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Genetic Analysis of Antimicrobial Resistance in *Salmonella* Typhi: An Extensive Surveillance Study with National and Global Relevance

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ABSTRACT

OBJECTIVE: This study aimed to determine the prevalence and antimicrobial susceptibility patterns of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Salmonella enterica* serovar Typhi (*S.* Typhi) strains across multiple districts of Punjab, Pakistan.

METHODOLOGY: A cross-sectional study was conducted across 15 districts in Punjab, Pakistan. A total of 7,993 blood cultures from febrile patients were obtained for the isolation and identification of *S.* Typhi, followed by antimicrobial susceptibility testing and screening for the resistance genes.

RESULTS: A total of 2,832 (35.4%) blood cultures were positive for any bacterial growth. Of these positive cases, 1346 (47.5%) were identified as *S.* Typhi. High resistance rates were observed against several antibiotics: ciprofloxacin (91.8%), trimethoprim-sulfamethoxazole (86.1%), doxycycline (79.5%), and third-generation cephalosporins (46.5%). However, all isolates remained susceptible to carbapenems and azithromycin. The prevalence of MDR and XDR strains was 24.2% and 46.4%, respectively, with significant geographic variations in resistance patterns. Overall, the bla_{TEM} gene was detected in 913 (67.8%) isolates. The resistance to cephalosporins was primarily mediated by the extended-spectrum beta-lactamase (ESBL) gene $bla_{\text{CTX-M-15,}}$ which was identified in 626 isolates (46.5%). The prevalence of $bla_{\text{CTX-M-15}}$ varied considerably across different districts, with the highest rate observed in Kasur district (72.9%).

CONCLUSION: This study reveals a significant public health concern, with a high prevalence of MDR and XDR *S.* Typhi strains, especially in certain districts. The findings support the use of azithromycin and carbapenems as empirical treatments in affected areas. Strengthened antimicrobial stewardship, targeted surveillance, and public awareness are crucial to address this rising threat.

KEYWORDS: Typhoid fever, *Salmonella* Typhi, XDR, Surveillance, Ceftriaxone, ESBL

INTRODUCTION

Typhoid fever poses a significant public health challenge in low- and middle-income countries, such as Pakistan. This illness is particularly prevalent in regions with poor sanitation and limited access to safe drinking water, including South Asia, Southeast Asia, and sub-Saharan Africa. Globally, typhoid fever affects an estimated 11 to 21 million people annually, leading to 128,000 to 161,000 deaths.[1, 2] According to the Global Burden of Disease report, an estimated 14 million cases of typhoid fever and 130,000 associated deaths occurred globally in 2017. [3] In the 1940s, chloramphenicol was the preferred treatment for typhoid fever. However, due to concerns about toxicity and the emergence of resistance, other antimicrobial agents, such as trimethoprim-sulfamethoxazole, began to be used in the 1970s. Subsequently, ampicillin replaced chloramphenicol as the treatment of choice, as it was comparatively safer. [4, 5] Despite the reduction in typhoid fever incidence due to antibiotic use, the pathogen has increasingly developed resistance to multiple drugs through various mechanisms. This has resulted in the emergence of multidrug-resistant (MDR) *Salmonella* Typhi strains, which are resistant to three first-line antibiotics: chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin.[6]

This situation led to a shift towards using fluoroquinolones, such as ciprofloxacin, for the treatment of typhoid fever. However, the emergence of resistance to fluoroquinolones, along with the acquisition of extended-spectrum beta-lactamases (ESBLs) resulting in resistance to third-generation cephalosporins, has led to the development of extensively drug-resistant (XDR) *S.* Typhi strains. This development has significantly limited treatment options for typhoid fever, leaving only a few viable alternatives, such as azithromycin and carbapenems. In Pakistan, there has been a notable rise in typhoid cases attributed to the dissemination of MDR and XDR *S.* Typhi, raising concerns about potential treatment failures.[7] Initially, XDR Typhoid cases were reported from the Sindh Province, 2016–2018, indicating that the region was experiencing an endemic situation.[8] There have been reports of emerging cases across the country, and international travel is playing a significant role in facilitating their spread.[5, 9, 10]

Punjab, the most populous province in Pakistan, has limited reports on XDR *S.* Typhi. Most existing studies focus on specific urban areas, creating a gap in comprehensive data. A study from Lahore revealed alarming trends, including XDR strains in 41.9% of isolates. [1] However, this urban-centric focus has created a significant gap in understanding the prevalence, distribution, and genetic mechanisms of antimicrobial resistance (AMR) in *S. enterica* serovar Typhi across the diverse districts of Punjab, Pakistan. Additionally, during the COVID-19 pandemic, azithromycin was widely used in Pakistan not only for its broad-spectrum antibacterial properties but also due to initial, unverified claims suggesting its potential antiviral efficacy against SARS-CoV-2.[11] Moreover, a study reported a case of carbapenem-resistant *Salmonella* Typhi in 2023,[12] which heightens concerns about the possible resistance to remaining treatment options, specifically azithromycin and carbapenems. Therefore, a larger surveillance study is necessary to determine the prevalence and antimicrobial susceptibility patterns of *Salmonella* Typhi across diverse regions. This study addresses a critical gap by conducting an extensive surveillance across 15 districts in Punjab, Pakistan, by collecting blood culture from febrile patients to map the prevalence of *Salmonella* Typhi. Additionally, the study evaluates AMR and provides a comprehensive genetic analysis of resistance mechanisms across a broad geographical area.

