

Antimicrobial susceptibility pattern and genotypic characterisation of quinolone and ceftriaxone resistant genes among *Salmonella typhi* from various regions of Pakistan

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Abstract

Objective: To determine the current antibiotic resistance patterns and identification of quinolone and ceftriaxone resistant genes among *Salmonella enterica* subspecies serovar Typhi.

Method: The prospective study was conducted from September 2018 to March 2019 and comprised samples collected from major hospitals and laboratories in Karachi, Quetta, Lahore, Kharia, Rawalpindi, Islamabad and Peshawar after approval from the institutional ethics review board of Hazara University, Mansehra, Pakistan. Antimicrobial susceptibility of isolates collected from the health facilities was checked using the Kirby Bauer disc diffusion method in line with the Clinical and Laboratory Standards Institute guidelines at the Department of Microbiology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. All isolates were subjected for identification of genes responsible for quinolone and ceftriaxone resistance using polymerase chain reaction followed by gel-electrophoresis.

Results: Among the 96 isolates, phenotypically, ceftriaxone was found resistant in 31(32.29%) and ciprofloxacin in 95(99%). Genotypically, blaCTX-M-15 (beta lactamase, CTX as its acronym, -M from Munich) gene for ceftriaxone resistance was found in all phenotypically resistant 31(32.29%) isolates, while QnrS (Quinolone resistance, S group), GyrA (DNA gyrase subunit A), and GyrB (DNA gyrase subunit B) genes responsible for ciprofloxacin resistance were found in different frequencies (percentages given in table 2).

Conclusion: The spread of extensively drug-resistant *Salmonella enterica* subspecies serovar Typhi strain to many big cities calls for urgent preventive measures.

Keywords: XDR *S. typhi* in Pakistan, AMR, Resistance pattern of *S. Typhi*, Ceftriaxone and ciprofloxacin resistant genes. (JPMA 72: 2166; 2022) DOI: <https://doi.org/10.47391/JPMA.163>

Introduction

Typhoid fever, a life-threatening febrile illness and significant public health issue of low- and middle-income countries (LMICs) is identified as the 4th fatal disease in Pakistan.¹ Worldwide, the disease is prevalent in Asia with highest incidence in southern parts of the continent. Since the emergence of multi-drug resistance (MDR) to ampicillin (AMP), chloramphenicol (C) and cotrimoxazole (SXT), and extensive drug resistance (XDR) additionally to ceftriaxone (CRO) and ciprofloxacin (CIP) strains, antimicrobial resistance (AMR) is the biggest threat causing not only increased morbidity and mortality, but also posing therapeutic challenges to clinicians globally. Since the outbreak of novel extensively drug-resistant (XDR) *Salmonella enterica* subspecies serovar (*S.*) *Typhi*, which is resistant to AMP, C, SXT, fluoroquinolone (FQL) and 3rd generation cephalosporin (CEF), in November 2016 in the Sindh province of Pakistan, about 1,000 cases have been confirmed by blood culture. The exact number is probably much higher as most typhoid cases are treated empirically due to limited microbiological culture services. The H58

clade *S. Typhi* involved in this outbreak contains IncY (incompatibility plasmid group Y) plasmid with resistant genes for FQLs, like QnrS, and for CROs, like blaCTX-M-15, in addition to chromosomally mediated AMR cassette for AMP, C and SXT.² Production of intracellular extended spectrum-β-lactamase (ESBL) enzymes by this novel strain is responsible for resistance to CRO and other ESBLs.³ CTX-M group ESBL classes 1-4 are commonly reported in *Salmonella strains* from Asia.⁴ Different classes of CTX-M reported in *Salmonella strains*, including CTX-M-15 from class 1, and CTX-M-9 and CTX-M-14 from class 4 are located on transmissible plasmids and can be easily transferred among members of Enterobacteriaceae.⁵ Quinolone resistance in *Salmonellae* is mainly due to double mutation in GyrA gene, single mutation in parC.⁶ efflux pumps and the presence of plasmid mediated quinolone resistance (PMQR) genes QnrA, QnrB, QnrD and QnrS.^{3,7} The chromosomal mutation and acquisition of AMR genes was associated with reduced susceptibility to FQLs but recent mutation of quinolone resistance-determining region (QRDR) has caused common resistance in deoxyribonucleic acid (DNA) gyrase GyrA and GyrB, and topoisomerase IV parC and parE, resulting in treatment failure.⁸ The emergence of XDR strain has led to a complex situation regarding effective empirical treatment of typhoid fever,

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thereby creating major public health issue in Pakistan.⁹ The current study was planned to determine the current phenotypic AMR pattern as well as genotypic identification of CIP and CRO resistant genes among *S. Typhi* from various regions of Pakistan.

