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Molecular characterization of extensively drug-resistant *Salmonella serovar Typhi* in patients with gastrointestinal complications in Quetta, Pakistan

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Abstract: Extensive drug resistance (XDR) *S. typhi* have been evaluated in patients with gastrointestinal disturbance who attended multiple tertiary care hospitals in Quetta, Balochistan, Pakistan. Blood samples of total of 480 patients were obtained and *S. typhi* was isolated and verified by PCR. Isolates were subjected to antimicrobial susceptibility testing and antimicrobial resistance (AMR) genes of 1st, 2nd line antibiotics, 3rd generation cephalosporin and azithromycin were identified by PCR. Among 65 PCR confirmed *S. typhi cases*, 18(27%) were Multidrug resistance (MDR), 25(38%) XDR, 13 (20%) Extended spectrum β-lactamase (ESBL) and only 4(6%) Azithromycin-resistant XDR *S. typhi*. The high frequency was observed for the antibiotics-resistant genes *catA1*, bla_{TEM-1} (100%), *dhfr7* (95%), *sul1* (98%), *gyrA, gyrB, parC* (93%) and *qnrS* and *parE* 100% each. The frequency of $bla_{CTX-M-15}$ and *acrB* were 78% and 6% respectively. We found high burden of MDR, XDR and ESBLs *S. typhi*. The AMR genes were similar to those of the regional countries. Azithromycin resistance was low could be a drug of choice against XDR *S. typhi* in the study area. The study provided the molecular profile of AMR *S. typhi* in Quetta, capital of Balochistan province of Pakistan.

Keywords: *S. typhi*; Multidrug resistant; Kirby-Bauer; Antimicrobial resistance; PCR.

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INTRODUCTION

Typhoid is a food and water born systemic infection of humen caused by *Salmonella typhi (S. typhi)* which is characterized by fever, abdominal pain, malaise, diarrhea, constipation (Crump *et al.,*, 2015; Sharvani *et al.,*, 2016) and is associated with the risk of myocarditis, hepatitis, encephalopathy and serious gastrointestinal disorders (Birkhold *et al.,*, 2020). It remains a serious public health problem in developing countries of African and South East Asia including Pakistan, Bangladesh and India. An estimated 7-12 million cases and death rate of 10% are reported annually from the developing countries (Mogasale *et al.,*, 2014; Subhani, 2017; Ugboko and De, 2014b) due to inadequate health policies, infrastructure, sanitation system, poverty, poor quality of drinking water, extensive self-medication habit and inadequate servullience (Antillón *et al.,*, 2017; Das *et al.,*, 2018; Garrett *et al.,*, 2022; Organization, 2018). The antimicrobial therapy is the main stay in controlling typhoid infections. (Ugboko and De, 2014a) but the indiscriminate and irrational usage of antimicrobials has led to the emergence and dissemination of resistance among the infectious strains of *S. typhi* making the treatment of typhoid infection challenging, expensive and complicated (Kadhiravan *et al.,*, 2005). *S. typhi* which simultaneously show resistance to 1st line of therapy (Trimethoprim-Sulfamethoxazole, Chloramphenicol and Ampicillin) are termed as multidrug-resistant (MDR) *S. typhi* (Shaikh *et al.,*, 2023) and the first case of MDR S. typhi was reported in 1980s (Rowe *et al.,*, 1997). Extensively drug-resistant (XDR) *S. typhi* demonstrates resistance to Chloramphenicol, Ampicillin, Fluoroquinolone and third generation Cephalosporin (Shaikh *et al.,*, 2023). A plethora of studies have been reporting the increasing prevalence of MDR and XDR *S. typhi* in local population of Kenya (Mutai *et al.,*, 2018), Burkina Faso (Dembélé *et al.,*, 2020), Vietnam (Holt *et al.,*, 2011), Iraq (Jubair *et al.,*, 2023), Bangladesh (Mina *et al.,*, 2023), Nepal (Maharjan *et al.,*, 2021), India (Balaji *et al.,*, 2018), China (Wang *et al.,*, 2022) and some highly populated cities of Pakistan (Fatima *et al.,*, 2021; Jabeen *et al.,*, 2023). The increase in international travel from Asian countries to abroad is likely to increase the danger of dissemination and burden of AMR worldwide in the near future (Butt *et al.,*, 2022). For better control of typhoid, it becomes necessary to understand the mechanism and spread of antibiotic-resistance among different strains of *S. typhi* (Dembélé *et al.,*, 2020).

In this scenario, azithromycin is the last choice left for controlling XDR and MDR *S. typhi* infection owing to

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high sensitivity to this antibiotic (Butt *et al.,*, 2022) but the recent reports of azithromycin-resistant XDR *S. typhi* in South East Asian countries has made the task challenging for health professionals to control typhoid fever (TF). However, the prevalence of resistance to azithromycin is still low (Sajib *et al.,*, 2021). Antibiotic susceptibility of *S. typhi* has been changing continuously (Shrestha *et al.,*, 2016) and instance of resistance to newer classes of antimicrobial are common. An effective surveillance and infection control programs are required to curb the problem of antimicrobial resistance (Maharjan *et al.,*, 2021)

Pakistan is among the countries with highest burden of typhoid infection with an estimated 11 million cases and 60000 death annually (Shaikh *et al.,*, 2023). The high prevalence of XDR *S. typi* in Pakistan is the matter of concern not only for the local authorities but also for many Middle East and European countries who are hosting a huge chunk of Pakistani skilled and non-skilled workers. The dissemination of XRD *S. typhi* in the local population of middle east and USA through Pakistani emigrants has already been reported in a case report (Bharathan and Kurian, 2021). Previous studies mainly focused on big cities of Pakistan but the economically and socially depressed region of Pakistan such as Balochistan, which is more vulnerable to typhoid infection because of poor quality of drinking water, poor sanitation condition, self-medication and poor health facilities, earned a very little attention. Furthermore, few studies conducted in Balochistan, only presented the data concerning the antimicrobial susceptibility but lacking the molecular mechanism of AMR in *S. typhi* isolates (Achakzai *et al.,*, 2017). The prime reason for the growing trend in AMR in Balochistan is attributed to the excessive and unnecessary use of antimicrobials (Nasir *et al.,*)

The present study was aimed to evaluate if there is re-emergence of MDR and XDR *S. typhi* against the commonly used antibiotics in Pakistan. The study included the patients attended tertiary care hospitals in Quetta city of Balochistan province of Pakistan. Quetta is the capital city of Balochistan and received patients all around the province. Blood samples of patients having gastrointestinal disturbance were included and *S. typhi* were identified. Confirmed cases were subjected to antimicrobial susceptibility evaluations and AMR resistance genes of various classes of antibiotics were identified.

