Original Article

Current status of fluoroquinolone and cephalosporin resistance in *Salmonella enterica* serovar Typhi isolates from Faisalabad, Pakistan

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ABSTRACT

Objectives: Currently fluoroquinolones and cephalosporins are at the forefront of typhoid treatment. The objective of this study was to assess the current drug resistance status of *S.* Typhi isolates from Faisalabad region by conventional and molecular methods.

Methodology: Drug resistance pattern of 30 clinical isolates (2011) against 8 drugs (nalidixic acid, ciprofloxacin, ofloxacin, gatifloxacin, cephradine, cefixime, ceftriaxone and cefpodoxime) was determined. MICs were noted by E-test. ESBL production was also tested. Relevant drug resistance genes bla_{TEM}, bla_{OXA}, *gyrA, gyrB, parC, parE, qnrS* and *qnrA* were targeted and QRDR regions of *gyrA, gyrB, parC,* and *parE* were sequenced for mutations.

Results: Nalidixic acid and ciprofloxacin resistance were seen in 30.0% and 10.0% of isolates respectively. No resistance was detected towards ofloxacin and gatifloxacin. Resistance to cephradine, cefixime, cefpodoxime and ceftriaxone was found in 46.7%, 13.3%, 16.7%, and 3.3% of isolates respectively. In ciprofloxacin resistant isolates a single mutation at codon Ser83 in *gyrA* gene was detected.

Conclusions: A slow increase in ciprofloxacin resistance was indicated. However, newer fluoroquinolones ofloxacin and gatifloxacin are still very effective. Among cephalosporins, ceftriaxone showed promising results but emerging resistance was evident. Fortunately no ESBL producing isolate was detected. No correlation between two groups was detected in emergence of drug resistance.

KEY WORDS: *S.* Typhi, Fluoroquinolone, Cephalosporin, Resistance.

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INTRODUCTION

Typhoid is a major disease in developing countries with 21 million cases each year.[1] It is the 4th largest killer disease in Pakistan.[2] The traditional antityphoidal drugs chlormaphenicol, ampicillin, and sulphamethoxazole have become outdated.

In recent years, fluoroquinolones, especially ciprofloxacin have been very successful in combating typhoid but unfortunately, resistant strains have emerged. There have been many reports of nalidixic acid resistant (NA^R) *S.* Typhi which exhibited decreased susceptibility to ciprofloxacin and showed in poor clinical response to fluoroquinolones.[3] The emergence of high-level ciprofloxacin resistance in

S. Typhi and *S.* Paratyphi A has also been reported in India.[4]

Antimicrobial resistance is usually conferred by certain genes.[5] A large number of resistance related genes have been reported for each group of antimicrobials. It is impossible to study all the reported genes, so most commonly isolated and reported genes were selected for this study.

Fluoroquinlones, especially ciprofloxacin are the most commonly used drugs for typhoid treatment. Reduced susceptibility to the fluoroquinolones group of antibiotics is usually linked with point mutations in the bacterial target genes *gyrA, gyrB* encoding DNA gyrase and/or *parC, parE* encoding DNA topoisomerase IV.

After the emergence of ciprofloxacin resistance in *S.* Typhi, cephalosporins especially ceftriaxone and cefixime are being extensively used for the treatment of enteric fever. Although resistance to third generation cephalosporins in non-typhoidal *Salmonella*e had been reported as early as 1989[6], resistance in *S.* Typhi remains rare. The first cases of reduced susceptibility or resistance to ceftriaxone were documented in Bangladesh and Kuwait in 2008.[7]

This study was focused on molecular mechanisms of development of resistance against different generations of quinolones/fluoroquinolones (nalidixic acid, ciprofloxacin, ofloxacin, and gatifloxacin) in local isolates of *S.* Typhi with emphasis on predictive value of nalidixic acid resistance. We have also tried to establish the significance of reduced MIC values. Similar framework was used for various generations of cephalosporins (ceftriaxone and cefpodoxime).