Materials and Methods

The prospective study was conducted from September 2018 to March 2019 at the Department of Microbiology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan, in collaboration with Combined Military Hospital (CMH), Rawalpindi, and Pak-Emirates Military Hospital (PEMH), Rawalpindi, and comprised samples collected from major laboratories in Karachi, Quetta, Lahore, Kharian, Rawalpindi, Islamabad and Peshawar.

After approval from the institutional ethics review board of Hazara University, Mansehra, Pakistan, the sample size was calculated using an online calculator (<https://www.calculator.net/sample-size-calculator.html>). Blood/bone marrow collected from *Salmonella* suspected patients without age discrimination were incubated (Automated BACTEC 9120 /BacT/ALERT 3D system) and further processed as per standard protocols. Isolates from various laboratories were transported in glycerol broth and were stored at -20°C till further processing. Automated blood culture positive samples and isolates from glycerol broth were sub-cultured on blood and MacConkey agar and were further identified by gram-staining, catalase, oxidase, analytical profile index (APIO 20E (BioMerieux, France) and serotyping using poly-valent and mono-valent *Salmonella* antisera (Denka Seiken Co. Ltd., Tokyo, Japan). Antibiotic susceptibility pattern was performed by Kirby Bauer (KB) disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) 2019 guidelines. Antibiotic disks, including ceftriaxone (CRO) 20µg, ciprofloxacin (CIP) 5µg, azithromycin (AZM) 15µg, chloramphenicol (C) 30µg, cotrimoxazole (SXT) 25µg, ampicillin (AMP) 10µg and meropenem (MEM) 10µg, were used. Susceptibility was also confirmed by automated Vitek-2 systems version 2.01. Deoxyribonucleic acid (DNA) extraction was done using a kit.¹⁰

Polymerase chain reaction (PCR) amplification of CIP and CRO resistant genes with primer sequences was done using in-house protocol. A total of 25µl reaction mixture was made by adding 1µl of each of forward and reverse primers (qnrS, gyrA, gyrB, blaCTX-M-15), 0.5µl of 10xPCR buffer, 0.4µl magnesium chloride (MgCl₂) 25mM, 0.1µl of 10mM deoxynucleoside triphosphate (dNTP) mix, 0.1µl of Taq DNA polymerase (5U/ul) and nuclease-free water. Thermo-cycler conditions were denaturation at 94°C for 2min, followed by 30 cycles of denaturation at 94°C for 1min, annealing temperature of 1 min was different for each primer (qnrS 46°C, gyrA 53°C, gyrB 54°C, blaCTX-M-15 at 56°C), extension at 72°C for 2min and final extension at 72°C for 5 min.

For gel-electrophoresis, 1% agarose gel was prepared. Initially, 50ml of 10X TBE (Tris-borate-EDTA) buffer was mixed with 450ml of distilled water to form 1X TBE buffer. Next, 60ml of 1X TBE Buffer mixed with 0.6gm of agarose powder was heated for 1min in a beaker and then poured into the gel tray. Ethidium Bromide 0.6µl was added and mixed well. The mixture was combed and kept at room temperature till solidification of gel After which 3.5µl 6x loading dye along with 6.5µl amplified product was added in reaction tube. Further, 0.5µl of 100bp and 10bp for blaCTX-M-15 leader was added in the first well, and 10µl samples were loaded in the successive wells. Amplified products were observed after one hour using trans-illuminator.

Results

Of the 96 isolates, 31(32.29%) were resistant to CRO, 95(98.95%) to CIP, 64(66.66%) to AMP, 70(72.91%) to C, and 64(66.66%) to SXT. All the 96(100%) isolates were sensitive to MEM and AZM. Most XDR strains were received from Sindh, followed by Baluchistan, Punjab, Khyber-Pakhtunkhwa and Islamabad/Rawalpindi (Table 1).