MATERIALS AND METHODS

Study design

The current study was a cross sectional study conducted at Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan Quetta, Pakistan, between March 2022 and 2023. The study was approved by an Institutional review board of CASVAB University of Balochistan Quetta and respective ethical committees of tertiary care hospitals in Balochistan, Pakistan with reference No.1-5/Estt/4382-83. In this study four hundred and eighty *(n=480)* blood samples were collected in culture bottles containing Brain Heart Infusion Broth (BHI) supplemented with 0.05% SPS as a routine procedures of hospitals and the supposition of typhoid was made on the basis of one of the given symptoms of typhoid fever (Maharjan *et al.,*, 2021). Prolonged fever and abdominal pain with diarrhea were the main clinical features considered. Suspected cases of typhoid were identified by the experienced medical practitioners and only those patients were included in study who provided informed consent. Those Patients those did not provide written consent, having mental disorder or receive antibiotic therapy more than 1 week prior to study were excluded from the study. Samples were transported to CASVAB, University of Balochistan Quetta, Pakistan, for further processing and only WIDAL positive samples were forwarded for further analysis.

Sample collection and processing

Blood samples of 10 mL volume were collected aseptically through vein puncture by experienced medical practitioners and samples were treated by the same way as reported by researchers (Javaid *et al.,*, 2012; Sabeetha *et al.,*, 2018). Serum was separated from the treated blood samples by centrifugation (Hettich, UK) at 3000 rpm for 10 min used for serological analysis.

WIDAL

The Widal test was performed by the tile method using antiserum to primary antigen O and secondary antigen H (TO & TH, Welcome, KS, USA) by the same way as reported in literature (Willke *et al.,*, 2002).

Isolation and Biochemical Identification of S. Typhi.

Inoculum from blood culture broth was streaked on *Salmonella-Shigella* agar (Sigma-Aldrich, Missouri, USA) and incubated at 37℃ for 24 hours. Next day, the bacterial morphology and biochemical analysis were performed. The identification of *S. typhi* was done by performing Gram's staining and the rapID one systempanel biochemical assay (Thermo Scientific, UK).

Extraction of DNA by CTAB Method

Extraction of bacterial DNA was performed by CTAB method as reported in literature (Jahan *et al.,* 2015). Briefly, *S. typhi* was isolated from a broth culture and the bacterial pellet was treated with a cocktail (TE buffer: 400 µL, 10% SDS: 10 µL, Proteinase-K: 50 µL) for 1 h at 60°C with continuous vortexing. Next, 5M NaCl and 10% CTAB 100 µL each, were added and vortexed for further 15 min at 60°C, briefly cooled at -70°C and again heated at 60°C. The other steps included, initial treatment with Phenol/Chloroform/iso-amyl alcohol (25:2:1), centrifuging at 12000 rpm, separating top most layer, mixing supernatant layer with pre-cooled isopropyl alcohol in a separate tube, shaking the tube for 25 min, cooling and then centrifuging at 15000 rpm for 15 min. The supernatant was discarded and the DNA-containing pellet was suspended in 70% ethanol and centrifuged for 15 min at 15000 rpm. Supernatant was discarded and tube was left open for few hours to allow the ethanol residues to evaporate the ethanol residues. The DNA pellet was finally vortexed with 100 µL of TE buffer and then stored at -20°C until further used.

Identification of Salmonella typhi through PCR

Since Widal-test sometimes provides false results due to cross-reactive antigens from previous exposure (Harris and Ryan, 2015), hence *S. typhi* were further confirmed by PCR. Bacteria were in nutrient broth and for 24 h at 37°C. The genomic DNA was extracted using CTAB method Isolation and purification of genomic DNA from approximately 200 µL of bacterial suspension was performed by the same way as reported in the literature (Al-Ansari *et al.,*, 2021). Molecular identification of *S. typhi* was performed through PCR by amplifying *aroC* and *fliC* genes sequences. The forward primer 5'GGCACCAGTATTGGCCTGCT3' and reverse primer 5'CATATGCGCCACAATGTGTTG3' were selected for *aroC* gene. The forward primer sequence 5' TATGCCGCTACATATGATGAG3' and reverse primer **5'** TTAACGCAGTAAAGAGAG 3' were selected for *fliC* gene (Macrogen, South Korea). The PCR conditions selected for *S. typhi* identification genes included 94°C for 1 min, 36 cycles of 94°C, 55°C for 1 min, 72°C for 2 min, a 72°C for 10 min analysis was performed by using thermocycler (Applied biosystem, thermofisher scientific, US). Molecular identification was performed for those cases that demonstrated a positive test on culture.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed by adopting by Kirby-Bauer disc diffusion method on Muller-Hinton agar plates (Merk, Germany). Antimicrobial susceptibility testing of *S. typhi* isolated serotypes was performed by disc diffusion method by exploiting a panel of antibiotics in accordance to the protocol of clinical and laboratory standards institute (CLSI, 2017). The following antimicrobials tested: Ciprofloxacin (10µg), Chloramphenicol (30µg), Ampicillin (30µg), Nalidixic acid (30µg), Cefixime (5µg), Ceftriaxone (30µg), Levofloxacin (5µg), Azithromycin (15µg), Trimethoprim+ Sulfamethoxazole (25µg), Meropenem (10µg). A Bacterial isolate was demonstrated to be multi-drug resistant (MDR) *S. typhi* if it showed resistant to three or more antibiotics.

Detection of Antimicrobial-resistant Genes by PCR

The expression of antibiotic resistance genes in MDR and XDR *S. typhi* isolates was evaluated through PCR analysis by detecting resistant genes. $bla_{CTX\text{-}M\text{-}15}$, *dhfr7*, *sul1*, and *catA1*genes were targeted to identify MDR *S. typhi* as possessing resistance against Ampicillin, Trimethoprim +Sulfamethoxazole and Chloramphenicol. *gyrA*, *gyrB*, *parC*, *parE,* $bla_{CTX\text{-}M\text{-}15}$ and *qnrS* were used to identify XDR *S. typhi* as possessing resistance to Levofloxacin, Nalidxic acid, Ciprofloxacin, Ceftriaxone and Cefixime. table 1. Provided the forward and reverse primer sequences of antibiotic-resistant genes amplified in *S. typhi* in the current study. PCR procedure was performed by following a reported method (Al-Ansari *et al.,*, 2021). The total reaction volume was 20 µL containing 10 µL of PCR master mix (GenScript, England), 1 µL of each primer (Macrogen, South Korea), 2 µL DNA template and 6 µL DNAase free water. PCR product was resolved by electrophoresis using 1% agarose gel and Gelpilot 100 bp ladder (Qiagen, Germany).