The aim of this study was to find the extent and nature of this emerging resistance in isolates from Faisalabad region with a population of more than 10 million.

METHODOLOGY

S. Typhi isolates: Thirty isolates of *S.* Typhi, collected recently (2011) from Faisalabad region, Pakistan, were taken from the culture collection of National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad. 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. Bacterial DNA was extracted by a DNA extraction kit (Fermentas, Hanover, MD, USA) and confirmation of *S.* Typhi isolates was done by targeting *fliC* gene as previously reported.[8]

Determination of drug resistance patterns: Drug sensitivity was checked by using disc diffusion method.[4] In fluoroquinolone group, nalidixic acid, a nontherapeutic drug was included to act as a stand-in for fluoroquinolones sensitivity assays. The other three drugs were ciprofloxacin, ofloxacin and gatifloxacin that belong to 1st, 2nd and 3rd generation fluoroquinolones respectively. First and third generation cephalosporins, cephradine, cefixime, ceftriaxone and cefpodoxime were also included.

ESBL production was tested by standard cephalosporin/clavulanate combination disc test.[9] For MIC determination; E test strips (AB Biodisk, Solna, Sweden) for the above mentioned drugs were used according to manufacturer's recommendations. MIC was determined for nalidixic acid, ciprofloxacin, ofloxacin and gatifloxacin, ceftriaxone and cefpodoxime.

Detection of antimicrobial drug resistance related genes: Molecular analysis of quinolone resistance determining region (QRDR) of *gyrA, gyrB*, *parC* and *parE* was performed first by amplifying the specific gene, and then by sequencing the amplified products. For the screening of plasmid-mediated resistance, *qnrS* and *qnrA* genes were also targeted. Targeted genes and relevant primers are listed in Table-I.

PCR conditions: In case of bla_{TEM}, and bla_{OXA}, each 100 µl of the reaction mixture contained 10x PCR buffer 10 µl, 25 mM $MgCl_2$, 25 microM of each dNTP, 25 pM each primer, 5 U of *Taq* polymerase, 10 ng of template DNA, and distilled water to make the volume. The thermal cycler conditions were: 94 °C for two minute followed by 30 cycles of 94 °C for one minute, 50 °C for one minute, 72 °C for one minute and a final extension of 72 °C for 5 min. The annealing temperature in case of *gyrA, gyrB, parC,* and *parE*; *qnr*A; and *qnrS* were 62°C; 53°C; and 48°C respectively. Other conditions were similar.

Sequencing of gyrA, gyrB, parC and parE genes: Three nalidixic acid resistant and three sensitive isolates chosen at random were studied in detail 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 given in Table-I. After amplifying the genes, sequencing was done. The amplimers were analyzed by direct sequencing to detect mutations. For this purpose, the unpurified samples were sent to a commercial vendor (Macrogen, Korea).

The nucleotide sequences for the genes *gyrA*, *gyrB*, *parC* and *parE* were BLAST at NCBI. Using a proteomic server, nucleotide sequences were

Table-I: Oligonucleotides used in the study.

Primer name	*Targeted gene*	*Oligonucleotide Sequences*	*Antimicrobial agent/ Genetic element*	*Amplicon size(bp)*
A1	*blaTEM-1*	GCACGAGTGGGTTACATCGA	Cephalosporins	311[10]
A2	*(Nested)*	GGTCCTCCGATCGTTGTCAG		
blt-F	*blaTEM-1*	CCCCTATTTGTTTATTTTTC	Cephalosporins	962[11]
blt-R		GACAGTTACCAATGCTTAAT		
OXA-F	*blaOXA*	ATGAAAAACACAATACATATCAACTTCGC	Cephalosporins	820[12]
OXA-R		GTGTGTTTAGAATGGTGATCGCATT		
GYRA/P1	*gyrA*	TGTCCGAGATGGCCTGAAGC	Quinolone	347[13]
GYRA/P2		TACCGTCATASGTTATCCACG		
StygyrB1	*gyrB*	CAAACTGGCGGACTGTCAGG	Quinolone	345[14]
StygyrB2		TTCCGGCATCTGACGATAGA		
StmparC1	*parC*	CTATGCGATGTCAGAGCTGG	Quinolone	270[15]
StmparC2		TAACAGCAGCTCGGCGTATT		
StmparE1	*parE*	TCTCTTCCGATGAAGTGCTG	Quinolone	240[15]
StmparE2		ATACGGTATAGCGGCGGTAG		
QnrS1	*qnrS*	ATGGAAACCTACAATCATAC	Quinolone	492[16]
QnrS2		AAAAACACCTCGACTTAAGT		
QnrA-F	*qnrA*	GATAAAGTTTTTCAGCAAGAGG	Quinolone	543[9]
QnrA-R		ATCCAGATCGGCAAAGGTTA		

