Induction of resistant mutants of *Salmonella enterica* serotype Typhi under ciprofloxacin selective pressure

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Background & objectives: Infection with *Salmonella enterica* serovar Typhi (hereafter *S.* Typhi) is an important public health problem in India. There has been an increase in the number of reported clinical failures to ciprofloxacin treatment but the data on possible mechanism of failure are limited. One mechanism that has been widely reported and found associated with ciprofloxacin resistance, is the mutations in target genes in QRDR (quinolone resistance determining region). It is hypothesized that mutations in DNA gyrase or topoisomerase IV result in therapeutic failure under selective pressure of antibiotic while the patient is on treatment. We undertook *in vitro* sequential selection studies to expose the clinical isolates of *S.* Typhi to different concentration of ciprofloxacin to study the role of antibiotic selective pressure in the development of mutations in QRDR.

Methods: Total 26 clinical isolates were divided in to two parts: part I included six isolates obtained from three patients with relapse of enteric fever and part II included 20 isolates with different ciprofloxacin MIC levels. For *in vitro* induction of mutation experiment, five *S.* Typhi isolates were selected which included three NAS (nalidixic acid sensitive) and 2 NAR (nalidixic acid resistant) *S.* Typhi. These isolates were grown under increasing concentrations of ciprofloxacin and mutations acquired in QRDR of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) were investigated by sequencing.

Results: For the isolates included in the part I of the study, it was found that the MIC to ciprofloxacin increased in the isolates obtained during the relapse of enteric fever as compare to the first isolate. All isolates had single mutation in *gyrA* gene at S83 without additional mutation in the second isolate. In the second part of the study, the nine isolates with varying MICs to ciprofloxacin also had single mutation in *gyrA* gene at S83 and another six had triple mutations, two mutations in *gyrA* gene (at S83 and D87) and one mutation in *parC* gene (at S80). In *in vitro* induction of mutation experiment, all mutated isolates showed triple mutation (two mutation in *gyrA* and one in *parC* gene) while no mutations were found in wild isolates.

Interpretation & conclusions: Upon exposure to the step-wise increased concentration of ciprofloxacin, isolates become more tolerant to the ciprofloxacin and showed 2-4 fold higher MICs without new mutation after 8 µg/ml. So the accumulation of mutations under continuous ciprofloxacin pressure and tolerance of the mutant isolates led to the clinical failure. These results also suggested that there could be another mechanism responsible for resistance.

Key words Fluoroquionolones - *in vitro* induction - Mutants - Quinolone resistance determining region - *S.* Typhi

The introduction of ciprofloxacin as the first line of treatment for enteric fever in the late 1980s followed by its wide use as the drug of choice led to the reports on decrease in the susceptibility of *Salmonella enterica* serovar Typhi (hereafter *S.* Typhi) to ciprofloxacin, with consequent therapeutic failure[1-6]. Two enzymes are the principal targets for the antibacterial activity of quinolones: DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*)[7,8]. One mechanism that has been widely reported and found associated with ciprofloxacin resistance, is mutations in target genes in QRDR[9-11]. However, these mutations are not able to impart *in vitro* resistance to ciprofloxacin.

With the initial isolates of *S.* Typhi from the blood culture of patient being susceptible to ciprofloxacin and still specific therapy resulting in clinical failure, it is hypothesized that mutations in DNA gyrase or topoisomerase IV result in therapeutic failure under selective pressure of antibiotic while the patient is still on treatment. This has been observed in animal isolates of *Salmonella* spp. by Giraud *et al*[12] and in *S.* Typhi and *S.* Paratyphi A by Hirose *et al*[13].

To study the role of antibiotic selective pressure in the development of mutations in QRDR (quinolone resistance determining region), we carried out *in vitro* sequential selection studies to expose the clinical isolates of *S.* Typhi from enteric fever patients with clinical failure to ciprofloxacin to different concentration of ciprofloxacin.