Results for genotypic identification of CIP and CRO resistant genes and their oligonucleotide sequences were noted (Table 2) and so were the amplified products of gel electrophoresis (Figure). All 31(32.29%) XDR isolates from different regions displayed phenotypically similar

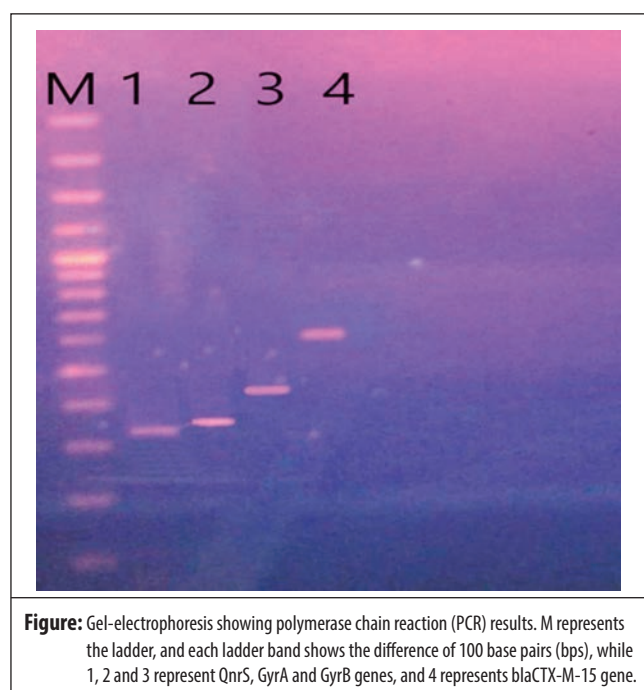
Table-1: Susceptibility pattern (% resistant) of *Salmonella enterica* subspecies Serovar Typhi (*S. Typhi*) isolates across various regions of Pakistan.

Region	Number of isolates	AMP n (%)	C n (%)	SXT n (%)	CIP n (%)	CRO n (%)	AZT	MEM
Punjab	25	17(68)	17 (64)	17 (56)	25 (100)	7 (28)	Nil	Nil
Khyber-Pakhtunkhwa (KP)	30	9(30)	14 (30)	9 (30)	30 (100)	6 (20)	Nil	Nil
Sindh	12	12 (100)	12 (83)	11 (91)	12 (100)	9 (75)	Nil	Nil
Balochistan	12	12 (100)	12 (91.66)	12 (100)	12 (100)	6 (50)	Nil	Nil
Islamabad & Rawalpindi	17	14 (82.35)	15 (88.23)	15 (88.23)	16 (88.23)	3 (17.64)	Nil	Nil
Total	96	64 (66.66)	70 (72.91)	64 (66.66)	95 (98.95)	31 (32.29)	Nil	Nil

AMP: Ampicillin, C: Chloramphenicol, SXT: Cotrimoxazole, CIP: Ciprofloxacin, CRO: Ceftriaxone, AZM: Azithromycin, MEM: Meropenem.

Table-2: Ciprofloxacin and ceftriaxone resistant genes detected among *Salmonella enterica* subspecies *Serovar Typhi* (*S. Typhi*) strains from various regions of Pakistan.

Primer	Gene	Oligonucleotide Sequence	Antimicrobial Agent	Amplicon Size (bp)	Gene detected in strain	Percent
QnrS1	QnrS	ATGGAACCTACAATCATCA	Ciprofloxacin	492	42	43.75
QnrS2		AAAAACACCTCGACTTAAGT				
GYRA/P1	GyrA	TGTCCGAGATGGCTGAAGC	Ciprofloxacin	347	75	78.83
GYRA/P2		TACCGTCATASGTTATCCAG				
StygyrB1	GyrB	CAAAGTGGCGGACTGTCAGG	Ciprofloxacin	345	66	68.75
StygyrB2		TTCCGGCATCTGACGATAGA				
blaCTX-M-15	blaCTX-M-15	ATGGTTAAAAATCACTGCG	Ceftriaxone	620	31	32.29
		TTACAAACCGTCGGTGAC				



resistance pattern and were also found positive genotypically for blaCTX-M-15 gene for CRO as well as for GyrA and QnrS genes for CIP. Of the 95(98.95%) isolates showing phenotypic resistance to CIP, 3(3.15%) did not reveal any band for CIP resistant genes used in the study.