translated, compared and aligned with *S*.Typhi strain CT18 (as reference strain).

RESULTS

Pattern of drug resistance in S. Typhi isolates: Eight drugs representing quinolones and cephalosporins were used against all 30 *S*. Typhi isolates. Detailed results are shown in Table-II. Among cephalosporins, significant resistance was observed against first generation cephradine (46.7%). It was considerable against third generation cefixime and cefpodoxime (13.3% and 16.7%) and negligible against third generation ceftriaxone (3.3%). Notable resistance was observed against nalidixic acid (30.0%). Three (10.0%) isolates showed resistance to ciprofloxacin whereas all the isolates were sensitive to ofloxacin and gatifloxacin. None of the 30 *S*. Typhi isolates produced ESBL.

MIC values: Nine isolates were resistant to nalidixic acid with MICs from 32µg/ml to >256µg/ml; 3 were intermediately susceptible with an MIC of 24µg/ml and 18 were sensitive with MICs from 1.5 to 16µg/ml. Maximum MIC value observed for ciprofloxacin was 4µg/ml which is interpreted as intermediately susceptible MIC value. It was observed in 3 isolates. Twenty seven isolates were sensitive with MICs in the range of 0.023µg/ml to 1µg/ml. No isolate was found to be totally resistant. All isolates showed cent percent sensitivity towards ofloxacin and gatifloxacin. MICs values for these anitimicrobial agents were found in the range of 0.064 to 2µg/ml and 0.023 to 4µg/ml respectively (Table-III). An E-test result is shown in Fig.1.

Both ceftriaxone and cefpodoxime are the 3rd generation cephlosporins. One isolate was resistant to ceftriaxone with a MIC value of >32µg/ml; 3 were intermediately susceptible with MICs of 32 and 12µg/ml; and 26 isolates were sensitive with MICs in the range of 0.19 to 8µg/ml. Against cefpodoxime, 5 isolates were resistant with MICs 12 to > 256 µg/ml; 12 were intermediately susceptible with MICs 4-12 µg/ml and remaining 13 were sensitive with MICs in the range of 1 -4µg/ml (Table-III).

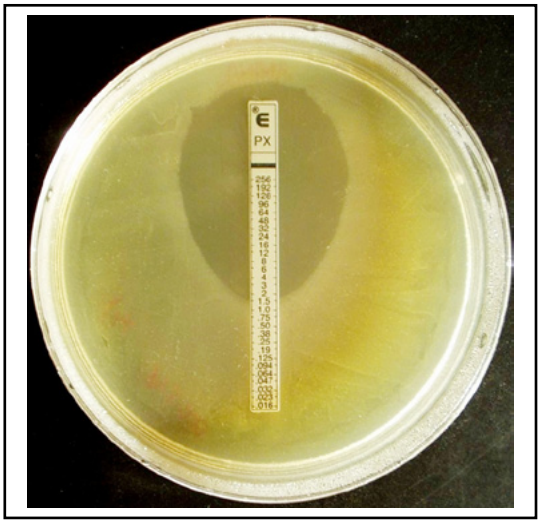

Fig.1: MIC determination by E-strip: The point where eclipse intersects the MIC value scale (in µg/ml) is the MIC (minimum inhibitory concentration). In this case it is 1.5 µg/ml.