The PCR conditions included 35 cycles. The initial denaturation at 95°C for 5 minutes, denaturation 95°C for 30 seconds, annealing of each primer was set to 30 seconds, 72°C for 30 seconds and final extension step ran at 72°C for 7 minutes (Farhan *et al.,*, 2018). The isolated gel bands of PCR product were photographed by using gel documentation system (InGenius3, UK)**.**

STATISTICAL ANALYSIS

Data was analyzed statistically using descriptive statistics and bivariate analysis. The relationship between the variables was evaluated using chi-square or χ^2 test. *P<0.05* was considered significant. Statistical calculation was performed using SPSS version 20.0.

RESULTS

Confirmation and Distribution of Human Isolates of S. typhi

Percentage of culture-positive cases were calculated from the Widal-positive cases and the percentage of PCR-positive cases were calculated from the number of culture positive cases. table 2 provided the total number of instances and corresponding percentage for each group. Out of total 480 samples, 57.5% (276/480) were male and 42.5 % (204/480) were female patients.

There were 67 males & 50 females among the 24.4% (117/480) total Widal positive cases. Approximately 55% (35/67) of the Widal positive male patients had positive development upon culture, whereas 64% of the Widal positive female patients demonstrated growth. Around 94% of male and female isolates that presented positive culture growth, were confirmed as *S. typhi* as represented by the expression of identification gene markers *aroC* and *fliC* in the (fig. 1A &B). Chi-square revealed a non-significant difference (*p:0.481*) in the number of positive cases of *S. typhi* between the male and female patients. The non-significant difference (*p: 0.736*) was also observed across the age groups. The age group of 29-41 years showed a slightly rising trend in the frequency of *S. typhi* cases in both male and female patients. The female of age group 42-54 years presented the highest number of *S. typhi* positive cases (21% of the total suspected female).

Antibiotic Resistance Profiling of S. typhi isolates

The antimicrobial susceptibility of the 65 PCR-confirmed *S. typhi* isolates were assessed against a panel of 10 antibiotic by performing the Kirby-Bauer (1961) disk diffusion method. All 65 *S. typhi* (100%) isolates exhibited strong resistance to both Ampicillin and Nalidixic acid. Chloramphenicol and Cefixime resistance were observed in 97% of isolates. The intermediate level of resistance to Levofloxacin was found to be 60% while the resistance to Azithromycin was found to be the lowest at 6%. We found maximum sensitivity to Azithromycin and Meropenem antibiotics that was in 94% and 81% of the isolates respectively (table 3). Out of 65 isolates that underwent antibiotic susceptibility testing, 38% were classified as XDR, 27% as MDR, 20% as extended spectrum beta-lactamase producing (ESBL) and 2% as fully resistant isolates. The CLSI and EUCAST guideline were consulted in order to assess each and every outcome. The antibiotic susceptibility pattern of different antibiotics are represented in the (fig. 1C & D).

Detection of Antibiotic Resistant Genes

Antibiotic-resistant genes were evaluated by PCR in all 65 confirmed *S. typhi* isolates that were previously screened for antibiotic susceptibility. Table 4 shows the frequency of isolates that expressed antibiotic-resistant genes. A total of 11 antibiotic resistant genes were targeted.

Antimicrobial-resistant gene profiling of S. typhi against 1^st^ line of antibiotics

Antibiotic resistant genes of 1st line of antibiotics were *catA1*, $bla_{\text{TEM-1}}$, *dhfr7* and *sul1*. All give genes were linked to MDR. *catA1*, and $bla_{\text{TEM-1}}$ were detected in 100% of the isolates followed by *sul1* in 98% of the isolates and the *dhfr*7 in 95% of the isolates (fig. 2A-D).

***Antimicrobial-resistant gene profiling of S. typhi* against Fluoroquinolones**

Genes such as *qnrS, gyrA* (fig. 2E & F) *parE*, *gyr B* and *parC* (fig. 3A-C) *are recognized as* Fluoroquinolones-resistant genes. *qnrS* and *parE* were detected in 100% of isolates and *gyrA, gyr B* and *parC* in 93% of isolates.

Antimicrobial-resistant gene profiling of S. typhi against 3^rd^ generation Cephalosporines

The extended spectrum β-lactamase resistance gene $bla_{\text{CTX-M-15}}$ demonstrated resistance to 2nd line and 3rd generation Cephalosporin, was expressed in n=51(78%) isolates. We detected the *acrB* gene in 6% of the isolates resistant to Azithromycin antibiotic (fig. 3D & E).

DISCUSSION

A high prevalence of MDR and XDR *S typhi* in Pakistan in the recent past have raised concerns among medical professionals and stake holders (Balaji *et al.,*, 2018) as MDR and XDR are implicated in failure of typhoid therapy which has severe economic ramifications (Kaljee *et al.,*, 2018). Diarrhea is the highly prevailing food born gastrointestinal complication caused by various types of G-ve bacteria most importantly the *S. typhi*. The irrational usage of antibiotics has been resulted in high prevalence of AMR in *S. typhi* we considered diarrhea as a disease indicator in suspected patients of typhoid (Diarra *et al.,*, 2024).The Burden of MDR and XDR *S. typhi* and AMR gene profile in local population of Balochistan were comparable to other regions of Pakistan and azithromycin resistance was still lower in the study area.