Discussion

AMR in Typhoidal *Salmonellae* has rapidly increased over the past many years, rendering first-line drugs, like AMP, SXT and C, and alternative drug CIP empirically ineffective. Currently, Pakistan is facing an outbreak of XDR strain of *S. typhi* in Sindh province, especially in Hyderabad and Karachi cities, rendering CRO also ineffective and thereby posing major therapeutic challenge for public health. There is impending threat of spread of this lethal strain to other parts of the country as well as globally. The current is the first to have collected and observed antimicrobial phenotypic resistance and corresponding resistant genes for CIP and CRO among *S. Typhi* strains from various regions

across Pakistan.

Of the 96 isolates, 31 were identified as XDR strains; 9(75%) from Sindh, followed by Baluchistan 6(50%), Punjab 7(28%), Khyber-Pakhtunkhwa 6(20%) and Islamabad/Rawalpindi 3(17.64%). Recent studies have shown XDR isolates mainly from Hyderabad and Karachi with sporadic cases from some other areas of Pakistan as well as globally.¹¹ The important finding in the current study is that all the XDR isolates from different regions had phenotypically

same antibiogram with almost identical zone sizes, Extended spectrum β -Lactamases (ESBL) production as well as being genotypically positive for blaCTX-M-15 gene for CRO and the GyrA and QnrS genes for CIP, confirming the spread across Pakistan of the same strain that was initially found in Hyderabad and Karachi.² The same XDR strain was also reported from the United Kingdom. CRO-resistant typhoid cases were also reported from Iraq, Bangladesh and India. The isolates from Bangladesh and Iraq also harboured blaCTX-M ESBL genes, but had an IncN plasmid (Incompatibility plasmid group-N), differing from the IncY plasmid identified in Pakistani strains.² In the current study, 2 XDR strains were also isolated from pus specimen of the same patient in addition to blood culture. One of the patients presented with fever and splenic abscess; splenectomy was performed which led to full recovery. The other patient was a young pregnant lady who had presented in emergency with perforated gall bladder, acute pancreatitis and peritonitis. The outcome was missed abortion, followed by death. Unusual presentation for XDR typhoid has also been reported earlier.¹² Out of the 96 isolates, 64(66.66%) were found resistant to AMP and SXT, while 70(72.91%) to C. Besides, 32(33.33%) isolates were detected as MDR strains resistant to AMP, SXT and C, 2(2%) isolates were sensitive to all the applied antimicrobials, while the remaining showed resistance in different combinations in the current study. Other local studies have reported comparable¹³ as well as higher resistance rates while a regional study showed lesser resistance rates to first-line drugs.¹⁴ CIP was found highly resistant (98.95%) in the current study. Higher resistance rates were also reported by other local studies.^{12,13} while lower rates were also reported by a regional study.¹⁴ Genotypically, GyrA gene was most commonly detected 75(78.83%), followed by GyrB 66(68.75%) and QnrS 42(43.75%). Both QnrS and GyrA genes were detected in all 31 isolates, exhibiting resistance to CRO and CIP phenotypically, and were positive for blaCTX-M-15 gene, while GyrB and GyrA were commonly detected either in combination or alone among most of the remaining isolates. Three isolates showing

phenotypic resistance to CIP did not reveal any band genotypically for gene sequences in the current study. Local and regional studies also commonly reported mutations in DNA gyrase genes (GyrA, GyrB) of quinolone resistance determining region (QRDR) region as well as acquisition of plasmid mediated Qnr genes for quinolone resistance.^{2,15,16} The emergence and spread of XDR *S. Typhi* are a demonstration of how a ubiquitous antibiotic resistance plasmid can be acquired by MDR *S. Typhi*, rendering it XDR, thereby limiting treatment options, increasing morbidity and mortality rate, and posing an impending threat of an epidemic-like spread.

Better strategies against typhoid are warranted, such as the introduction of preventive measures, including vaccines, improved sanitation and antibiotic stewardship. New point-of-care test, like Gene-expert Mycobacterium tuberculosis/resistance to Rifampicin (TB/RIF), is suggested to be designed for early detection of *Salmonella* as well as CRO-resistant genes simultaneously for early diagnosis and prompt treatment to reduce morbidity and mortality.

Conclusion

The spread of XDR *S. Typhi* strain to many big cities of Pakistan signals impending threat of its spread to other parts of the world, and, therefore, it needs urgent preventive measures to limit its spread.

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Conflict of interest: None.

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