Table-II: Levels of antimicrobial resistance of *S.* Typhi isolates (n=30).

Antimicrobial	*Level of Resistance n (%)*
Cephradine	14 (46.7)
Cefixime	4 (13.3)
Ceftrioxone	1 (03.3)
Cefpodoxime	5 (16.7)
Ciprofloxacin	3 (10.0)
Nalidixic acid	9 (30.0)
Ofloxacin	0
Gatifloxacin	0

Detection of drug resistance related genes: For the detection of cephalosporin resistance, bla_{TEM-1} gene was amplified in 14 (46.67%) isolates; another primer pair A1/A2 nested in the same gene, amplified bla_{TEM-1}(Nested) in 13 (43.33%) isolates. The bla_{OXA} was also targeted but there were no positive results.

For quinolone resistance, chromosomal genes *gyrA, gyrB, parC*, and *parE* were detected as expected. However, no amplification was observed for *qnrA* and *qnrS* genes that are associated with plasmid mediated resistance.

Analysis of the quinolone resistance determining regions (QRDRs) sequencing results: In this study, the only mutation found in NA resistant isolates was at codon Ser83 in *gyrA* gene. This single nucleotide transition from C to T changes the amino acid from serine to phenylalanine. 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 sensitive isolates in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* genes.

Table-III: MICs of *S.*Typhi isolates by E-test strip method.

MIC value µg/ml	*No. of isolates for each antimicrobial*					
	Nalidixic acid[a]	*Ciprofloxacin*[b]	*Ofloxacin*[b]	*Gatifloxacin*[b]	*Ceftriaxone*[b]	*Cefpodoxime*[a]
>256*	7	0	0	0	0	1
256	0	0	0	0	0	0
128	1	0	0	0	0	0
96	1	0	0	0	0	1
>32*	0	0	0	0	1	0
32	1	0	0	0	1	0
24	2	0	0	0	0	0
16	1	0	0	0	1	2
12	1	0	0	0	1	2
8	1	0	0	0	2	2
6	2	0	0	0	3	2
4	2	3	0	1	3	8
3	6	0	0	0	4	6
2	4	0	1	0	1	3
1.5	1	0	0	0	1	2
1	0	1	0	1	2	2
0.75	0	1	1	2	1	0
0.5	0	0	2	2	1	0
0.38	0	3	2	2	4	0
0.25	0	4	5	3	2	0
0.19	0	8	5	3	2	0
0.125	0	4	4	4	0	0
0.094	0	3	7	4	0	0
0.064	0	2	3	4	0	0
0.047	0	1	0	4	0	0
0.032	0	0	0	1	0	0
0.023	0	2	0	1	0	0

a; E-test strip MIC range 0.016-256µg/ml, b; Etest strip MIC range 0.002-32µg/ml
* no zone was observed and MIC was considered > the highest value on the strip

DISCUSSION

Typhoid fever is a serious health problem particularly in developing countries including Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, and central Vietnam which are also home to more than 80% of the world's typhoid cases.[1] *Salmonella* infections due to *S*. Typhi strains resistant to multiple antibacterial drugs have rapidly increased over the past 20 years in the South Asian region and have now spread widely to the Middle East, Africa, and Asia. The emergence and spread of drug resistance to newer and more potent agents used in treatment of *Salmonella* species is a major therapeutic challenge.[17]

As the conventional antityphoid drugs have become outdated, cephalosporins and fluoroquinolones are the main treatment for typhoid. However, resistance is emerging against these drugs gradually which is very alarming.[18]

Very interesting observations were made regarding cephalosporins. As expected, significant resistance was found against first generation cephalosporin, cephradine (46.7%) but third generation cephalosporins gave heterogenous results. A considerable number of isolates were resistant to cefixime (13.3%) and cefpodoxime (16.7%), but ceftriaxone was very effective as only 1 (3.3%) isolate was resistant. This is consistent with the findings of Pontali et al.[6] MIC values were consistent with disc diffusion results.