The overall percentage of *S. typhi* was comparable to previous studies in Balochistan providence of Pakistan (Naeem Khan *et al.,*, 2013) but in conflict with findings from Pakistan's densely populated metropolitan regions, including Lahore (Jabeen *et al.,*, 2023), Karachi (Yousafzai *et al.,*, 2020), Khayber Pakhtunkhwa (Hussain *et al.,*, 2019) and Islamabad (Ahmad *et al.,*, 2020). This implies that the burden of *S. typhi* in Balochistan is lower than other regions of the country and that it is not expected to rise in future. The emergence and dissemination of AMR in a particular region is the interplay between environment factors and socioeconomic status of the local population (Allel *et al.,*, 2020). The compelling reasons for the higher frequency of *S. typhi* in densely populated regions of Pakistan than Balochistan include overcrowding, poor hygiene, lack of access to clean drinking water, contamination of municipality water by the sewage line and large scale exodus people from the economically deprived areas of Pakistan to the big cities due to political unrest and poverty (Ahmad *et al.,*, 2023; Organization, 2018). We found relatively low percentage of *S. typhi* cases in neighboring nations such as Nepal (Khadka *et al.,*, 2021), India (Bhumbla *et al.,*, 2022), Bangladesh (Mina *et al.,*, 2023) and Iran (Abbasi and Ghaznavi-Rad, 2021) due to variation in the natural reservoirs of *S. typhi*, geographical locations, climate changes, socioeconomically factors, ecological factors, personal hygiene conditions, prescription trends and the degree of water/ food contamination (Abbasi and Ghaznavi-Rad, 2021).

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Even though we found insignificant correlation between gender and typhoid cases, the frequency of cases in male patients was marginally higher than female which is consistent with a few published reports from Pakistan (Ashfaq *et al.,*, 2024; Hasan *et al.,*, 2023; Yousafzai *et al.,*, 2020) and also in India (Bhumbla *et al.,*, 2022), Nepal (Maharjan *et al.,*, 2021), Bangladesh (Begum *et al.,*, 2018) and Iran (Abbasi and Ghaznavi-Rad, 2021).

Table 1: Primers used to detect antibiotic-resistant genes in *S.typhi*

1	*bla*$_{TEM-1}$	CAGCGGTAAGATCCTTGAGA ACTCCCCGTCGTGTAGATAA	55	643	(Adesiji, *et al.,* 2014).
2	*bla*$_{CTX-M-15}$	CACACGTGGAATTTAGGGACT GCCGTCTAAGGCGATAAACA	55	996	(Saeed *et al.,* 2020).
3	*dhfr7*	GTGTCGAGGAAAGGAATTTCAAGCTC TCACCTTCAACCTCAACGTGAACAG	59.6	191	(Imran *et al*., 2010).
4	*qnrS*	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	54.2	417	Ramachandran, Shanthi & Sekar, 2017).
5	*catA1*	CGCCTGATG AATGCTCATCCG CCTGCCACTCATCGCAGTAC	58	456	(Ren *et al*., 2020).
6	*sul1*	CTTCGATGAGAGCCGGCGGC GCAAGGCGGAAACCCGCGCC	65.5 67.5	430	(Phan *et al.,* 2009).
7	*gyrB*	AAGCGCGATGGCAAAGAAG AACGGTCTGCTCATCAGAAAGG	55.9	1500	(Shaheen *et al*., 2013).
8	*gyrA*	*TACCGTCATAGTTATCCACGA* *GTACTTTACGCCATGAACGT*	51.4	*313*	(Holt *et al.,* 2011).
9	*Pare*	TCTCTTCCGATGAAGTGCTG ATACGGTATAGCGGCGGTAG	54.2	240	(Acheampong *et al.,* 2019).
10	*parC*	CTATGCGATGTCAGAGCTGG TAACAGCAGCTCGGCGTATT	54.2	270	(Shaheen *et al*., 2013).
11	*acrB*	AcrB-UFP -F GCTGGATGAGGTCACGGATT ACRb-MAMA-R TTCCAGACCGTTAGGGCG	58.4	397	(Sajib *et al.*,2021).

Table 2: Gender wise and age wise distribution of suspected and confirmed cases of *S. typhi.*

Age	Total subjects		Widal		Culture		PCR	
(years)	M	F	M *n (%)*	F *n (%)*	M *n (%)*	F *n (%)*	M *n (%)*	F *n (%)*
3-15	72	68	17 (23.6)	17 (25.0)	10 (58.8)	9 (52.9%)	10 (100.0)	7 (77.7)
16-28	68	48	16 (23.5)	11 (22.9)	10 (62.5)	7 (63.6%)	09 (90.0)	7 (100.0)
29-41	44	48	11 (25.0)	12 (25.0)	07 (63.6)	9 (75.0%)	07 (100.0)	9 (100.0)
42-54	48	28	12 (25.5)	07 (25.0)	05 (41.6)	6 (85.7%)	05 (100.0)	6 (100.0)
55-57	44	12	11 (25.0)	03 (25.0)	05 (45.5)	1 (33.3%)	04 (80.0)	1 (100.0)
Total	276	204	67 (24.3)	50 (24.5)	37 (55.2)	32 (64.0)	35 (94.5)	30 (93.7)
	480		117 (24.4)		69 (58.9)		65 (94)	

M= Male; F= Female; PCR= Cases confirmed by polymerase chain reaction; *n*= number of cases.

Table 3: Antibiotic susceptibility profile of *S. typhi* against various antibiotics (Kirbay-Bauer method)

Antibiotics	Disk (µg)	Antibiotic resistance profile *(n= 65)*		
		Sensitive (%)	Intermediate (%)	Resistant (%)
CIP	10 µg	10	6	84
C	30 µg	3	0	97
AMP	30 µg	0	0	100
NA	30 µg	0	0	100
CFM	5 µg	3	0	97
CRO	30 µg	8	0	92
LEV	5 µg	30	10	60
AZM	15 µg	94	0	6
SXT	25 µg	30	0	70
MEM	10 µg	81	0	19

CIP: Ciprofloxacin; CHL: Chloramphenicol; AMP: Ampicillin; Nalidixic acid; CFM: Cefixime; CRO: Ceftriaxone; LEV: Levofloxacin; AZM: Azithromycin; SXT: Sulfamethoxazole-Trimethoprim; MEM: Meropenem;

Table 4: Antibiotic resistance genes detected in *S. typhi* isolates directed against various group of antibiotics.

Genes Resistant to 1st Lab		Genes Resistant to FQAb		Genes Resistant to 3rd Gab	
Genes	Present n (%)	Genes	Present n (%)	Genes	Present n (%)
dhfr7	62 (95%)	*qnrS*	65 (100%)	*bla*$_{CTX-M-15}$	51 (78%)
catA1	65 (100%)	*parE*	65 (100%)	*acrB*	4 (6%)
bla$_{TEM-1}$	65 (100%)	*gyrA*	61 (93%)	--	--
sul1	64 (98%)	*gyrB*	61 (93%)	--	--
--	--	*parC*	61 (93%)	--	--

LAb: Line of antibiotics; FQAb: Fluoroquinolone antibiotics; n: number of isolates.