METHODOLOGY

Study Design and Sampling

This descriptive cross-sectional study was conducted at the Institute of Microbiology, Government College University, Faisalabad, in collaboration with Jinnah Hospital Lahore and Test Zone Diagnostic Centre. The study was carried out from July 2022 to December 2023. A total of 7,993 blood cultures were collected from febrile patients across various tertiary care hospitals in 15 districts of Punjab, Pakistan. The districts included Faisalabad (FSD), Gujranwala (GRW), Gujrat (GRT), Jhang (JNG), Jhelum (JLM), Kasur (KSR), Lahore (LHR), Mandi Bahauddin (MBD), Multan (MLN), Narowal (NWL), Okara (OKR), Sahiwal (SWL), Sialkot (SKT), Toba Tek Singh (TTS), and Vehari (VHR).

Culturing and Identification of Bacteria

Blood cultures were collected from febrile patients clinically suspected of typhoid fever. Patients who had received antibiotics within 72 hours before sample collection were excluded to minimize the risk of false-negative results. For blood cultures, the BacT/ALERT 3D Microbial Detection System (BioMérieux, Lyon, France) was employed. Briefly, the blood samples (10 mL) were collected after disinfecting the venipuncture site with 70% isopropyl alcohol. The flip caps from the BacT/ALERT culture vials were removed, and the tops of the vials were cleaned with an alcohol swab and allowed to dry. The blood was then transferred into the BacT/ALERT vials and promptly transported to the laboratory for incubation. Automated alerts from the system identified positive cultures. Bottles flagged as positive by the BacT/ALERT 3D system were subsequently sub-cultured onto MacConkey and blood agar plates. The inoculated plates were incubated in an inverted position at 37°C for 18–24 hours under aerobic conditions. To identify the organism, standard laboratory procedures were employed, including an initial assessment of colonial morphology, Gram staining, and biochemical tests using the VITEK2® system (BioMérieux, France) according to the manufacturer's guidelines. For serovar confirmation, agglutination assays were conducted using antisera (Pro Lab Diagnostics Inc., Canada) following the user guidelines.

Molecular Characterization

Molecular confirmation of the isolates was performed at the Institute of Microbiology, Government College University, Faisalabad, Pakistan, using PCR with specific primers (forward primer: 5'ACTCAGGCTTCCCGTAACGC3' and reverse primer: 5'GGCTAGTATTGTCCTTATCGG3') targeting the fliC gene of the *S.* Typhi strain. The extraction of bacterial DNA was performed using the FavorPrep™ Genomic DNA Extraction Kit (Favorgen Biotech Corporation, Taiwan) following the manufacturer's instructions. The extracted DNA was stored at -20°C until further use. Before PCR, the DNA was run on a 1% agarose gel, stained with ethidium bromide, and visualised under UV light to confirm the extracted DNA's conformation. The PCR was performed with a total reaction volume of 50 µL, including 25 µL of 2X master mix (Thermo Fisher Scientific Inc., USA), 1 µL each primer and 1 µL of DNA using the T100™ thermal cycler (Bio-Rad Laboratories Inc., Hercules, California, USA). The amplification protocol included: an initial cycle of 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds, 50°C for 45 seconds, and 72°C for 45 seconds; and a final extension at 72°C for 7-10 minutes. The resulting amplicons were separated by 1.2% agarose gel electrophoresis, stained with ethidium bromide, and visualized using a UV transilluminator.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of *S.* Typhi clinical strains was assessed using the disc diffusion method by Clinical and Laboratory Standards Institute (CLSI) 2023 guidelines. The antibiotics discs (Oxoid, Hampshire, UK) used included ampicillin [10 µg], trimethoprim-sulfamethoxazole [1.25/23.75 µg], chloramphenicol [30 µg], ceftriaxone [30 µg], cefotaxime

[30 µg], ciprofloxacin [5 µg], imipenem [10 µg], meropenem [10 µg], doxycycline [30 µg], and azithromycin [15 µg].

The minimum inhibitory concentrations (MICs) of the antibiotics were determined using the broth microdilution method by Clinical and Laboratory Standards Institute (CLSI) 2023 guidelines. The tested antibiotics included ampicillin (0.5–256 µg/mL), trimethoprim-sulfamethoxazole (0.5/9.5–256/4864 µg/mL), ceftriaxone (0.25–128 µg/mL), cefotaxime (0.25–128 µg/mL), ciprofloxacin (0.06–32 µg/mL), imipenem (0.06–32 µg/mL), meropenem (0.06–32 µg/mL), doxycycline (0.5–256 µg/mL), and azithromycin (0.06–32 µg/mL). Twofold serial dilutions of each antibiotic were prepared in cation-adjusted Mueller-Hinton broth. Bacterial suspensions were adjusted to a 0.5 McFarland turbidity standard and further diluted to achieve a final inoculum density of approximately 5×10^5 CFU/mL. Following 18 hours of incubation at 35°C, MIC values were recorded as the lowest antibiotic concentrations that completely inhibited visible bacterial growth. Quality control was performed using *Escherichia coli* (ATCC-25922) and *Pseudomonas aeruginosa* (ATCC-27853).

Isolates exhibiting resistance to first-line antibiotics (ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol) were classified as MDR. Those demonstrating additional resistance to fluoroquinolones (e.g., ciprofloxacin) and third-generation cephalosporins (ceftriaxone, cefotaxime) were categorised as XDR, consistent with definitions established by the World Health Organisation (WHO) and the Centres for Disease Control and Prevention (CDC) [9].

