Original Article

Molecular evaluation of drug resistance in clinical isolates of *Salmonella enterica* serovar Typhi from Pakistan

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Abstract

Introduction: This study aimed to determine the drug susceptibility patterns and genetic elements related to drug resistance in isolates of *Salmonella enterica* serovar Typhi (*S*. Typhi) from the Faisalabad region of Pakistan.

Methodology: The drug resistance status of 80 isolates were evaluated by determining antimicrobial susceptibility, MICs, drug resistance genes involved, and the presence of integrons. Nalidixic acid resistance and reduced susceptibility to ciprofloxacin were also investigated by mutation screening of the *gyrA, gyrB*, *parC,* and *parE* genes.

Results: Forty-seven (58.7%) isolates were multidrug resistant (MDR). Among the different resistance (R) types, the most commonly observed (13/80) was AmChStrTeSxtSmzTmp, which is the most frequent type observed in India and Pakistan. The most common drug resistant genes were $bla_{\text{TEM-1}}$, *cat*, *strA-strB*, *tetB*, *sul1*, *sul2,* and *dfrA7*. Among the detected genes, only *dfrA7* was found to be associated in the form of a single gene cassette within the class 1 integrons.

Conclusions: MIC determination of currently used drugs revealed fourth-generation gatifloxacin as an effective drug against multidrug-resistant *S*. Typhi, but its clinical use is controversial. The Ser83→Phe substitution in *gyrA* was the predominant alteration in nalidixic acid-resistant isolates, exhibiting reduced susceptibility and increased MICs against ciprofloxacin. No mutations in *gyrB*, *parC*, or *parE* were detected in any isolate.

Key words: *Salmonella* Typhi; multidrug resistance; fluoroquinolone resistance

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Introduction

Typhoid fever remains a major public health problem in developing countries. The estimated incidence is approximately 33 million cases each year [1]. Over the years, the traditional antityphoid drugs, chloramphenicol, ampicillin, and trimethoprim-sulfamehtoxazole (cotrimoxazole) have become ineffective, leading to a rapid increase in multidrug-resistant (MDR) *S*. Typhi. As a result, quinolones/fluoroquinolones and third-generation cephalosporins have emerged as current front-line drugs against typhoid. However, resistance against nalidixic acid has emerged rapidly in recent years [2].

Among fluoroquinolones, ciprofloxacin, ofloxacin, and pefloxacin are currently in popular use. Ciprofloxacin, in particular, is the drug of choice for the treatment of typhoid. But fluoroquinolones are not registered for use in children on a routine basis [3,4]. In addition, resistance to ciprofloxacin is being reported with increasing frequency. As a result, third-generation cephalosporins have become popular, especially in the treatment of children. Although resistance to third-generation cephalosporins in non-typhoidal *Salmonellae* had been reported as early as 1989 [5], resistance in *S.* Typhi remains rare. The first cases of reduced susceptibility or resistance to ceftriaxone were documented in Bangladesh in 1999 and in Kuwait in 2008 [6,7].

Mutations in quinolone resistance determining regions (QRDR) of some genes are the basis of resistance development against ciprofloxacin and other fluoroquinolones. Mutations in QRDR of *gyrA* gene are reported to be at various locations, but the most common are at codons encoding serine at position 83, and aspartate at position 87 [8]. Other mutations in the QRDR of *gyrB, parC,* and *parE* genes of *Salmonella* isolates have been described as well [9].

The rapid spread of drug resistance is mainly due to the horizontal transfer of genes; it may also be caused by mutations in some of the key bacterial

genes, as is the case with quinolones. In some cases, alterations in a single gene can confer resistance, whereas in the majority of cases, a consortium of genes is involved in the development of resistance against a particular drug. The most common vehicles for this horizontal transfer are integrons. Class 1 integrons are found extensively in clinical isolates, and most of the known drug-resistance gene cassettes belong to this class. By contrast, only six different resistance cassettes have been found that are associated with class 2 integrons [10].

The presence of *dfrA7* gene is very important for determining the ancestry of an *S*. Typhi isolate. The first *dfrA7* cassette within the class 1 integron of *S*. Typhi isolates was reported by Wain *et al.* from a collection of MDR plasmids isolated after 1994 from Vietnam [11]. The *dfr* gene reported in two incHI plasmids pHCM1 and R27 (precursor of pHCM1) isolated before 1994 was *dfrA14*. The presence of a different *dfr* gene in MDR *S*. Typhi strains isolated after 1994 may explain the historical data. In a separate study on the role of integrons in the global dissemination of antimicrobial drug resistance, a single gene cassette encoding the *dfrA7* gene within the class 1 integron was reported among non-typhoidal *Salmonella* serovars of same sequence types (STs) from Uganda and South Africa, which provides an example of clonal expansion [12].