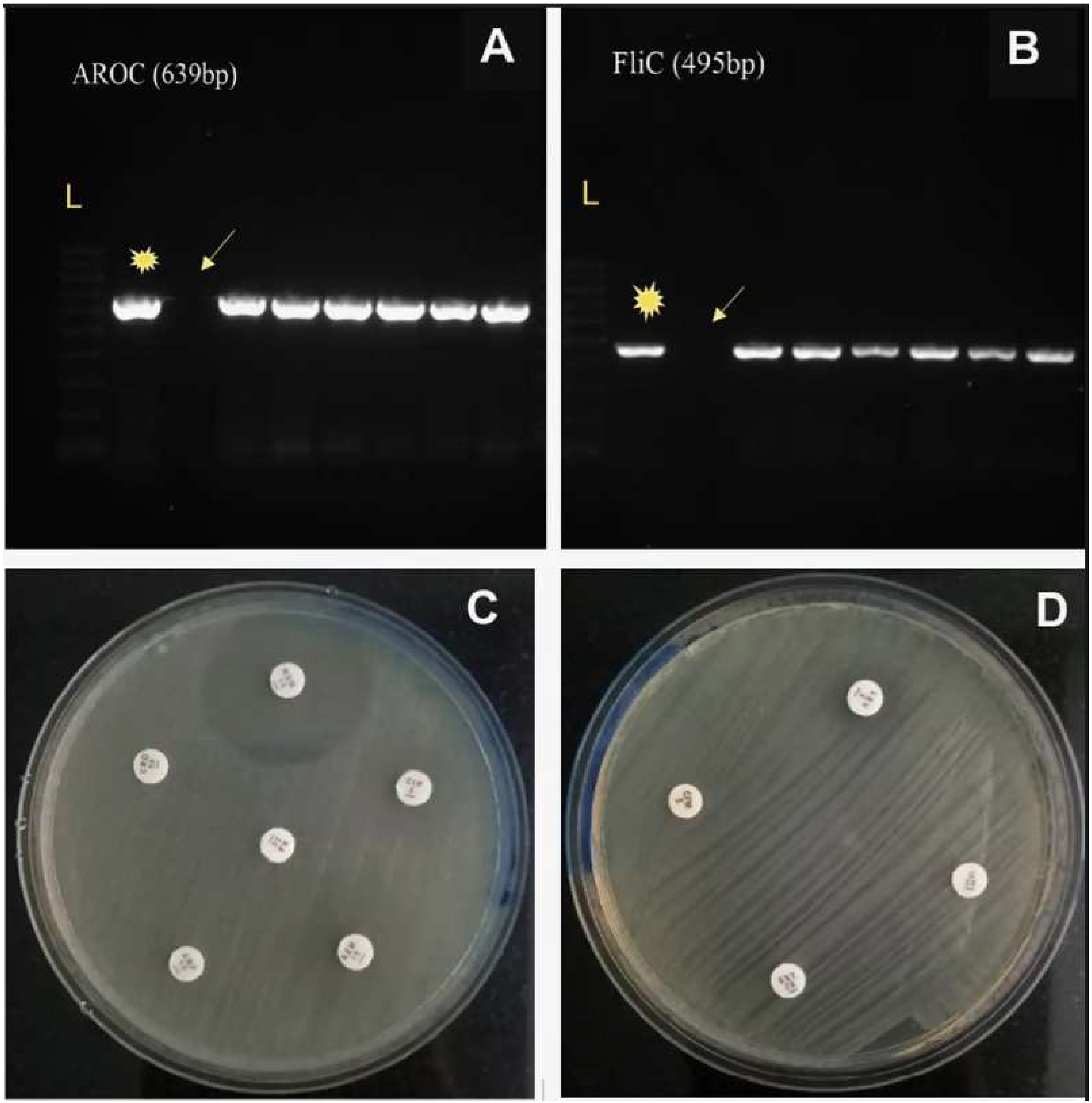

Fig. 1: Identification of *Salmonella* isolates. A: Indicates the existence of 693-base pair (bp) *aroc* gene. B: Indicates the existence of 495bp *fliC* gene. L: Indicate the ladder of 1000bp. Arrows: indicate the negative control and the steric sign indicates the positive control. All other bands belong to *S. typhi* isolates. C: showed sensitivity of isolates to Meropenem and resistance against Ampicillin, Azithromycin, Nalidixic acid, Ciprofloxacin and Ceftriaxone pattern of antibiotic D: showed resistance against Cefixime, Levofloxacin, Sulfamethoxazole-Trimethoprim and Chloramphenicol. Antibiotic susceptibility performed by Kirby Bauer Disc Diffusion.