Molecular detection of antimicrobial resistant determinants

All isolates, including susceptible and resistant strains, were screened via PCR to detect antimicrobial resistance genes (ARGs) using primers reported in prior studies. The ARGs include ESBLs, i.e., blaTEM, blaSHV, and blaCTX-M genes [9,13], sulfonamide resistance genes including sul1, sul2, *sul*3, and *dfr*A7[1,14], the catA1 gene encoding chloramphenicol acetyltransferase [1], and the plasmid-mediated quinolone resistance determinants (*qnr*A, *qnr*B, and *qnr*S) [15]. For isolates positive for $bla_{\text{CTX-M}}$, further characterization was performed to identify specific $bla_{\text{CTX-M}}$ types, including $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-10}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{CTX-M-15}}$, using species-specific primers [9]. PCR amplification was carried out in a 30 µL reaction volume comprising 15 µL of 2X master mix, 200 nM of each ESBL primer, and 1 µL of template DNA, using a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., California, USA). Positive controls (reference strains with confirmed ARGs) and negative controls (no-template DNA) were included in each PCR run to validate amplification specificity. Following amplification, 5 µL of each PCR product was separated by 1.2% agarose gel electrophoresis at 120 V in 1X TAE buffer for 35 minutes and visualized with a UV transilluminator. PCR products from representative isolates were purified, sequenced by Macrogen Inc. (Seoul, South Korea), and confirmed using the NCBI BLAST tool.

Statistical Analysis

The data were analyzed using Microsoft Excel and Statistical Package for Social Sciences (SPSS) software, version 27. Categorical data are presented as frequencies and percentages. Comparative analyses among various groups were conducted using the Chi-square test. Statistical significance was determined with a threshold P-value of ≤ 0.05.

RESULTS

Distribution of positive blood cultures and S. Typhi

From a total of 7,993 blood cultures that were analyzed in this study, 2,832 (35.4%) were positive for microbial growth. Among these, *S.* Typhi accounted for 1,346 isolates (47.5% of positive cultures). The Sahiwal district reported the highest positive blood culture ratio, with 231 out of 522 (44.3%) cultures being positive. This was followed by Okara, where 201 out of 461 (43.6%) cultures were positive, and Jhelum, with 131 out of 310 (42.3%) positive cultures. In contrast, Sialkot had the lowest positivity rate at 27.9%, followed closely by Lahore at 28.3% and Kasur at 29.6% (**Figure 1**). Sialkot showed the highest percentage of *S.* Typhi isolates among positive cultures at 56.7%, followed by Gujrat (53.4%), Okara (52.2%), and Jhang (51.4%). On the other hand, Mandi Bahauddin and Lahore had the lowest percentage at 40.7% and 41.2% respectively (**Figure 2**).

Antimicrobial Sensitivity Testing

The analysis of *S.* Typhi isolates from 15 districts in Punjab, Pakistan, revealed high resistance rates to first-line antimicrobial agents. Resistance was most prevalent to trimethoprim-sulfamethoxazole (86.1%), ciprofloxacin (91.8%), ampicillin (78.5%), and chloramphenicol (76.4%). Notably, ciprofloxacin resistance exceeded 90% in nearly all districts, with the highest rate observed in Okara (94.8%). Resistance to doxycycline was also widespread, affecting 79.5% of isolates. Third-generation cephalosporins, including ceftriaxone and cefotaxime, showed moderate but concerning resistance rates (46.5% for both), with significant regional variation ($p = 0.006$). For example, resistance to these cephalosporins was highest in Kasur (72.9%) and Gujrat (57.3%), whereas lower rates were recorded in Gujranwala (44.6%) and Toba Tek Singh (44.2%). Chloramphenicol resistance varied between 70.5% (Vehari) and 87.1% (Kasur). Statistical analysis indicated no significant differences in resistance rates for most antimicrobials ($p > 0.05$), except for ceftriaxone and cefotaxime, where resistance varied markedly across districts ($p = 0.006$) (**Table I**).

For ampicillin (resistance ≥32 µg/mL), most isolates exhibited high MICs: 30.3% (n=414) at ≥256 µg/mL, 18.0% (n=246) at 128 µg/mL, and 15.4% (n=211) at 32 µg/mL. Cephalosporins showed lower MICs, with 28.2% (n=379) and 40.2% (n=542) of isolates at 0.5 µg/mL for ceftriaxone and cefotaxime, respectively (resistance ≥4 µg/mL). Trimethoprim-sulfamethoxazole (resistance ≥4/76 µg/mL) resistance was prevalent, with 53.5% (n=721) at 16/304 µg/mL and 25.6% (n=345) at 8/152 µg/mL. Ciprofloxacin resistance (≥4 µg/mL) was observed in 38.5% (n=518) at 4 µg/mL. Carbapenems (imipenem/meropenem) showed universal susceptibility (MICs ≤0.25 µg/mL; resistance ≥4 µg/mL). For doxycycline (resistance ≥16 µg/mL), 33.6% (n=453) exhibited MICs of 32 µg/mL. All isolates were susceptible to azithromycin (resistance ≥32 µg/mL), with 61.9% (n=833) at 2 µg/mL (**Figure 3**).

Among the total 1,346 cases, 393 (29.1%) were classified as non-MDR, 327 (24.2%) as MDR, and 626 (46.4%) as XDR. District Kasur has the highest percentage of XDR cases, with 51 out of 70 cases (72.9%) classified as XDR followed by Jhang (57.3%) and Narowal (53.4%). Conversely, Sialkot has the lowest XDR percentage, with 38 out of 97 cases (39.2%) classified as XDR, followed by Lahore (48.5%) and Multan (45.5%) (**Figure 4**).

Overall, resistance to third-generation cephalosporins was observed in 46.5% of the isolates; however, resistance rates varied significantly across the districts, with isolates from Kasur exhibiting the highest resistance (72.9%) and Gujrat showing the lowest (40.8%) ($p=0.006$). Although there were fluctuations in resistance patterns to ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol, ciprofloxacin, and doxycycline, no statistically significant differences were found among these antibiotics (**Table I**).