Typhoid accounts for the fourth largest number of disease-related deaths in Pakistan [13]; MDR typhoid is becoming a very complicated problem. There have been instances of resistance to even fluoroquinolones and third-generation cephalosporins. In this study, we evaluated the drug resistance status of *S*. Typhi isolates from the Faisalabad region (with a population of more than 10 million) of Pakistan with special emphasis on contemporary front-line drugs, and also identified the related genetic elements responsible for the drug resistance.

Methodology

S. Typhi isolates

Eighty isolates of *S*. Typhi, collected between 2010 and 2011 from the Faisalabad region of Pakistan, were taken from the culture collection of the National Institute for Biotechnology and Genetic Engineering (NIBGE) in Faisalabad. Initially, these isolates were obtained from cultures of blood taken at high temperatures from suspected typhoid patients admitted to various public hospitals in the region, following reported procedures [14]. The isolates were stored at -20°C in tryptic soy broth (Merck, Darmstadt, Germany) containing 10% dimethyl sulfoxide, and subcultured on MacConkey agar to get isolated colonies.

Two well-characterized *S*. Typhi strains, STK1 and STK2, which were a gift from University of Karachi, Pakistan, were used as quality control (QC) organisms.

Bacterial DNA was extracted using a DNA extraction kit (Fermentas, Hanover, USA), and confirmation of *S.* Typhi isolates was done by targeting the *fliC* gene as previously reported [14,15].

Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested using the Kirby-Bauer disk diffusion method and interpreted following Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. The drugs included nalidixic acid, a non-therapeutic drug to act as a stand-in for fluoroquinolones susceptibility assays [17,18], and ciprofloxacin, which belongs to second-generation fluoroquinolones. Traditional anti-typhoidal drugs ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole were also included. In addition, cephradine, cefixime, ceftriaxone, cefoparazone/sulbactum, aztreonam, streptomycin, gentamicin, amikacin, and tetracycline were also tested. These drugs are representative of six major groups of antimicrobials. Extended-spectrum β-lactamase (ESBL) production was measured by standard cephalosporin/clavulanic acid combination disk test [16]. This test is based on the measurement of susceptibility to two cephalosporin agents in the presence of clavulanic acid, a β-lactamase inhibitor. Disks containing cefotaxime (30g), cefotaxime/clavulanic acid (30/10g), ceftazidime (30g), and ceftazidime/clavulanic acid (30/10g) were purchased from Oxoid, (Oxoid, Basingstoke, UK).

For the quantitative estimation of antimicrobial susceptibility, E-test strips (AB Biodisk, Solna, Sweden) were used following the manufacturer's recommendations. The E-test is a simple, accurate, and reliable method to determine the MIC for a wide spectrum of infectious agents. E-strips for selected drugs belonging to the fluoroquinolone (nalidixic acid, ciprofloxacin, ofloxacin, and gatifloxacin) and cephalosporin (ceftriaxone and cefpodoxime) groups, which are currently at the forefront of typhoid treatment, were used. The strips were a gift from Dr. John Wain of the Norwich Medical School NRP Innovation Centre, UK.