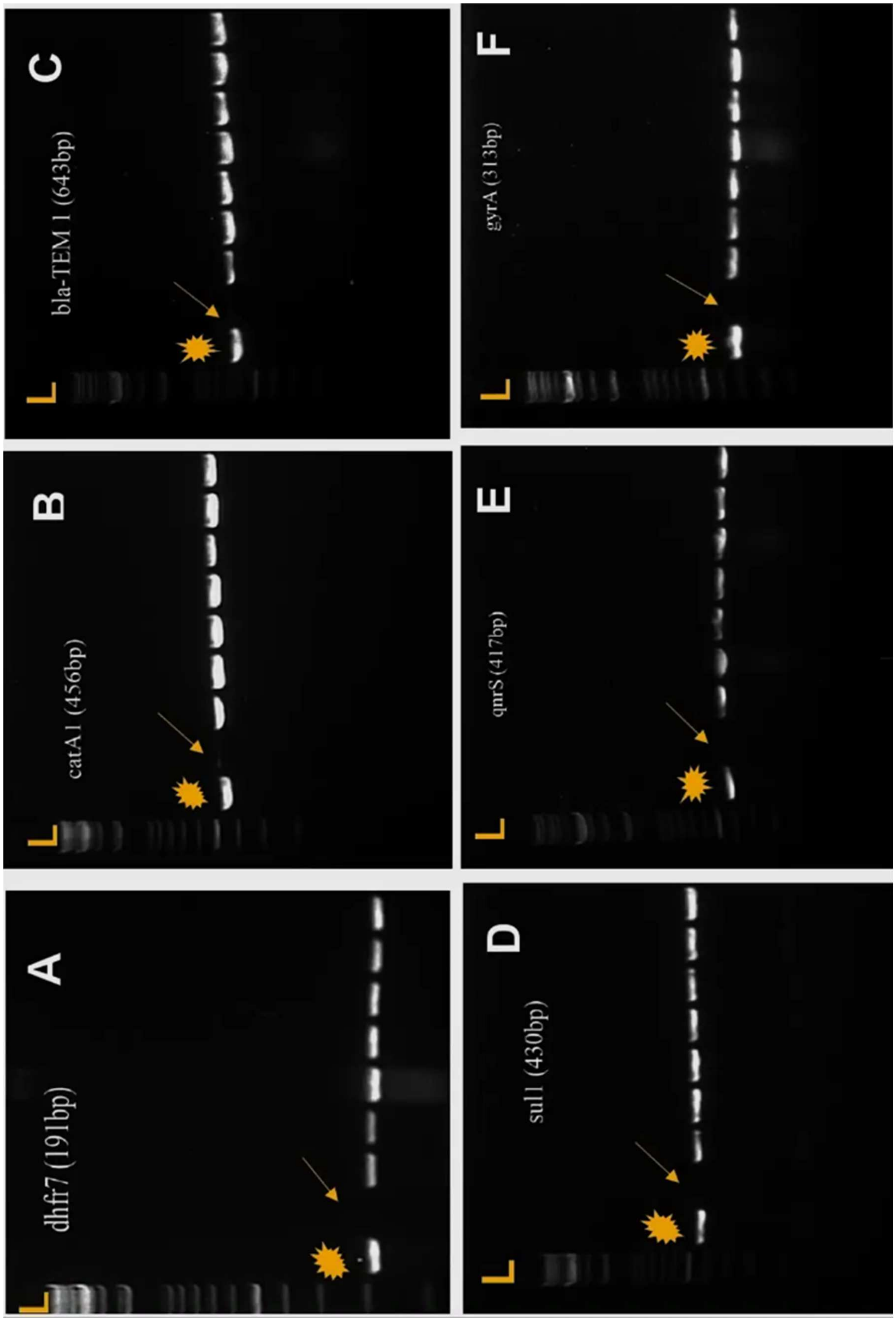

Fig. 2: PCR for the detection of resistance genes in *S. typhi* against 1st line of antibiotics. A: Indicates the presence of *dhfr7* (191bp) gene against Cotrimoxazole. B: Indicates the presence of *catA1* (456bp) gene directed against Chloramphenicol. C: Indicates the presence of bla_{TEM-1} (643bp) gene directed against Ampicillin. D: Indicates the presence of *sul1(430bp)*. E: Indicates the presence of *qnrS* (417bp). F: Indicates the presence of *gyrA* (313bp). L: in all images indicate the ladder of 1000bp. Arrows: In all images indicate the negative control and the steric indicates the positive control. All other bands belong to *S. typhi* isolates.

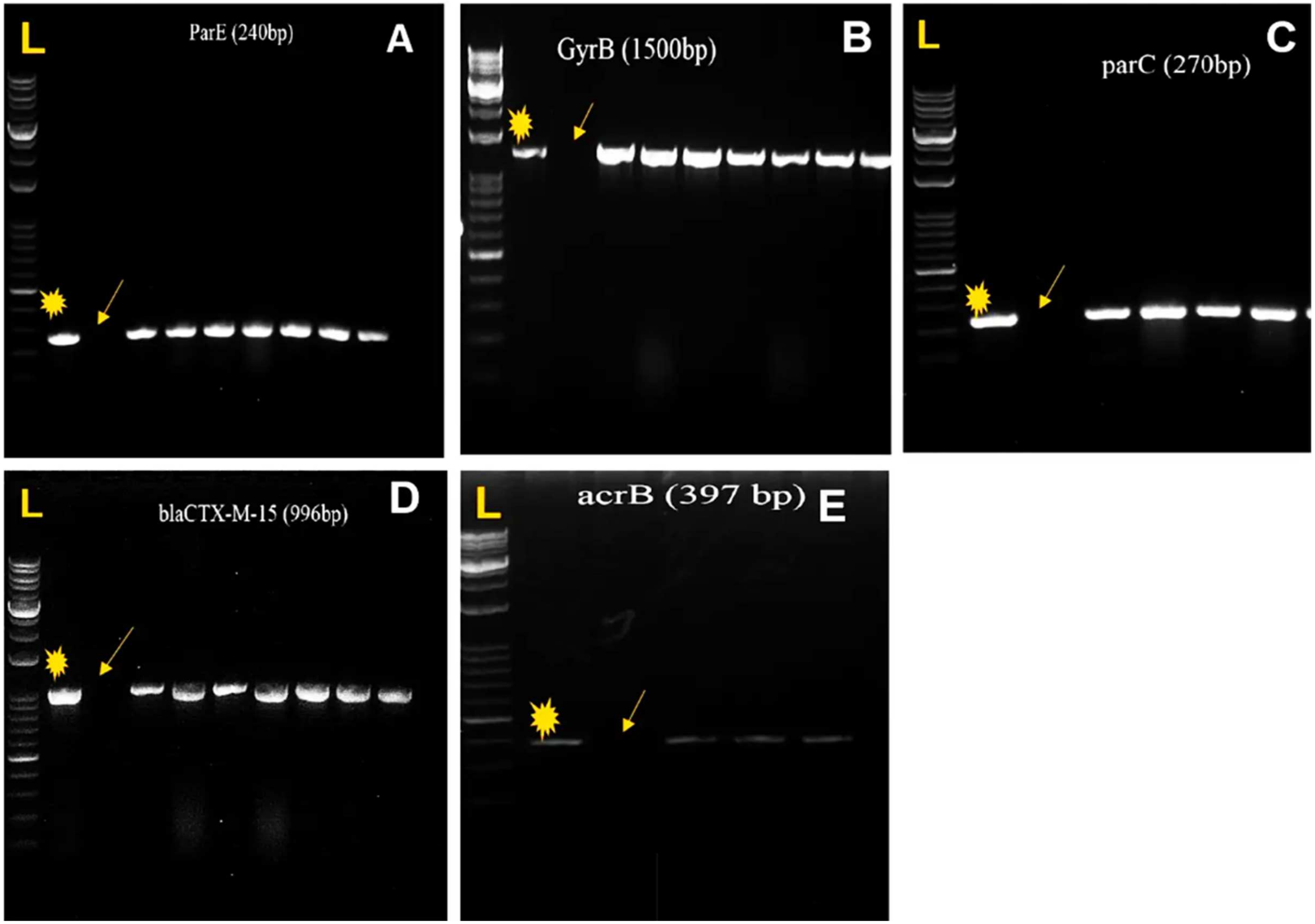

Fig. 3: PCR for the detection of resistance genes in *S. typhi* against Fluoroquinolone, 3rd generation cephalosporin and azithromycin antibiotics. A: Indicates the presence of *parE* (240bp) B: Indicates the presence of *gyrB* (1500bp).C: Indicate the presence of *parC* (270bp). D: Indicates the presence of *bla*$_{CTX-M-15}$ (996bp) gene directed against 3rd generation Cephalosporin. E: Indicates the presence of *acrB* (397bp) gene directed against Azithromycin antibiotic. L: in all images indicate the ladder of 2000bp. Arrows: In all images indicate the negative control and the steric indicates the positive control. All other bands belong to *S. typhi* isolates.