Molecular Basis of Resistance

Among the antibiotic resistance genes conferring resistance to first-line antimicrobial agents, *sul*1 (86.1%) and *sul*2 (79.9%), as well as the *dfr*A7 gene (84.2%), were the most prevalent genes associated with resistance to sulfamethoxazole and trimethoprim. The bla_{TEM} gene was present in 913 (67.8%) of *S.* Typhi isolates. Additionally, the plasmid-mediated quinolone resistance gene *qnr*S was found in 1236 (91.8%) of *S.* Typhi isolates (**Table I, Figure 5**). Resistance to third-generation cephalosporins was linked to the $bla_{CTX\text{-}M}$ gene, which was detected in 46.5% of isolates, and all these isolates harbored the $bla_{CTX\text{-}M\text{-}15}$ variant. The $bla_{CTX\text{-}M\text{-}1}$ gene was identified in 20.3% (n=273) of *S.* Typhi isolates (**Table I**).

The prevalence of ARGs in *S.* Typhi isolates was assessed across 15 districts of Punjab, revealing distinct regional patterns. Notably, the prevalence of $bla_{CTX\text{-}M\text{-}15}$ genes varied significantly among districts (p=0.006). The highest positivity for this gene was found in the isolates from Kasur district (72.9%), while Sialkot had the lowest (39.2%). In contrast, no statistically significant differences were observed across districts for other ARGs, including *sul*1, *sul*2, *dfr*A7, and *qnr*S (**Table I**).

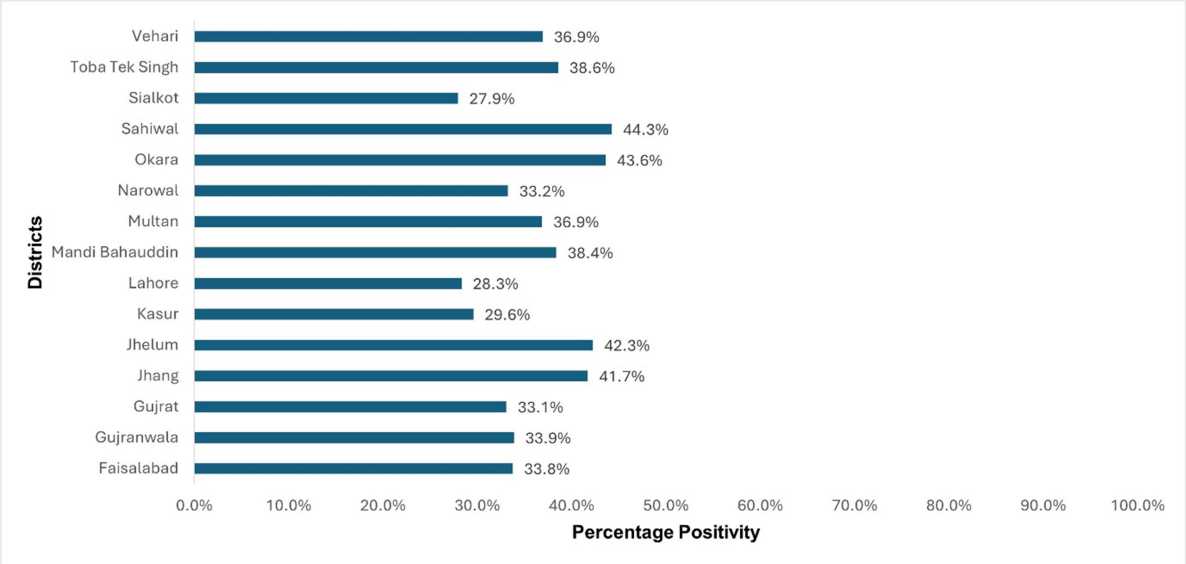

Figure I: Percentage of Positive Blood Cultures from Various Districts in Punjab, Pakistan

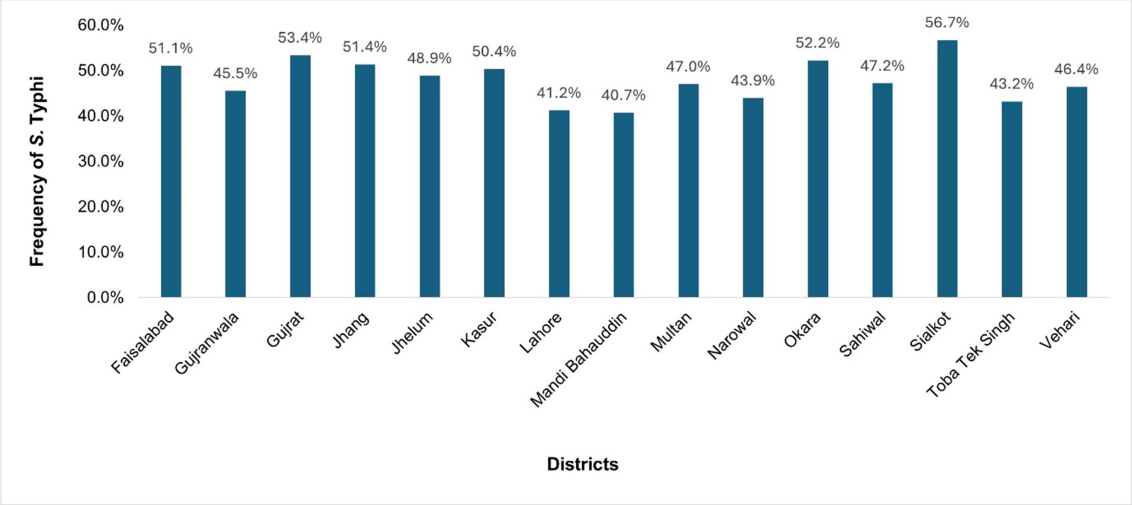

Figure II: Frequency of *Salmonella* enterica Serovar Typhi detection in Positive Blood Cultures