Table 1. Oligonucleotides used in the study

Primer	Gene	Oligonucleotide Sequence (5′→3′)	Antimicrobial agent/Genetic element	Amplicon size in bp [reference]
A1	bla_{TEM-1}	GCACGAGTGGGTTACATCGA	Ampicillin + Cephalosporins	311 [44]
A2	(Nested)	GGTCCTCCGATCGTTGTCAG		
blt-F	bla_{TEM-1}	CCCCTATTTGTTTATTTTTC	Ampicillin + Cephalosporins	962 [45]
blt-R		GACAGTTACCAATGCTTAAT		
OXA-F	bla_{OXA}	ATGAAAAACACAATACATATCAACTTCGC	Ampicillin	820 [46]
OXA-R		GTGTGTTTAGAATGGTGATCGCATT		
cat-F	*Cat*	CCTGCCACTCATCGCAGT	Chloramphenicol	623 [47]
cat-R		CACCGTTGATATATCCC		
TB-F	*tetB*	CTCAGTATTCCAAGCCTTTG	Tetracycline	416 [48]
TB-R		CTAAGCACTTGTCTCCTGTT		
sulI-F	*sul1*	GGATGGGATTTTTCTTGAGCCCCGC	Sulfonamide	308 [11]
sulI-R		ATCTAACCCTCGGTCTCTGGCGTCG		
sul-3	*sul2*	TCAACATAACCTCGGACAGT	Sulfonamide	707 [49]
sul-4		GATGAAGTCAGCTCCACCT		
DHFR1-F	*dfrA7*	GTGTCGAGGAAAGGAATTTCAAGCTC	Trimethoprim	191 [11]
DHFR1-R		TCACCTTCAACCTCAACGTGAACAG		
DHFR2-F	*dfrA14*	TTTGATGTCCAACCTGAGCGGG	Trimethoprim	189 [11]
DHFR2-R		TGCGAAAGCGAAAAACGGCG		
aadA-F	*aadA*	TGATTTGCTGGTTACGGTGAC	Streptomycin	284 [50]
aadA-R		CGCTATGTTCTCTTGCTTTTG		
aadA2-F	*aadA2*	TGTTGGTTACTGTGGCCGTA	Streptomycin	526 [51]
aadA2-R		GCTGCGAGTTCCATAGCTTC		
aadA1a-F	*aadA1a*	GTGGATGGCGGCCTGAAGCC	Streptomycin	526 [47]
aadA1a-R		ATTGCCCAGTCGGCAGCG		
strA	*strA-strB*	ATGGTGGACCCTAAAACTCT	Streptomycin	891 [52]
strB		CGT-CTAGGATCGAGACAAAG		
aacC2-F	*aacC2*	GGCAATAACGGAGGCAATTCGA	Gentamicin	450 [53]
aacC2-R		CTCGATGGCGACCGAGCTTCA		
GYRA/P1	*gyrA*	TGTCCGAGATGGCCTGAAGC	Quinolone	347 [54]
GYRA/P2		TACCGTCATASGTTATCCACG		
StygyrB1	*gyrB*	CAAACTGGCGGACTGTCAGG	Quinolone	345 [55]
StygyrB2		TTCCGGCATCTGACGATAGA		
StmparC1	*parC*	CTATGCGATGTCAGAGCTGG	Quinolone	270 [9]
StmparC2		TAACAGCAGCTCGGCGTATT		
StmparE1	*ParE*	TCTCTTCCGATGAAGTGCTG	Quinolone	240 [9]
StmparE2		ATACGGTATAGCGGCGGTAG		
QnrS1	*qnrS*	ATGGAAACCTACAATCATAC	Quinolone	492 [56]
QnrS2		AAAAACACCTCGACTTAAGT		
QnrA-F	*qnrA*	GATAAAGTTTTTCAGCAAGAGG	Quinolone	543 [57]
QnrA-R		ATCCAGATCGGCAAAGGTTA		
Int1-F	*intI1*	ATCATCGTCGTAGAGACGTCGG	Class 1 integron	892 [58]
Int1-R		GTCAAGGTTCTGGACCAGTTGC		
Int2-F	*intI2*	GCAAATGAAGTGCAACGC	Class 2 integron	467 [59]
Int2-R		ACACGCTTGCTAACGATG		
Int3-F	*intI3*	GCAGGGTGTGGACGAATACG	Class 3 integron	760 [60]
Int3-R		ACAGACCGAGAAGGCTTATG		
qacE1Δ-F	*qacE1Δ/sul1*	ATCGCAATAGTTGGCGAAGT	3′CS	800 [61]
sulI-B		GCAAGGCGGAAACCCGCGCC		
5′CS-F	Gene cassette	GGCATCCAAGCAGCAAGC	Gene cassette	Variable [62]
3′CS-B		AAGCAGACTTGACCTGAT		

Selection of antimicrobial drug resistance related genes

Resistance to several classes of antimicrobial agents is conferred by the presence of certain genes and related mutations, as observed in different studies [19]. For each group of antimicrobials, a large number of genes that can be responsible for resistance phenotype have been reported. It is impossible to study all the reported genes. The most commonly isolated and reported genes related to resistance against each antimicrobial group were selected for this study. These included $bla_{\text{TEM-1}}$, bla_{OXA} (β-lactams) *cat* (chloramphenicol) *tetB* (tetracycline), *sul1, sul2* (sulfonamides), *dfrA7, dfrA14* (trimethoprim) *aadA, aadA1a, aadA2, aacC2,* and *strA-strB* (aminoglycosides) genes.

QRDR of *gyrA, gyrB*, *parC,* and *parE* was PCR amplified and sequenced for detection of mutations; *qnrS* and *qnrA* genes were targeted for the screening of plasmid-mediated resistance. Oligonucleotide primer pairs used in this study are given in Table 1.

PCR conditions

For $bla_{\text{TEM-1}}$, bla_{OXA}, *cat, tetB, sul2,* and *aadA* genes, each 100 µL of the reaction mixture contained 10x PCR buffer 10 µL, 25 mM $MgCl_2$, 0.7 nM each dNTP, 25 pM each primer, 5 U of *Taq* DNA polymerase, 10 ng of template DNA, and sterile water to increase the volume. The thermal cycler conditions were initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 1 minute, and a final extension of 72°C for 5 minutes. The annealing temperature for *sul1, dfrA7, dfrA14* was 57.5°C; for *aadA1a*, 60°C; for *aadA2, strA-strB, aacC2*, 55°C; for *gyrA, gyrB, parC,* and *parE*, 62 °C; for *qnr*A, 53°C; and for *qnrS*, 48°C.