The patients of age group 29-41 years accounted for largest proportion of typhoid cases in our study. These results are in line with reports published in different regions of Pakistan (Ahmad *et al.,*, 2020; Ashfaq *et al.,*, 2024; Hussain *et al.,*, 2019; Naeem Khan *et al.,*, 2013), India, Indonesia (Chen *et al.,*, 2007), Bangladesh (Begum *et al.,*, 2018). The attributing factors for the high frequency of typhoid in adults are those mentored for the male patients (Khadka *et al.,*, 2021). The increase in frequency of *S.typhi* cases in male was might be attributed to the increased outdoor activities of male, preference for outdoor eating and visit to hospitals than female (Kalsoom *et al.,*, 2014).

Antibiotic resistance

The MDR *S typhi* were countered by fluoroquinolone and 3rd generation cephalosporin but the emergence of XDR *S typhi* in many countries left very few choices to counter typhoid (Syed Asim Ali Shah, 2020) since XDR *S typhi* possess simultaneous resistance to antibiotics of first line, fluoroquinolone and the 3rd generation cephalosporin. Both MDR and XDR strains of *S typhi* are sensitive to azithromycin and meropenem (Butt *et al.,*, 2022) so currently these antibiotics are the main stay in the treatment of typhoid involving XDR (Syed Asim Ali Shah, 2020). Fortunately, prevalence of azithromycin resistance in countries like Bangladesh and Pakistan is very low (Hooda Y *et al.,*, 2019).

In our study, frequency of XDR was substantially higher and the frequency of azithromycin was lower and comparable to frequency reported in Karachi (Syed Asim Ali Shah, 2020), and Lahore Pakistan (Zakir *et al.,*, 2021). Contrary to our findings, some studies published in Pakistan reported exceptionally high frequencies of MDR and XDR in Lahore (Ahmad *et al.,*, 2023) , Karachi (Khan *et al.,*, 2012) and Hyderabad (Fatima *et al.,*, 2021). Polypharmacy, self-medication, poor drinking water quality, abysmal sewage system and over population were the major attributes to the high frequency of MDR and XDR in some clinical settings of Pakistan (Ahmad *et al.,*, 2023; Dalton, 2018). Frequency of MDR and XDR in neighboring countries such as Nepal (Maharjan *et al.,*, 2021) and India (Veeraraghavan *et al.,*, 2021) was lower due to the decreasing trends in the prescription of 1st line of antibiotics. Azithromycin resistance in general was found lower in various regions of Pakistan (Carey *et al.,*, 2021) so is still viewed as drug of choice against XDR *S .typhi* infections in Pakistan but strict control on drug utilization is prerequisite to stop the dissemination of Azithromycin resistance in future.

Molecular basis of resistance to antibiotics

Antibiotic resistance genes were investigated to ascertain the molecular basis of resistance in our AMR *S typhi* isolates. *catA1, dhfr7,* $bla_{\text{TEM-1}}$ and *sul1* antibiotic resistant genes carried out by IncHI1 region of plasmid or chromosome of *S. typhi* are normally categorized as MDR resistant genes (Jabeen *et al.,*, 2023). *CatA1* provides resistance against chloramphenicol, $bla_{\text{TEM-1}}$ against ampicillin, *dhfR7* and *sul1* against Cotrimoxazole. The point mutations in the quinolone resistance determination region (QRDR) of IncY plasmid in *S typhi* harboring genes for Topoisomerase-II (*gyrA, gyrB*) and Topoisomerase-IV (*parC, parE*) and are implicated in Fluoroquinolone resistance. $bla_{\text{CTX-M-15}}$ gene is related to 3rd generation cephalosporin. Fluoroquinolone and cephalosporin resistance genes are expressed by *XDR S. typhi* (Kim *et al.,*, 2021). *acrB* gene is linked to *a*zithromycin resistance in *S. typhi* (Duy *et al.,*, 2020). A study from Lahore, Pakistan reported the expression of all above mentioned genes in XDR *S. typhi* isolates (Kim *et al.,*, 2021). We identified each of $bla_{\text{TEM-1}}$ and *catA 1* genes in 100% of *S. typhi* isolates. *dhfr7* and *sul1* genes were identified in 95% and 98% of *S. typhi* isolates respectively. Our findings are comparable to a study published in Pakistan that reported the occurrence of *catA1* and $bla_{\text{CTX-M-15}}$ genes in MDR and XDR *S. typhi* isolated from blood samples of patients (Mumtaz *et al.,*, 2024) and an Indian investigation which reported the expression of $bla_{\text{TEM-1}}$, *catA1*, and *sul1* genes in 90%, 90% and 80% of the MDR *S. typhi* isolates respectively (Katiyar *et al.,*, 2020). Consistent with our results, a Nigerian study reported the expression of *sul1* gene in 100% of MDR *S. typhi* isolates (Adesiji *et al.,*, 2014) and a Pakistani study published from Lahore reported the expression of *cat1, sul1* and *dhfr7* genes in 86%, 70% and 56% of *S. typhi* isolates respectively(Jabeen *et al.,*, 2023). Studies from Lahore, Pakistan reported the expression of $bla_{\text{TEM-1}}$ in 73% of MDR isolates in different clinical settings (Jabeen *et al.,*, 2023; Saeed *et al.,*, 2020). The prevalence of resistant genes to 1st line of antimicrobial therapy in our antimicrobial-resistant *S. typhi* isolates was relatively higher than those reported from other areas of Pakistan (Jabeen *et al.,*, 2023). The absence of these resistant genes in MDR and XDR *S. typhi* strains of the published studies was might be due to the involvement of alternative pathways of resistance (Wang *et al.,*, 2022).

