics and strengthened surveillance systems remain essential to mitigate resistance development (Moro *et al.,* 2024).

CONCLUSION

In conclusion, widespread resistance to antibiotics like ofloxacin**,** oxacillin**,** amoxicillin**,** azithromycin and erythromycin along with the identification of resistance genes highlight the complexity of the resistance pattern in *S. pneumoniae* within targeted area of study. The results reveal that serotype 19A, which is resistant to several common antibiotics, was most prevalent serotype. Moreover, 36% of the strains were classified as NVTs, pointing to a significant shift in pneumococcal serotype. However, there is no clear evidence of high phenotypic penicillin resistance, so *pbp* gene particularly *pbp1a, pbp2b* and *pbp2x* sequencing is encouraging. Based on results of our study, to effectively counter the growing risk of AMR, joint action by healthcare experts, policy formulators and society is essential.

Limitations and future recommendations

This study had limitations that must be acknowledged. Firstly, 106 recovered isolates out of 450 samples might not completely represent the strain diversity and resistance profile of the entire province. Cross-sectional study, capturing the resistance pattern at single point without any follow up of capturing potential temporal shifts in resistance is also another limitation of study. Moreover, the potential genetic resistance mechanisms such as gene mutations by sequencing were not explored which limits the understanding of resistance dynamics. Despite these limitations, the study provides valuable insights into the resistance patterns in key areas of Pakistan, offering a foundation for future, more extensive national-level research. Based on findings and limitations of the study, number of recommendations were made to address increasing issue of antibiotic resistance in *S. pneumoniae.* Despite of vaccine implementation, continues monitoring of vaccine effectiveness and surveillance of circulating serotypes is crucial. Enhanced surveillance of resistance patterns in *S. pneumoniae* and its serotypes should be implemented countrywide to detect potential serotype replacement and guide vaccine policy. Where needed, the consideration of introducing higher-valent vaccines, such as PCV15 or PCV20, should be explored to expand protection. Future studies should incorporate larger and

more geographically diverse sample sizes, along with longitudinal monitoring to assess trends over time. Incorporation of advanced diagnostic tools, such as PCR and whole-genome sequencing, will provide deeper insights into resistance mechanisms, including emerging mutations in genes like *pbp1a*, *pbp2x* and efflux pumps. Strengthening antimicrobial stewardship programs remains crucial to curbing the overuse and misuse of antibiotics, particularly in outpatient settings. Finally, public health campaigns should focus on educating the community about the importance of completing vaccination schedules, avoiding self-medication and seeking proper diagnostic testing to improve patient outcomes and reduce antibiotic misuse.

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Author's contributions
Maria Rukan: Conceptualization, methodology, investigation, data assessment, graphical illustration, writing original draft-review and editing. Muhammad Ali Syed: Conceptualization and supervision, resource management, review and editing of manuscript, Muhammad Mumtaz Khan: Supervision, writing-review and editing. Uroosa Ejaz: Data analysis, formal analysis, and help in writing up and editing. Adil Shakil Ahmed: Contributed to molecular analysis, visualization, formal analysis, and editing.

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The study was approved by "Research Ethics Committee" of the University of Haripur (Approval number UOH/DASR/2024/2234). Prior to registration, all participants and their legal guardians provided informed written consent.

Data availability statement
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Conflict of interest
All authors declare no conflict of interest.

Supplementary data

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