Material & Methods

Total 26 clinical isolates of *S.* Typhi recovered from children presenting with enteric fever and documented clinical failure to ciprofloxacin at the Paediatric Outpatients department, as also inpatients, All India Institute of Medical sciences (AIIMS), New Delhi, India, from 2005-2010 were included in the study. All the isolates were identified by standard biochemical tests and serotyped by using specific antisera (dH and O9) (Murex Diagnostics Ltd, UK), in the microbiology laboratory at AIIMS. The isolates were stored in glycerol at -70ºc till the time of use. The study was divided in to 2 parts: - Part I and II.

Part I included characterization of six isolates, obtained from three patients with relapse of enteric fever who were culture positive both in first and second episode for *S.* Typhi (2 patients) and *S.* Paratyphi (1 patient). During the first episode, these patients were treated with ciprofloxacin for 10 days. No other antimicrobial was used initially because they all showed response to the treatment in terms of defervascence of fever in 5-6 days of initiating treatment.

However, these patients had recurrence of fever after 10-14 days and this second episode was defined as relapse because the fever reappeared after completion of first line treatment. All these patients were treated with intravenous ceftriaxone for 14 days during the second episode.

Part II of the study included 20 isolates, 3 NAS (nalidixic acid sensitive) and 17 NAR (nalidixic acid resistant) with different levels of MIC (minimum inhibitory concentration) to ciprofloxacin against *S.* Typhi.

Susceptibility testing: Antimicrobial susceptibility was determined to chloramphenicol (30 μg), amoxicillin (10 μg), co-trimoxazole (1.25/23.75 μg), ceftriaxone (30 μg), cefexime (30 μg), nalidixic acid (30 μg) and ciprofloxacin (5 μg) (Hi-Media Labortories limited, Mumbai, India) by disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) guidelines, 2011[14]. *Escherichia coli* ATCC 25922 was used as reference strains for quality control. MIC to ciprofloxacin was determined using the E-test (AB Bio disk, Solna, Sweden) following the manufacturer's instructions.

DNA isolation: DNA was isolated by boiling method as earlier described in our laboratory[15]. Briefly, DNA of each sample was isolated from one millilitre of overnight culture ($OD_{600} = 5$) equivalent to approximately 10^9 bacterial cells. Culture was centrifuged at 14000 *g* for 5 min and DNA extraction yielded about 2 μg DNA (elution volume was 100 μl). So 100 ng of DNA (per 5μl) was used in the PCR as template.

PCR amplification and DNA sequencing of the QRDR: PCR for QRDR of all subunits for DNA gyrase (*gyrA* and *gyrB* gene) and topoisomerase IV (*parC* and *parE*) was run using published primers[15-17]. DNA sequence analysis for mutation detection was done as described earlier[15].

Briefly, PCR was performed on genomic DNA of each isolates in a total volume of 50 μl, which contained 5 μl of supernatant, 5 pmol of each primer, 200 μM deoxynucleoside triphosphates and 2.5 U of Taq polymerase. The details of primers and PCR reactions are given in Table I. Multiple DNA sequencing reactions were performed for each QRDR of individual isolates using the Applied Biosystems Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA)

Table I. Details of primer and PCR conditions for the amplification of QRDRs

QRDR target	Primer sequence	PCR conditions (30 cycles)	PCR product	References
gyrA	5' –ATGAGCGACCTTGCGAGAGAAATTACACCG- 3' F 5' -TTCCATCAGCCCTTCAATGCTGATGTCTTC- 3'R	94°C 30 sec Denaturation 70 °C 60 sec Annealing 72 °C 90 sec Extension	630 bp	15
gyrB	5'-GCGCTGTCCGAACTGTACCT-3'F 5'-T GATCAGCGTCGCCACTTCC-3'R	94°C 30 sec Denaturation 63 °C 60 sec Annealing 72 °C 90 sec Extension	181 bp	16
parC	5'-ATGAGCGATATGGCAGAGCG-3' F 5'-TGACCGAGTTCGCTTAACAG-3' R	94°C 30 sec Denaturation 56 °C 60 sec Annealing 72 °C 90 sec Extension	~412 bp	17
parE	5'-TCTCTTCCGATGAAGTGCTG-3' F 5'-ATACGGTATAGCGGCGGTAG-3'R	94°C 30 sec Denaturation 64 °C 60 sec Annealing 72 °C 90 sec Extension	~240 bp	16