Sequencing of gyrA, gyrB, parC and parE genes

Following the results of drug susceptibility test for the quinolone group, the isolates were segregated into two groups, nalidixic acid susceptible (NA^S) and nalidixic acid resistant (NA^R). Three isolates were chosen randomly from each group for DNA sequencing. The chosen isolates were studied for mutations in *gyrA, gyrB, parC,* and *parE* genes. Regions covering the QRDR of *gyrA* (Asp36 to Gly151), *gyrB* (Gly405 to Glu520), *parC* (Val46 to Leu133), and *parE* (Glu449 to Ile529) were amplified with primers, as shown in Table 1. The amplimers were sent to a commercial vendor (Macrogen, Seoul, Korea) for DNA sequencing.

To detect mutation at the amino acid level, the nucleotide sequences obtained for the genes *gyrA*, *gyrB*, *parC,* and *parE* were translated and aligned with a reference *S.* Typhi strain, CT18.

PCR for detection of integrons

The primers listed in Table 1 were used to detect the conserved regions of the integron-encoded integrase genes (*intI1*, *intI2,* and *intI3*). Each 100 µL of the reaction mixture contained 10x PCR buffer 10 µL, 25 mM $MgCl_2$, 0.7 nM each dNTP, 25 pM each primer, 4 U of *Taq* DNA polymerase, 10 ng of template DNA, and sterile water to increase the volume. The thermal cycler conditions were initial denaturation at 96°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 2 minutes, and a final extension of 72°C for 7 minutes.

Restriction analysis and sequencing of dfrA7, intI1 genes and gene cassettes

Confirmation of *dfrA7* and *intI1* genes was done by digesting with DNA endonuclease. The restriction mixture (30 µL) included 8 µL PCR product (0.5 µg of DNA), 1× recommended buffer for restriction enzyme, and 5U of restriction enzyme (*Alw*261 for *dfrA7*, NsbI and Eco521 for *intI1*; Fermentas, (Fermentas-Thermo Fisher Scientific Inc., Waltham, USA) and sterile water to increase the volume. Restriction products were fractionated by gel electrophoresis (2% agarose in 1× TAE buffer with 0.5 µg ethidium bromide/mL), and the length of the restriction products was determined by comparing with a 100 bp ladder.

Amplified products of *dfrA7, intI1,* and gene cassettes were gel purified using Qiagen gel extraction kit following the manufacturer's instructions, and sequenced at Technology Facility, Department of Biology, University of York.

Results

Antimicrobial susceptibility testing of S. Typhi isolates

Antimicrobial susceptibility tests were performed for all 80 *S.* Typhi isolates using 16 drugs representing six major groups of antimicrobials. Among the tested antimicrobials, sulfonamide was the least active drug (73.7% resistance), whereas the most active antimicrobial agent was cefoparazone/sulbactam (2.5% resistance). Nalidixic acid (NA) and ciprofloxacin were included in the quinolone group for disk diffusion testing.

Table 2. Antimicrobial susceptibility profile of 80 *S.* Typhi isolates by disk diffusion test

Antimicrobial	Number (%) of *S.* Typhi isolates		
	Resistant	Intermediate	Susceptible
Ampicillin	45 (56.2)	0	35 (43.7)
Cephradine	31 (38.7)	47 (58.7)	02 (02.5)
Cefixime	10 (12.5)	14 (17.5)	56 (70.0)
Ceftriaxone	09 (11.2)	26 (32.5)	45 (56.2)
Cefoparazone/sulbactum	02 (02.5)	19 (23.7)	59 (73.7)
Aztreonam	12 (15.0)	12 (15.0)	56 (70.0)
Streptomycin	42 (52.5)	16 (20.0)	22 (27.5)
Gentamicin	05 (06.2)	05 (06.2)	70 (87.5)
Amikacin	12 (15.0)	14 (17.5)	54 (67.5)
Sulfonamide	59 (73.7)	19 (23.7)	02 (02.5)
Trimethoprim	23 (28.7)	0	57 (71.2)
Trimethoprim-sulfamethoxazole	24 (30.0)	0	56 (70.0)
Nalidixic acid	19 (23.7)	10 (12.5)	51 (63.7)
Ciprofloxacin	10 (12.5)	52 (65.0)	18 (22.5)
Chloramphenicol	26 (32.5)	04 (05.0)	50 (62.5)
Tetracycline	23 (28.7)	05 (06.2)	52 (65.0)