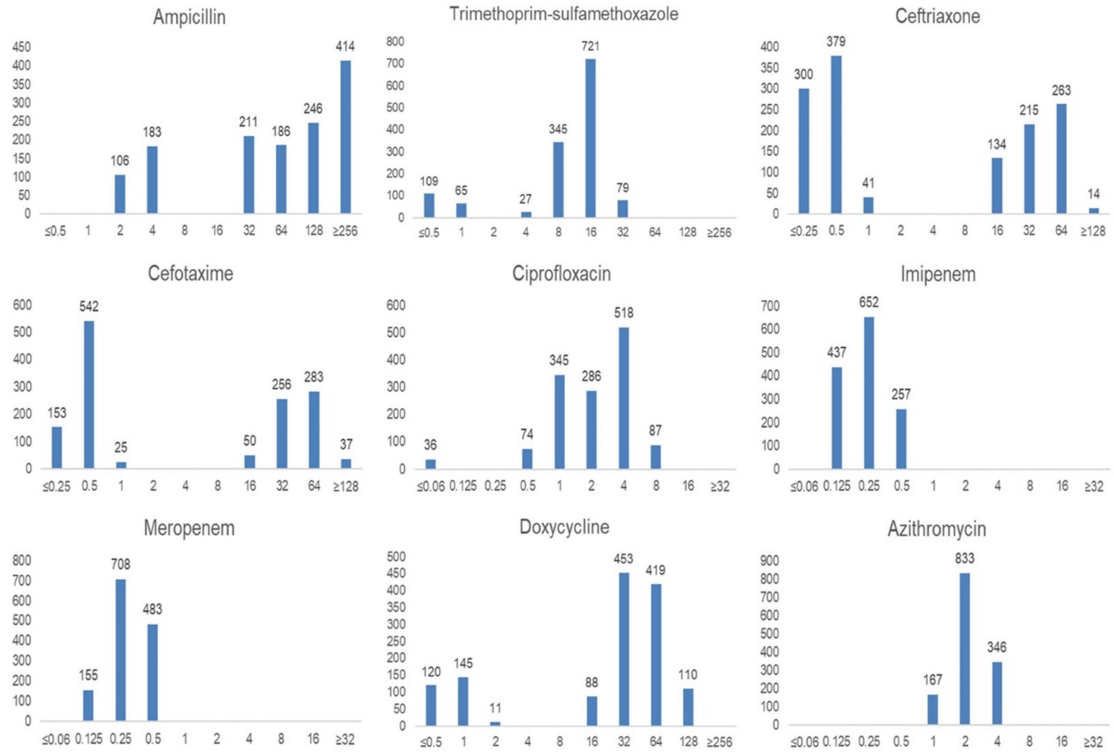

Figure III: MIC distribution of *S.* Typhi isolates against various antimicrobial agents

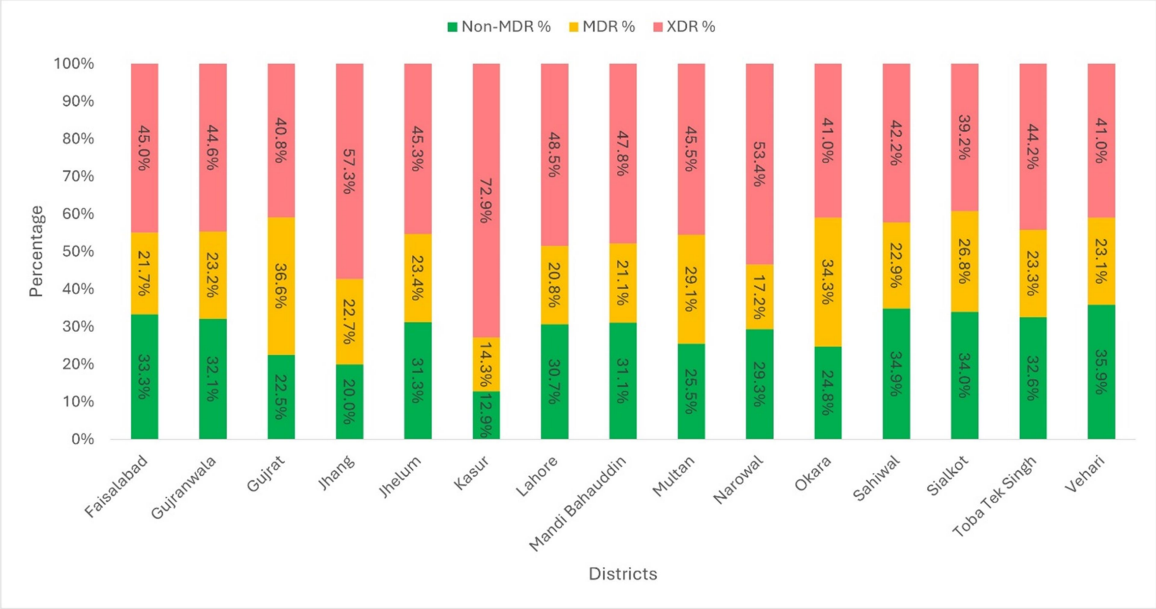

Figure IV: Percentage prevalence of non-MDR, MDR and XDR *S.* Typhi isolates in various districts of Punjab, Pakistan