for cycle sequencing reactions on 'Gene Amp PCR system 2400' thermal cycler (Applied Biosystems, USA) and ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Applied Biosystems, USA). AmpliTaq Gold DNA polymerase (Applied Biosystems, USA) was used for PCR, which is a modified form of AmpliTaq DNA Polymerase.

Sequence analysis: Reference sequences for all QRDRs (*gyrA, gyrB, parC* and *parE*) were obtained from *http://www.ncbi.nlm.nih.gov/gene* and compared with the sequence of *S.* Typhi strain CT18 (accession no. AL627274). Sequence chromatograph files were analyzed using BioEdit ver 5.0.9[18] to resolve nucleotide ambiguities. Global alignment of sequences was done using the software Clustal X ver 1.8 and Gene Doc version 2.1.02[19].

In vitro induction of mutation

Of the 20 isolates included in the second part of the study, five (all the three NAS and two NAR) were selected based on their MIC to ciprofloxacin for the *in vitro* induction experiment[12,13,20]. These were designated as wild type (Table II). The isolates selected after ciprofloxacin exposure designated as mutants.

Multistep induction and selection of resistant mutants: Wild isolates were grown in antibiotic-free brain heart infusion broth (Difco Laboratories, USA) at 37°C for 18 h[12]. To start with about 1x10^8 cfu/ml (0.5 McFarland standard) of each isolates (1 ml) was inoculated into 2 ml of cation-adjusted Muller Hinton broth (Difco Laboratories, USA) containing ciprofloxacin concentration equal to MIC of corresponding wild isolates. The ciprofloxacin concentrations used for

mutant induction ranged from 2- to 4-fold higher the MIC for the wild isolates or sub-wild mutant isolates resulting from the prior induction step[13,20]. After an overnight incubation at 37°C, cells growing in the tubes with the highest ciprofloxacin exposure concentration were selected for mutants with increased MICs by plating onto ciprofloxacin containing cation-adjusted Muller Hinton agar plates at concentrations of 0, 2, 4, 8 and 16 mg/l, or higher when necessary. After each two subcultures the organism was confirmed by MIC and serotyping. MICs were determined in cation-adjusted Muller Hinton agar medium without drugs. Three colonies were randomly selected for MIC determination, and isolates with the highest MIC were subjected to sequence analysis and further induction. This was repeated when a higher exposure concentration was used for the next step of the induction/selection cycle until mutants with significantly high MICs were selected. When MICs of mutant isolates increased up to

Table II. Phenotypic characteristics of the five isolates selected for *in vitro* induction

ID no.	Ciprofloxacin MIC (µg/ml)	Nalidixic acid sensitivity
6509	0.016	S
23522	0.094	S
9425	0.250	R
3269	0.19	S
23460	≥32	R
ATCC *E. coli* 25922	0.008	S

S, sensitive; R, resistant

four-fold higher than wild type, and mutant isolate stop growing in a particular concentration of ciprofloxacin, it was considered as "significantly high MICs". The stability of the selected resistant mutants was confirmed by sub-culture onto antibiotic-free sheep blood agar plates for 10 serial passages.

Susceptibility testing, MIC determination, DNA isolation, PCR amplification and sequencing of QRDR region were done as described above.