(%) = Percentage

Table 3. Antimicrobial drug resistance patterns of MDR *S.* Typhi

Resistance to antimicrobial groups	No of isolates	Resistance to antimicrobial drugs (n)	Pattern
6	02	10	AmCeChNACipStrTeSxtSmzTmp (2)
5	21	10	AmCfmCroAtmChStrTeSxtSmzTmp (2)
		08	AmChNACipStrSxtSmzTmp (4)
		07	AmChStrTeSxtSmzTmp (13)
		05	CeCnNATeSxt (2)
4	08	08	AmCeCroAtmNAStrAkSmz (1)
		07	AmCeCfmCroNAStrSmz (2)
		07	AmCeAtmNAStrAkSmz (1)
		05	CeAtmChStrSmz (2)
		04	ChStrTeSxt (2)
3	16	08	AmCeCfmCroAtmStrAkSmz (2)
		07	AmCeCesCfmAtmCnSmz (2)
		05	AmCeCfmNASmz (2)
		05	AmCeStrAkSmz (3)
		04	AmCeStrSmz (4)
		04	AmN.ACipAk (1)
		03	AmStrSmz (2)
2	14	03	AmNACip (2)
		03	AmCeSmz (2)
		03	CeCroSmz (2)
		02	AkSmz (1)
		02	AtmSmz (2)
		02	CeSmz (4)
		02	ChTmp (1)
1	14	02	CeCfm (2)
		02	NACip (2)
		01	Cn (2)
		01	Smz (7)
		01	Str (1)
0	05	0	(0)

Am: ampicillin; Ce: cephradine; Ces: cefoparazone/sulbactum; Cfm: cefixime; Cro: ceftrioxone; Atm: Aztreonam (β-lactams); Smz: sulfonamide; Sxt: trimethoprim-sulfamethoxazole; Tmp: trimethoprim (antifolates); Ak: amikacin; Cn: gentamicin; Str: streptomycin (aminoglycoside); Cip: ciprofloxacin; NA: nalidixic acid (quinolone/fluoroquinolone); Te: tetracycline; Ch: chloramphenicol; Figures in parenthesis indicate individual isolates having that specified pattern

Nineteen isolates were resistant to nalidixic acid and only seven NA^R isolates were intermediately susceptible to ciprofloxacin. Of the 80 isolates, 52 were intermediately susceptible to ciprofloxacin. Detailed results are shown in Table 2.

MDR pattern of S. Typhi

Among 80 isolates of *S.* Typhi, 47 (58.7%) were MDR, *i.e.,* resistant to three structurally different drugs. Twenty-one (44.6%) of these isolates were simultaneously resistant to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole (traditional antityphoidal drugs). Only five isolates were resistant to both nalidixic acid and ciprofloxacin but susceptible to ceftriaxone. Twenty-three isolates were resistant to five or more antimicrobial agents, and the most prevalent pattern observed was AmChStrTeSxtSmzTmp; it was found in 13 isolates. Only two isolates were resistant to at least one member of all six antimicrobial groups, and five were found susceptible to all antimicrobial agents. Detailed results are shown in Table 3. None of the 80 *S.* Typhi isolates produced ESBL when checked using the standard cephalosporin/clavulanic acid combination disk test.

E-test results

After a general overview of the drug resistance status of local isolates was established, MIC values were determined for six antimicrobial agents representing two major groups of antimicrobials now at the forefront of typhoid treatment, *i.e.,* cephalosporins and fluoroquinolones.

Twenty-one isolates were resistant to nalidixic acid, with MICs ranging from 32 µg/mL to > 256 µg/mL; three were intermediately susceptible with an MIC of 24 µg/mL, and 56 were susceptible with MICs ranging from 1.5 to 16 µg/mL. Nine isolates were resistant to ciprofloxacin, and the maximum MIC value observed for ciprofloxacin was 3 µg/mL (7); only two isolates were found with an MIC of 1 µg/mL. Fifty-four isolates showed intermediate susceptibility to this antimicrobial, with MICs in the range of 0.125 µg/mL to 0.75 µg/mL. Only seven isolates were susceptible, with MICs in the range of 0.023 to 0.094 µg/mL. The MICs against ofloxacin for all the isolates were in the range of 0.064 to 2 µg/mL. In gatifloxacin, 78/80 isolates were fully susceptible, with MICs in the range of 0.023 to 1 µg/mL; only two with an MIC of 4 µg/mL was intermediately susceptible against gatifloxacin.

Among NA^R isolates only, the MICs against ofloxacin and gatifloxacin were in the range of 0.064 to 2 µg/mL and 0.125 to 1 µg/mL, respectively.

Both ceftriaxone and cefpodoxime are third-generation cephalosporins. Twenty-three isolates were resistant to ceftriaxone with MICs of > 32 µg/mL (1), 32 µg/mL (2), 16 µg/mL (2), 12 µg/mL (3), 8 µg/mL

Table 4. Prevalence of antimicrobial drug resistance related genes in *S.* Typhi isolates