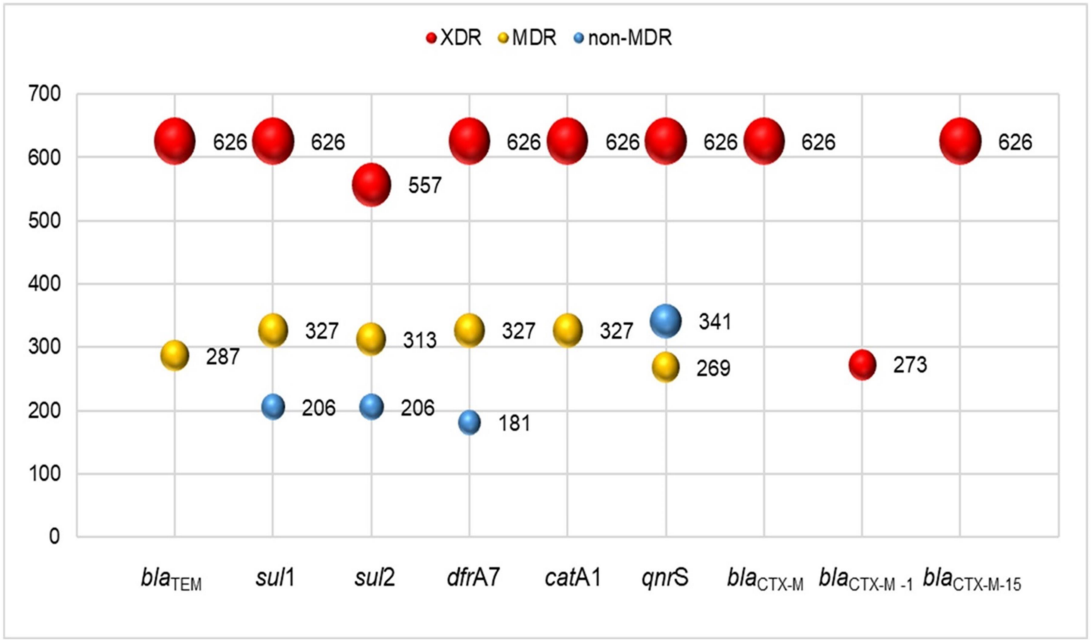

Figure V: Frequency of antibiotic resistance genes among non-MDR, MDR and XDR *Salmonella* Typhi isolates

Table I: Antimicrobial resistance and antibiotic resistance genes in *Salmonella* Typhi isolates across various districts of Punjab, Pakistan

Resistance Traits		Total n(%)	Districts															p-value
			FSD n(%)	GRW n(%)	GRT n(%)	JNG n(%)	JLM n(%)	KSR n(%)	LHR n(%)	MBD n(%)	MLN n(%)	NWL n(%)	OKR n(%)	SWL n(%)	SKT n(%)	TTS n(%)	VHR n(%)	
Antimicrobial Agents	Ampicillin	1057 (78.5)	90 (75)	85 (75.9)	62 (87.3)	63 (84)	50 (78.1)	64 (91.4)	78 (77.2)	69 (76.7)	89 (80.9)	47 (81)	86 (81.9)	81 (74.3)	71 (73.2)	65 (75.6)	57 (73.1)	0.163
	trimethoprim - sulfamethoxazole	1159 (86.1)	104 (86.7)	96 (85.7)	65 (91.5)	66 (88)	52 (81.3)	65 (92.9)	86 (85.1)	76 (84.4)	96 (87.3)	50 (86.2)	91 (86.7)	94 (86.2)	82 (84.5)	72 (83.7)	64 (82.1)	0.884
	Chloramphenicol	1028 (76.4)	87 (72.5)	83 (74.1)	60 (84.5)	63 (84)	48 (75)	61 (87.1)	75 (74.3)	67 (74.4)	87 (79.1)	46 (79.3)	84 (80)	78 (71.6)	71 (73.2)	63 (73.3)	55 (70.5)	0.712
	Ceftriaxone	626 (46.5)	54 (45)	50 (44.6)	29 (40.8)	43 (57.3)	29 (45.3)	51 (72.9)	49 (48.5)	43 (47.8)	50 (45.5)	31 (53.4)	43 (41)	46 (42.2)	38 (39.2)	38 (44.2)	32 (41)	0.006
	Cefotaxime	626 (46.5)	54 (45)	50 (44.6)	29 (40.8)	43 (57.3)	29 (45.3)	51 (72.9)	49 (48.5)	43 (47.8)	50 (45.5)	31 (53.4)	43 (41)	46 (42.2)	38 (39.2)	38 (44.2)	32 (41)	0.006
	Ciprofloxacin	1236 (91.8)	111 (92.5)	103 (92.0)	64 (90.1)	70 (93.3)	58 (90.6)	66 (94.3)	92 (91.1)	82 (91.1)	101 (91.8)	55 (94.8)	97 (92.4)	100 (91.7)	89 (91.8)	78 (90.7)	70 (89.7)	1
	Imipenem	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Meropenem	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Doxycycline	1070 (79.5)	97 (80.8)	89 (79.5)	57 (80.3)	62 (82.7)	47 (73.4 %)	61 (87.1)	80 (79.2)	72 (80)	89 (80.9)	44 (75.9)	82 (78.1)	87 (79.8)	74 (76.3)	68 (79.1)	61 (78.2)	0.966
	Azithromycin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ARGs	*Sul*1	1159 (86.1)	104 (86.7)	96 (85.7)	65 (91.5)	66 (88)	52 (81.3)	65 (92.9)	86 (85.1)	76 (84.4)	96 (87.3)	50 (86.2)	91 (86.7)	94 (86.2)	82 (84.5)	72 (83.7)	64 (82.1)	0.884
	*Sul*2	1076 (79.9)	97 (80.8)	89 (79.5)	59 (83.1)	61 (81.3)	48 (75)	59 (84.3)	78 (77.2)	71 (78.9)	90 (81.8)	45 (77.6)	86 (81.9)	89 (81.7)	77 (79.4)	67 (77.9)	60 (76.9)	0.992
	*dfr*A7	1134 (84.2)	99 (82.5)	92 (82.1)	64 (90.1)	66 (88)	52 (81.3)	64 (91.4)	85 (84.2)	75 (83.3)	95 (86.4)	49 (84.5)	91 (86.7)	89 (81.7)	79 (81.4)	71 (82.6)	63 (80.8)	0.805
	*cat*A1	953 (70.8)	80 (66.7)	76 (67.9)	55 (77.5)	60 (80)	44 (68.8)	61 (87.1)	70 (69.3)	62 (68.9)	82 (74.5)	41 (70.7)	79 (75.2)	71 (65.1)	64 (66)	58 (67.4)	50 (64.1)	0.074
	bla_{TEM}	913 (67.8)	77 (64.2)	73 (65.2)	52 (73.2)	57 (76)	42 (65.6)	60 (85.7)	67 (66.3)	59 (65.6)	97 (71.8)	40 (69)	76 (72.4)	68 (62.4)	61 (62.9)	55 (64.0)	47 (60.3)	0.077
	bla_{CTX-M}	626 (46.5)	54 (45.0)	50 (44.6)	29 (40.8)	43 (57.3)	29 (45.3)	51 (72.9)	49 (48.5)	43 (47.8)	50 (45.5)	31 (53.4)	43 (41)	46 (42.2)	38 (39.2)	38 (44.2)	32 (41)	0.006
	$bla_{CTX-M-1}$	273 (20.3)	23 (19.2)	20 (17.9)	10 (14.1)	20 (26.7)	13 (20.3)	22 (31.4)	22 (21.8)	20 (22.2)	23 (20.9)	12 (20.7)	20 (19)	20 (18.3)	16 (16.5)	17 (19.8)	15 (19.2)	0.675
	$bla_{CTX-M-15}$	626 (46.5)	54 (45.0)	50 (44.6)	29 (40.8)	43 (57.3)	29 (45.3)	51 (72.9)	49 (48.5)	43 (47.8)	50 (45.5)	31 (53.4)	43 (41)	46 (42.2)	38 (39.2)	38 (44.2)	32 (41.0)	0.006
	*qnr*S	1236 (91.8)	111 (92.5)	103 (92.0)	64 (90.1)	70 (93.3)	58 (90.6)	66 (94.3)	92 (91.1)	82 (91.1)	101 (91.8)	55 (94.8)	97 (92.4)	100 (91.7)	89 (91.8)	78 (90.7)	70 (89.7)	0.999