Fortunately, not a single isolate was ESBL positive. However, ESBL positive isolates have been reported elsewhere and these have been attributed to transfer of an extended-beta-lactamase gene to *S*. Typhi from non-Typhi *Salmonella* strains.[19]

The identification of a bla_{TEM-1} gene as the determinant of beta-lactam resistance in 14 (46.67 %) of our *S*. Typhi isolates, is perhaps not surprising because this beta-lactamase has been found extensively.[20] Though not a single *S*. Typhi isolate was ESBL producer but presence of TEM-1 (encoded by bla_{TEM-1}) has been described to have clinical implications because this beta-lactamase is recognized as the progenitor to many extended-spectrum beta-lactamases and inhibitor-resistant beta-lactamases.[21]

In our study, 6 isolates were identified that were susceptible to tested drugs despite the presence of bla_{TEM-1}. The distal location from the integron promoter could be the result of decreased expression of gene cassettes in these isolates.[22]

For predicting low-level resistance, with a high MIC of ciprofloxacin among *S*. Typhi and also an indicator of treatment failure to ciprofloxacin, nalidixic acid resistance acts as a marker,[23] and any isolate that shows resistance to nalidixic acid may be reported as intermediately susceptible to ciprofloxacin.[24] In our study, nalidixic acid resistance was observed in 9 (30.0%) isolates with MICs ≥ 96 µg/ml (Table-III); most were also having reduced susceptibility to ciprofloxacin and were associated with increase in MIC to this drug from 0.19 to 4 µg/ml, which is in accordance with results reported by Nagshetty et al.[25] This trend of increase in MIC of ciprofloxacin was also observed in some nalidixic acid sensitive and intermediately susceptible isolates but these isolates were sensitive to other two fluoroquinolones gatifloxacin and ofloxacin with MICs of ≤ 2µg/ml. Trend in the MIC values of NA^R isolates was not so different from NA^S isolates in case of these 3rd generation fluoroquinolones.

This disparity in the MIC levels of different tested quinolones can be attributed to differences in the additional fluoro group and other substitutions in their chemical structure.[4] Parry CM et al[26] has reported that on using ofloxacin for treatment of typhoid fever, a significantly higher MIC≥0.25µg/ml for this drug is associated with treatment failure and in our series 30% isolates showed MIC in the range of 0.25 to 2µg/ml (Table-III) that is of serious concern.

Reduced susceptibility to fluoroquinolones is usually associated with point mutations in the bacterial target genes encoding DNA gyrase and/or DNA topoisomerase IV within the 'quinolone resistance determining region' (QRDR).[27] 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 being observed as occasional second substitutions.[15]

Sequence analysis of some of our nalidixic acid resistant isolates 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 form Ser83- position to Phe. This is supported by the study of Turner et al[28] who reported that single amino acid substitutions in GyrA was sufficient for resistance to the quinolones nalidixic acid and cinoxacin, but resistance to the fluoroquinolones (gatifloxacin, ofloxacin, ciprofloxacin, enrofloxacin and moxifloxacin) required two substitutions in GyrA and one in ParC.

Fortunately, it was found that 8 out of 9 NA^R isolates, all with slightly higher MIC value (0.19 to 4µg/ml) for ciprofloxacin, were not resistant to ceftriaxone. Although increasing ciprofloxacin resistance was evident, ofloxacin and gatifloxacin were effective against all isolates (MICs 0.064-2 µg/ml).

In conclusion we can say that the clinical isolates of *S*. Typhi isolated from Faisalabad region are showing increasing ciprofloxacin resistance as indicated by increase in nalidixic acid resistant isolates. However, newer fluoroquinolones like ofloxacin and gatifloxacin are still very effective. Among third generation cephalosporins, ceftriaxone showed promising results but emerging resistance was evident. Unlike some other global reports, fortunately no ESBL producing isolate was detected. Another significant finding was that nalidixic acid resistance was not corresponding to cephalosporin resistance which means that the process of emerging drug resistance against these two major groups is independent.

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