Drug group and their members		Isolates resistant to antimicrobials* N (%)	Targeted gene	Number of positive isolates
Aminoglycoside	Streptomycin	42 (52.5)	*aadA*	0
			aadA1a	0
	Gentamicin	05 (6.2)	*aadA2*	0
			aacC2	0
	Amikacin	12 (15.0)	*strA-strB*	21
Antifolate	Sulfonamide	59 (73.7)	*sul1*	24
			sul2	54
	Trimethoprim	23 (28.7)	*dfrA7*	30
			dfrA14	0
Chloramphenicol		26 (32.5)	*cat*	21
Beta-lactam	Ampicillin	45 (56.2)	$bla_{\text{TEM-1}}$	35
	Cephradine	31 (38.7)		
	Cefixime	10 (12.5)	bla_{OXA}	0
	Ceftriaxone	09 (11.2)		
Quinolone	Nalidixic acid	19 (23.7)	*qnrA*	0
	Ciprofloxacin	10 (12.5)	*qnrS*	0
Tetracyclines		21 (26.2)	*tetB*	28

* Isolates phenotypically resistant through disk diffusion method

(4), 6 µg/mL (5) and 4 µg/mL (6); 19 were intermediately susceptible with MICs of 3 µg/mL (17) and 2 µg/mL (2); and 38 were susceptible with MICs in the range of 0.19 to 1 µg/mL. Against cefpodoxime, 12 isolates were resistant with MICs > 356 µg/mL (1), 16 µg/mL (3), 12 µg/mL (4), and 8 µg/mL (4); 40 were intermediately susceptible with MICs of 6 µg/mL (2), 4 µg/mL (18), and 3 µg/mL (20); the remaining 28 were susceptible with MICs in the range of 1 µg/mL to 2 µg/mL. Isolates resistant to these third-generation cephalosporins were not ESBL producers when checked using the standard cephalosporin/clavulanic acid combination disk test.

Molecular identification of drug resistance-related genes

Penicillin and cephalosporin resistance-related gene $bla_{\text{TEM-1}}$ was amplified in 35 (43.7%) isolates. No amplification was observed for the bla_{OXA} gene. Genes responsible for conferring resistance to chloramphenicol (*cat*) and tetracycline (*tetB*) were detected in 21 (26.2%) and 28 (35.0 %) isolates, respectively. Genes related to sulfonamide resistance (*sul1* and *sul2*) were detected in 24 (30.0 %) and 54 (67.5 %) isolates, respectively.

As already mentioned, the *dfrA7* gene related to trimethoprim resistance has special importance. The gene was detected in 30 (37.5%) isolates. The results were confirmed by digestion of amplicons with endonuclease Alw261. No isolate was found positive for the *dfrA14* gene, which was most frequently reported in *S*. Typhi isolates before 1994.

With reference to aminoglycoside resistance, no amplification was detected for *aadA, aad1A, aadA2,* and *aacC2* genes. However, a gene specifically responsible for streptomycin resistance (*strA-strB*) was amplified in 21 (26.2%) isolates.

No amplification was observed for *qnrA* and *qnrS* genes that are associated with plasmid mediated quinolone resistance. The detailed results are shown in Table 4.

Analysis of the quinolone resistance determining regions (QRDRs)

In this study, the only mutation found in NA^R isolates was at codon encoding Ser83 in the *gyrA* gene. A single nucleotide transition from C to T changes the amino acid from serine to phenylalanine. The five NA^R isolates were resistant to ciprofloxacin, as determined through disk diffusion testing; the MICs for ciprofloxacin were relatively high (0.75 µg/mL to 3 µg/mL) as compared to NA^S isolates (0.094 µg/mL to 1 µg/mL), which were ciprofloxacin susceptible as determined through disk diffusion testing. Other mutations most commonly reported among *Salmonella* isolates in the QRDR of *gyrB, parC,* and *parE* genes were not detected. As expected, no mutation was found in any of the NA^S isolates in the QRDRs of *gyrA, gyrB, parC,* and *parE* genes.

Detection of integrons and associated elements

Among 80 *S*. Typhi isolates tested for conserved regions of the integron-encoded integrase genes (*intI1*, *intI2,* and *intI3*), the results of the current study showed that only 24 isolates carried gene for class 1 integrase, whereas no evidence was found for the presence of class 2 and 3 integrase genes. The results were confirmed by restriction digestion of PCR amplified products of *intI1* gene with Eco521 and NsbI.

Isolates yielding a PCR product with the class 1 *intI1* gene were also found positive for the 3'-conserved segment, amplified using primers targeting *qacEΔ1-F* and *sul1-B* (Table 1). These 24 isolates, when further amplified with primer pair 5′CS/3′CS (Table 1), yielded specific product, indicating that all had same gene contents within the 5'-CS and the 3'-CS region.

Sequencing of selected amplified products

Purified PCR products for *dfrA7*, *intI1,* and gene cassette were sequenced at the Technology Facility, Department of Biology, University of York. The deduced nucleotide sequences were BLAST searched against Genbank database of NCBI and were found to have similarities with *dfrA7*, integrase gene of class 1 integron (Accession HQ832469.1) and dihydrofolate reductase type 7 *(dhfr7)* gene (Accession HQ132376.1), respectively.