DISCUSSION

AMR represents one of the most pressing global public health crises of the 21st century, threatening to undermine decades of medical progress by rendering lifesaving antibiotics ineffective [16]. This challenge is exemplified by the emergence of MDR and XDR *Salmonella* Typhi strains, which have become a formidable threat in resource-limited settings [9, 10]. Notably, an XDR typhoid outbreak caused by *Salmonella enterica* serovar Typhi began in 2016 in Sindh province of Pakistan and has since spread to other parts of the country. The XDR strain is resistant to five classes of antibiotics, including first-line drugs (chloramphenicol, ampicillin, trimethoprim-sulfamethoxazole), and fluoroquinolones, as well as third-generation cephalosporins. As of June 2021, a total of 14,360 XDR typhoid cases have been reported in Karachi, and 5,741 cases in other districts of Sindh province [6]. The surveillance studies are crucial, particularly involving areas other than the provincial capitals, considering the ongoing challenges posed by the XDR typhoid outbreak. This study presents a comprehensive analysis of antibiotic resistance patterns in *Salmonella* Typhi isolates obtained from febrile patients across 15 districts in Punjab, Pakistan. The substantial number of positive blood cultures (35.4%) and the significant prevalence of *S.* Typhi (47.5%) among these cultures highlight the continued burden of typhoid fever in the region.

The observed high resistance rates to ciprofloxacin (91.8%) and trimethoprim-sulfamethoxazole (86.1%) are particularly alarming, as they indicate a concerning shift in resistance patterns that may complicate treatment strategies. These findings align with a previous study from Karachi, which reported a similarly high resistance rate of 91% to ciprofloxacin among *S.* Typhi and S. Paratyphi A isolates [17]. Furthermore, resistance to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole in *S.* Typhi was found to be 79%, 82%, and 83%, respectively, which is comparable to our results of 76.4%, 78.5%, and 86.1%. These data collectively highlight a concerning increase in resistance to first-line antibiotics, particularly trimethoprim-sulfamethoxazole. It is important to note that potential discrepancies in resistance rates between our study and the study from Karachi may be attributed to methodological differences. While the Karachi study employed the Kirby-Bauer method for susceptibility testing, our study incorporated both Kirby-Bauer and MIC determination. In case of disagreements between the two methods, the testing was repeated.

A study from Lahore, the capital of Punjab, reported an exceptionally high resistance rate of 97.3% to chloramphenicol and trimethoprim-sulfamethoxazole among a cohort of 150 extensively drug-resistant (XDR) isolates [1]. While these rates surpass those observed in our overall isolate population (n=1346), it is essential to note that the study from Lahore exclusively focused on XDR isolates. When compared to our XDR isolate cohort (n=626), resistance rates to chloramphenicol (96.6%) and trimethoprim-sulfamethoxazole (100%) were similarly high. The sul1 and sul2 genes primarily mediate resistance to sulfonamides in S. Typhi. Among the 1,346 *S.* Typhi isolates studied, the prevalence of the *sul*1 and *sul*2 genes was 86.1% and 79.9%, respectively.