Results

The results for part 1 of the study are shown in Table III. In *S.* Typhi MIC was increased from 0.38 and 0.125 to 0.50 in both the isolates, respectively. These isolates showed mutation at S83 to F/Y (serine to phenylalanine, or tyrosine), respectively in *gyrA* gene. The MIC in *S.* Paratyphi A was increased from 0.75 to 1 in subsequent isolate and the mutation was at S83 to F (serine to phenylalanine) in *gyrA* gene only and no mutations were found in *gyrB*, *parC* and *parE* genes in all the isolates.

In part II of the study, nine isolates had single mutation in *gyrA* gene at S83 to F/Y while six had triple mutation (double mutation in *gyrA* gene at S83 to F/Y and D87 to N with single mutation in *parC* gene at S80 to I) (Figure). All these clinical isolates with triple mutations were ciprofloxacin resistant and showed higher MIC (>4 µg/ml) (Table IV). S83 to F/Y was the most common mutation except in one isolate where only single mutation was detected at D87 to N.

In the *in vitro* induction of mutation experiment, MIC of wild and mutant isolates is shown in Table V. With each set of wild/mutant, the MIC of ciprofloxacin for mutants was increased following induction/selection cycles. All mutants reached MICs of ciprofloxacin >32 µg/l after six to eight cycles of induction/selection except in one strain that is reached up to 16 µg/l .

The isolates with single *gyrA* mutation were less resistant to fluoroquinolones than those with additional *parC* mutations. Bacterial isolates acquired mutations (triple mutations) thus exhibiting MICs (> 4µg/ml) except in one isolate. No mutations were detected in the *gyrB* and *parE* in our study.

Discussion

Enteric fever remains to be an important public health problem despite the availability of vaccine and effective antibiotics particularly in endemic countries and this has been compounded by the global emergence

Table III. Phenotypic and genotypic characteristics of bacteria isolated from the patients with relapse of enteric fever (Part I)

S. no	Age/sex of patient	Isolate from first episode of fever					Isolate from second episode of fever				
		Organism	Phenotype	MIC of CIP (ciprofloxacin) (µg/ml)	*gyrA* mutation	*parC* mutation	Organism	Phenotype	MIC of CIP (Ciprofloxacin) (µg/ml)	*gyrA* mutation	*parC* mutation
1.	3 yr/F	*S.* Typhi	NAR	0.38	Ser83 → Phe	NP	*S.* Typhi	NAR	0.50	Ser83 → Phe	NP
2.	11 yr/F	*S.* Typhi	NAR	0.125	Ser83 → Tyr	NP	*S.* Typhi	NAR	0.50	Ser83 → Tyr	NP
3.	22 yr/M	*S.* Paratyphi A	NAR	0.75	Ser83 →Phe	NP	*S.* Paratyphi A	NAR	1.0	Ser83 → Phe	NP

NP, not present
NAR, nalidixic acid resistant
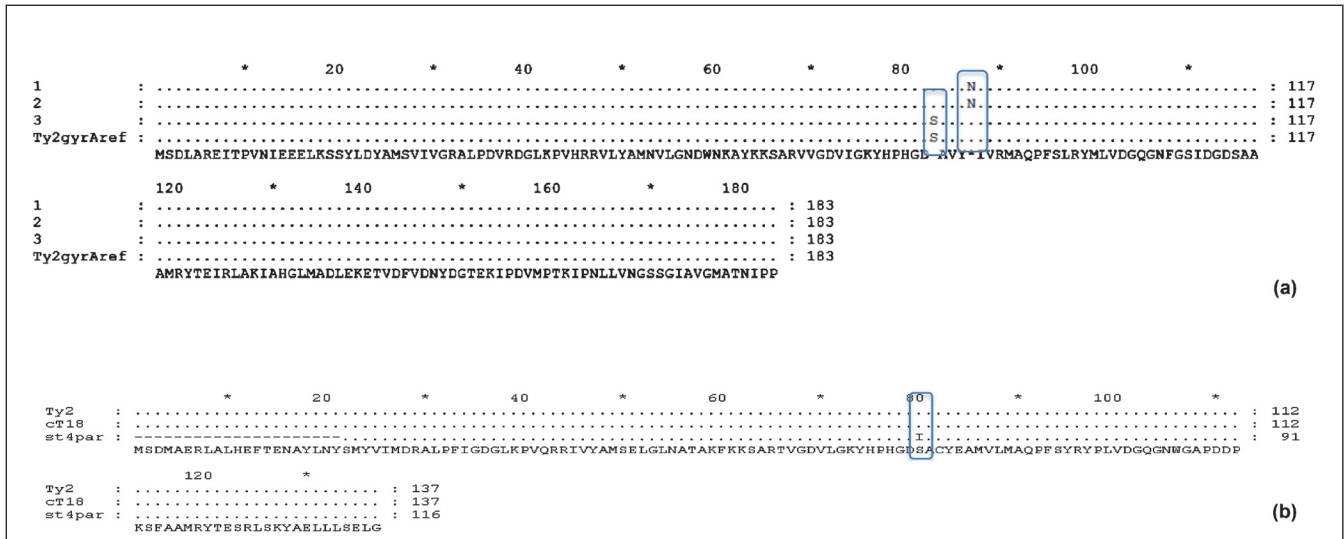
Fig. a. Double mutation in *gyrA* gene at S83 and D87. **Fig. b.** Mutation in *parC* gene at S80. **Fig. a** and **b** show mutations, found in a clinical isolate of *S.* Typhi.