Discussion

Infections due to *S*. Typhi strains resistant to multiple antimicrobial drugs have rapidly increased over the past 20 years in the South Asia and have now spread widely to the Middle East, Africa, and Asia [20]. The emergence and spread of drug resistance to newer and more potent agents used in treatment of *Salmonella* species is a major therapeutic challenge [21].

In this study, we observed that 47 (58.7%) of *S*. Typhi isolates were MDR. They were resistant to at least three and up to six different groups of drugs, which is alarming. Among the MDR isolates, a considerable number of isolates (44.6 %) was resistant

to traditional antityphoidal drugs (ampicillin, chloramphenicol, and cotrimoxazole).

Among aminoglycosides, first-generation cephalosporins, sulfonamides, tetracyclines, and nalidixic acid, the maximum resistance was observed towards sulfonamide; 59 (73.7%) isolates were resistant, 19 (23.7%) were intermediately susceptible, and only 2 (2.5%) were fully susceptible. Resistance to cotrimoxazole (a combination of trimethoprim-sulfamethoxazole) was seen in 24 (30.0%) isolates (Table 2). This is in contrast with the studies of Onyango *et al.,* from Kenya observed no resistance against sulfamethoxazole and 66.7% resistance against cotrimoxazole in *S.* Typhi isolates [22]. Glynn *et al.,* reported 56% resistance against sulfamethoxazole and 2% resistance against trimethoprim-sulfamethoxazole [23].

Consistent with the report of Randall *et al.* [24] there were several instances in this study when an isolate was resistant to an antimicrobial drug, but the identity of the gene conferring resistance was not ascribed with the primers used in the study. For example, 10 of 45 ampicillin-resistant isolates did not contain $bla_{\text{TEM-1}}$ gene; 5 of 26 chloramphenicol-resistant isolates were negative for *cat* gene; 7 of 28 tetracycline-resistant isolates did not show presence of *tetB* gene; and 21 of 42 streptomycin-resistant isolates were negative for *aadA, aadA1a, aadA2* and *strA-strB* (Table 4). This discrepancy exists because there are usually many genes related to the development of phenotypic resistance to a single drug. It is impossible to cover all reported genes in one study. There were also some isolates in which drug resistance-related genes were detected but corresponding phenotypic resistance was absent (Table 4). Such anomalies are common because the genes may be inactive due to an incomplete or missing part of the gene sequence important for imparting resistance [25, 26].

As shown in Table 3, only five isolates were susceptible to all drugs, and two were resistant to at least one member of the six drug groups tested. Twenty-one isolates were found resistant to first-line anti-typhoidal drugs (AmChSxt) with additional resistance to other drugs as well. The most common R type AmChStrTeSxtSmzTmp was found in 13 isolates (Table 3). This is the most frequent R type observed in isolates from India and Pakistan [27]. Four isolates showed R type AmCeStrSmz, and three were also resistant to amikacin (R-type AmCeStrAkSmz). Other isolates showed several different patterns. Among the MDR isolates, the most commonly encountered drugs in most of the R type were ampicillin, sulfamethoxazole, and streptomycin. This is not surprising, as these drugs have been used extensively for treatment of typhoid, which resulted in the development of resistance.

The *S.* Typhi isolates resistant to traditional antityphoidal drugs (chloramphenicol, ampicillin, sulfamethoxazole) and nalidixic acid are emerging problem that severely restrict the therapeutic options for patients with typhoid fever. Chau *et al.*, in 2007, found this combination in 4.3% to 30% of *S.* Typhi isolates representative of different regions [28]. However, in this study, this combination was detected in only 6.3% isolates. It was also found that 17 of 19 NA^R isolates had higher MIC values (0.19 µg/mL to 3 µg/mL) for ciprofloxacin.

Besides fluoroquinolones, third-generation cephalosporins are currently at the forefront for typhoid treatment. In this study, among third-generation cephalosporins, 45 (56.2 %) and 26 (32.5%) isolates showed full and intermediate susceptibility towards ceftriaxone, respectively, through a disk diffusion assay (Table 2). It was also found that 12 out of 19 NA^R isolates were not resistant to ceftriaxone (both from disk diffusion assay and E-test), whereas some resistance was observed against cefpodoxime when analyzed by E-test.

Although resistance to third-generation cephalosporins is thought to be rare among MDR *S.* Typhi isolates, in this study it was observed in 12 (15.0 %) of our isolates against cefpodoxime and in 23 (28.7%) of the isolates against ceftriaxone when checked using the E-test. Only nine isolates were resistant to both of these cephalosporin drugs. Such resistant isolates were not ESBL-positive when checked using the standard cephalosporin/clavulanic acid combination disk test. However, rare ESBL-positive *S.* Typhi isolates have been reported from Pakistan (unpublished data).