In contrast, among the XDR isolates, *sul*1 was present in 100% of cases, and *sul*2 was found in 89%. A previous study, which screened only for the *sul*1 gene, reported a prevalence of 70% among *S.* Typhi isolates. Moreover, this study identified the *cat*A gene, which is essential in conferring chloramphenicol resistance among 86.6% of *S.* Typhi isolates [1]. This finding is comparable to our study, which observed a 70.8% prevalence of the *cat*A gene.

Sporadic cases of typhoid fever caused by ceftriaxone/cefotaxime-resistant *Salmonella* Typhi have been reported globally, with notable occurrences in Africa, Asia, some European countries, and Latin America [9, 10, 18, 19]. Reports from India, Bangladesh, and Pakistan have documented *S.* Typhi isolates resistant to third-generation cephalosporins [20]. A study conducted in Karachi, Pakistan, between 2009 and 2011 indicated a minimal resistance to

third-generation cephalosporins, with only 0.08% of *S.* Typhi isolates showing resistance [21]. In Pakistan, ceftriaxone is commonly used as empirical treatment for typhoid fever. Our study found an overall resistance rate of 46.5% to third-generation cephalosporins, which is comparable to the 48% resistance rate reported in Karachi [17].

The studies have suggested that resistance to third-generation cephalosporins (3GCs) is predominantly driven by the extended-spectrum beta-lactamase (ESBL) gene $bla_{\text{CTX-M}}$ among *Salmonella enterica* serovar Typhi, with the $bla_{\text{CTX-M-15}}$ variant emerging as the dominant subtype responsible for widespread cephalosporin resistance [1, 9, 10]. In this study, all XDR isolates were found to harbor the $bla_{\text{CTX-M-15}}$ gene, whereas the $bla_{\text{CTX-M-1}}$ gene was detected in 43.6% of the isolates. A recent study reported 83.9% prevalence of $bla_{\text{CTX-M-15}}$ gene among XDR isolates [22]. Genomic analyses of *S.* Typhi isolates from Peshawar, the capital of Khyber Pakhtunkhwa (KPK), revealed that all XDR isolates carried the $bla_{\text{CTX-M-15}}$ gene [23]. The *bla*TEM gene is another essential ESBL gene, which is associated with resistance to various beta-lactam antibiotics, including ampicillin and some cephalosporins.

The co-occurrence of bla_{TEM} (conferring penicillin resistance) and $bla_{\text{CTX-M}}$ (an ESBL gene) significantly increases the MICs of beta-lactam antibiotics by broadening hydrolytic activity against these drugs [24]. In our study, 67.8% of *S.* Typhi isolates were found to harbor the bla_{TEM} gene. A study from Lahore, Pakistan, reported the presence of the bla_{TEM} gene in 72.6% of XDR *S.* Typhi isolates [1]. The identification of MDR and especially XDR strains underscores the urgent need for effective surveillance and intervention measures. The molecular characterization further elucidates the genetic underpinnings of resistance, with prevalent genes such as *sul*1, *sul*2, *dfr*A7, and *qnr*S contributing to the observed resistance profiles. These findings underscore the necessity for continuous monitoring of resistance trends and the implementation of tailored treatment regimens to address the growing challenge of antibiotic resistance in *S.* Typhi. Moreover, despite all isolates in this study remained susceptible to azithromycin, the emergence of resistance genes such as *mphA* and *ermB* in *S. Typhi* were reported in the recent studies which underscores the need for sustained vigilance.[12, 25] These genes are associated with macrolide resistance, therefore, pose a potential threat to the clinical use of azithromycin which necessitates ongoing surveillance to preempt resistance trends.

CONCLUSION

This surveillance study reveals critical insights into the evolving AMR landscape of *S.* Typhi in Punjab, Pakistan, with implications for national and global typhoid management. The dominance of XDR strains harboring the $bla_{\text{CTX-M-15}}$ gene, which was found in all XDR isolates, suggests a clonal expansion of XDR strains. Notably, resistance to ciprofloxacin (91.8%) and trimethoprim-sulfamethoxazole (86.1%) renders these therapies obsolete in Punjab. The identification of key resistance genes such as sul1, sul2, catA, $bla_{\text{CTX-M}}$, and bla_{TEM} in a considerable proportion of the isolates emphasises the need for robust and continuous surveillance, particularly in rural areas, to track the resistance patterns. These findings indicate an urgent shift toward novel treatment paradigms and proactive containment strategies to avert a pan-drug resistant typhoid epidemic.

Recommendations

It is strongly recommended to prioritize district-specific interventions based on the resistance patterns to combat the escalating threat of XDR S. Typhi effectively. High-burden areas may adopt azithromycin as first-line empirical therapy while restricting cephalosporins to culture-confirmed cases supported by rapid diagnostics targeting *bla*$_{\text{CTX-M-15}}$ genes. The surveillance networks must be established at the district level to detect emerging resistance among the isolates. Concurrently, typhoid conjugate vaccines should be urgently scaled in regions with increased 40% XDR prevalence.

Limitations of the study

Although the study encompasses 15 districts in Punjab, Pakistan, it does not include data from other provinces or regions. This limits the generalizability of the findings to the entire country or neighboring regions with potentially different epidemiological trends. The cross-sectional nature of the study makes it difficult to assess temporal trends in AMR. Moreover, Advanced sequencing techniques, such as whole-genome sequencing, were not used, which might have provided a more comprehensive understanding of resistance mechanisms and strain diversity.

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AUTHOR CONTRIBUTION

Ahmad M: Conceptualization, study design, data collection, and manuscript drafting.

Rasool MH: Conceptualization, Supervision, methodology validation.

Rasheed F: Methodology, Data analysis, interpretation of results, and critical revision of the manuscript.

Aslam B: Literature review, manuscript editing and revisions.

Khurshid M: Manuscript drafting, molecular analysis and final approval of the version to be published.

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