Concerning the existence of Fluoroquinolone resistance genes, we identified *qnrS, parE* in 100% of *S. typhi* isolates and each of *gyrA, gyrB* and *parC* in *93%* of isolates. Our findings were closely aligned with the previous research which reported the high frequency of these genes in XDR *S. typhi* isolates in Pakistan (Jabeen *et al.,*, 2023), Nicobar and Northern India (Carey *et al.,*, 2021; Katiyar *et al.,*, 2020; Thamizhmani *et al.,*, 2012) and Nepal (Khadka *et al.,*, 2021). Jubair and associates reported the expression of *qnrS* gene in Fluoroquinolone resistant *S typhi* from patients of Iraq (Jubair *et al.,*, 2023). Another study confirmed the phylogenic similarity between the AMR *S. typh* from Pakistan and India (Thamizhmani *et al.,*, 2012).

CTX is a resistant determinant against 3rd generation Cephalosporin antimicrobials (Jabeen *et al.,*, 2023). There are several variants of CTX. $bla_{CTX-M-15}$ is highly prevalent in XDR *S. typhi* from Pakistan (Kim *et al.,*, 2021; Zahid *et al.,*, 2022), China (Wang *et al.,*, 2022), Bangladesh (Lima *et al.,*, 2019) and India (Saeed *et al.,*, 2020). According to a published study from Karachi, Pakistan, XDR *S. typhi* harboring $bla_{CTX-M-15}$ gene occurred with an extremely high frequency (85%) (Sohail *et al.,*, 2024). XDR *S. typhi* expressed gene $bla_{CTX-M-15}$ were similar to those reported elsewhere in Pakistan (Rasheed *et al.,*, 2020).

In the present study, the frequency of $bla_{CTX-M-15}$ gene was 78% which closely resembles to the previously published study from Karachi, (Sohail *et al.,*, 2024), Lahore (Rasheed *et al.,*, 2020) and other areas of Punjab, Pakistan (Saeed *et al.,*, 2020). Contrary to our findings, some studies conducted in Pakistan have found comparatively lower frequency of $bla_{CTX-M-15}$ in XDR *S. typhi* isolates (Jabeen *et al.,*, 2023). The low frequency of this genes in Cephalosporin-resistant *S. typhi* reported by published studies is attributed to the occurrence of alternative pathways of cephalosporin resistance (Kim *et al.,* 2021) and may be the changing prescription trends in different regions. Additionally, the high frequency of $bla_{CTX-M-15}$ have also been reported in XDR *S. typhi* isolates from Andaman and Nicobar islands of India (Thamizhmani *et al.,*, 2012) UAE and Kuwait (Rotimi *et al.,*, 2008). Single point mutation in *acrB* efflux pump is the molecular basis of Azithromycin resistance (Sajib *et al.,*, 2021). All of our azithromycin-resistant isolates were positive for *acrB* gene which is in agreement with the published studies from Pakistan (Jabeen *et al.,*, 2023).

Taken together, the frequency of resistance against 1st line, 2nd line and Fluoroquinolone antibiotics in our *S. typhi* isolates was although high, still lower than some regions of Pakistan (Ahmad *et al.,*, 2023; Fatima *et al.,*, 2021; Khan *et al.,*, 2012; Zakir *et al.,*, 2021), Bangladesh (Ghurnee *et al.,*, 2021; Mina *et al.,*, 2023) and Kenya (Ghurnee *et al.,*, 2021; Mutai *et al.,*, 2018) and the molecular mechanism of AMR evaluated in our study was not different in cases reported from Pakistan (Jabeen *et al.,*, 2023; Rasheed *et al.,*, 2020; Sohail *et al.,*, 2024), China (Kim *et al.,*, 2021), India (Thamizhmani *et al.,*, 2012) and Kuwait (Rotimi *et al.,*, 2008). The resistance against azithromycin antibiotic in our study is still very low when compared to the other parts of Pakistan (Aziz and Malik, 2018) and the neighboring countries (Sharma *et al.,*, 2018; Taneja *et al.,*, 2021). Azithromycin is the last oral antibiotic left for treating typhoid infection. Due to great reliance on azithromycin for typhoid therapy, there is concern that the resistance will increase soon and it would not be a viable option for typhoid therapy (Duy *et al.,*, 2020).

The frequent emergence of MDR and XDR *S. typhi* calls for the limited use of antimicrobial agents and nationwide real time surveillance to identify the mechanism of resistance and effective measures to counter the dissemination of resistance genes at an early stage of the problem (Yan *et al.,*, 2016). The best strategy to combat antibiotic resistance in fighting against typhoid fever is to combine two or more antimicrobial agents in wise able manner to avoid the risk of adverse drug reaction (Booker *et al.,*, 2005). As indiscriminate usage of antibiotic is one of the major reasons of the emergence of AMR in Baluchistan, there is need to limit the availability of antibiotic as On the Counter. Additional inputs include provision of health facilities in affordable price and public health educational program. AMR in *S. typhi* most commonly through point mutation in the quinolone resistance-determining region (QRDR) harboring the genes for DNA gyrase *gyrA* and *gyrB* and topoisomerase IV *parC* and *parE* result in quinolone-resistant. Horizontal gene transfer (HGT) through plasmid, transposon and phage trigger the dissemination of AMR in *S. typhi* via conjugation, transduction and transformation respectively. Plasmid mediated HGT is prominent in this regards so there is a need to track plasmid mediated AMR in future (McMillan *et al.,*, 2020). HGT allows the AMR to be disseminated among the other bacteria species (Yusof *et al.,*, 2022)

CONCLUSION

The given study revealed the occurrence MDR and XDR *S. typhi* in Quetta, Balochistan. The study has also reported the occurrence of azithromycin-resistant *S. typhi* although in low frequency. The genes linked to antibiotic resistance were identical to those found in prior studies conducted in Pakistan and Southeast Asian countries. The molecular evaluations performed in the present study to decipher the genes implicated in AMR should be the part of surveillance screening performed in the future. Azithromycin might still be the drug of choice for treating typhoid in Balochistan and other parts of Pakistan, but should be used by following strict protocol to avoid the emergence of resistance. Future studies should span a large geographic area in this region of Pakistan and incorporate all available demographic data to provide comprehensive picture of AMR *S. typh*i load. The determination of MIC may offer better estimation of AMR than disc diffusion alone. Better provision of diagnostic facilities, strict nationwide legislation and planes are prerequisite to limit the availability of antibiotics as OTC products. Limitation of current study is the less geographical coverage. Prospective researchers are suggested to include additional sampling area thus to present the better picture of AMR in Balochistan.

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Conflict of interest

The authors declare that there is no conflict of interest regarding this manuscript.

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