In terms of fluoroquinolones, nalidixic acid resistance acts as a marker for predicting low-level resistance, with a high MIC of ciprofloxacin among *S.* Typhi; it is also an indicator of ciprofloxacin treatment failure [29]. Any isolate that shows resistance to nalidixic acid may be reported as intermediately susceptible to ciprofloxacin [30]. In this study, nalidixic acid resistance was observed in 19 (23.7%) isolates, with MICs $\geq$ 96 µg/mL; most also had reduced susceptibility to ciprofloxacin and were associated with an increase in MIC to this drug from 0.19 µg/mL to 3 µg/mL, which is in accordance with results reported by Nagshetty *et al*. [31] All NA^R isolates were apparently susceptible to two other

fluoroquinolones, gatifloxacin and ofloxacin, with MICs of ≤ 2 µg/mL. But Parry *et al.* [32] reported that MICs of ≥ 0.25 µg/mL for ofloxacin do not respond well to ofloxacin therapy. In our study, 17/19 NA^R isolates had MICs of ≥ 0.25 µg/mL, which suggests that ofloxacin could not be an option for treating NA^R isolates. Gatifloxacin can be a good option in such cases, as there are no reports that challenge the current criteria for gatifloxacin breakpoint. However, its clinical use is controversial because of the potential risk of dysglycemia in elderly and diabetics persons [33,34].

Not all NA^R isolates were susceptible to ceftriaxone and cefpodoxime, most showing intermediate susceptibility toward these two cephalosporins, further reducing options for these two drugs to be used against typhoid caused by NA^R isolates.

Reduced susceptibility to fluoroquinolones is usually associated with point mutations in the bacterial target genes encoding DNA gyrase and/or DNA topoisomerase IV. Point mutations in the topoisomerase genes are generally restricted to certain codons within the QRDR [8]. In *Salmonella*, some of the more common point mutations found to be associated with resistance to quinolones occur in the *gyrA* gene, resulting in substitutions at the Ser-83 position, often to Tyr, Phe, or Ala, and Asp-87 substitutions to Asn, Gly, or Tyr. The most common amino acid substitution reported in ParC is Thr-57→Ser, with Thr-66→Ile or Ser-80→Arg observed as occasional second substitutions [9].

Sequence analysis of some randomly selected NA^R isolates in this study revealed that the reason for resistance to nalidixic acid and increase in MIC of ciprofloxacin is associated with a single point mutation that resulted in amino acid change from Ser83-position to Phe. This is supported by the study of Turner *et al.* [35], who reported that a single amino acid substitution in *gyrA* was sufficient for resistance to the quinolones nalidixic acid and cinoxacin, but resistance to other fluoroquinolones (gatifloxacin, ofloxacin, ciprofloxacin, enrofloxacin, and moxifloxacin) required two substitutions in *gyrA* and one in *parC*.

Resistance genes are often associated with integrons [36,37]. Studies of selected clinical bacterial populations have shown that 59% to 75% of drug-resistant isolates contain class 1 integrons [38,39]. In this study, 30.0% (24 of 80) drug-resistant isolates contained class 1 integrons, and as expected, these isolates were less susceptible to antimicrobial drugs than the integron-negative isolates. Among integron-positive isolates, 28.7% (23 of 80) were MDR-resistant to β-lactams, especially ampicillin and, in most cases, also to chloramphenicol, streptomycin, trimethoprim, sulfonamides, cotrimoxazole, and tetracycline. Only two integron-positive isolates were resistant to chloramphenicol and trimethoprim only.

This study did not reveal any class 2 and 3 integrons. This is not surprising, as these integrons are less common or absent in *Salmonella* spp [40].

Class 1 integrons possess two conserved segments separated by a variable region that includes integrated antimicrobial resistance genes or gene cassettes with unknown functions [41]. The 3'conserved segment of class 1 integrons is characterized by the *qacEΔ1* and *sul1* genes, which impart resistance to disinfectants and sulfonamides, respectively [42]. This segment was amplified in all class 1 integron-positive *S.* Typhi isolates in this study. Further characterization of the integrons revealed that all contained a > 700 bp insert in the variable region. DNA sequencing showed the inserted DNA to be comprised of a *dfrA7* gene that encodes resistance to trimethoprim. These results are consistent with the findings of a study by Mulvey *et al.* [43].

In conclusion, this study revealed high frequency of MDR *S.* Typhi isolates. This is alarming, since resistance to first-line drugs will require more expensive drugs for effective treatment of typhoid fever and may pose a major challenge to the health care system. Since traditional anti-typhoidal drugs are losing ground, the emerging resistance against ciprofloxacin and disputed breakpoint for ofloxacin are big problems. In this scenario, the susceptibility exhibited to gatifloxacin, especially by NA^R isolates, provides a valuable option in the treatment of MDR typhoid fever; however, its clinical use is controversial. It is encouraging that none of the isolates produced ESBLs.

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