of multidrug resistant *S.* Typhi leading to treatment failures[1-6]. The data on the status of *Salmonella* strains in a patient on documented treatment failure are limited because the sensitivity of blood culture is very poor in a patient already on ciprofloxacin treatment, although there is no clinical response. Subsequent treatment with ceftriaxone or cefexime helps in curing the patient.

The isolates from the three patients who relapsed also showed an increased MIC to ciprofloxacin during the relapse. It is possible that the MIC increases while the patient is on treatment with ciprofloxacin, which results in clinical non-response. It may be hypothesized that during the first stages of antibiotic treatment, a subpopulation of *S.* Typhi with reduced susceptibility to ciprofloxacin may be able to survive, replicate and acquire substitutions conferring resistance to higher concentrations of drug, while the antimicrobial level is still inhibitory to the remaining population. In *in vitro* induction of mutation experiment, isolates acquired an increased MIC to ciprofloxacin under sequential increase of ciprofloxacin concentration. Further, single point mutation in *gyrA* gene from S83 to F/Y was associated with decreased susceptibility to ciprofloxacin, while accumulation of mutations in *gyrA* gene (mainly at S83 to F/Y and D87 to G/N/Y) and *parC* gene (at S80 to I) led to the increased MIC to ciprofloxacin and complete resistance. We found triple mutations in all isolates after 2-3 initial induction/ selection cycles in *gyrA* and *parC* gene which resulted in increased MIC. While Hirose *et al*[13] found that the

Table IV. Phenotypic and genotypic characteristics of isolates included in Part II study

Isolate no	Ciprofloxacin MIC (µg/ml)	*gyr A* mutation	*parC* mutation
13787	0.250	S83→ F	NP
21949	0.380	S83→ Y	NP
23436	0.380	S83→ F	NP
4797	0.380	S83→ F	NP
6652	0.380	D87→ N	NP
14000	0.75	S83→ F	NP
8583	0.750	S83→ Y	NP
CH-1	1.0	S83→ F	NP
CH-2	3.0	S83→ F	NP
13279	8	S83→ F D87→ N	S80→ I
13848	8	S83→ F D87→ N	S80→ I
12669	10	S83→ F D87→ N	S80→ I
7541	12	S83→ F D87→ N	S80→ I
17062	16	S83→ F D87→ G	S80→ I
24238	≥32	S83→ F D87→ N	S80→ I

Table V. Phenotypic and genotypic characteristics of wild and mutated isolates of *S.* Typhi obtained after selection by ciprofloxacin

Wild/mutant in steps[*]	Ciprofloxacin MIC (μg/ml)	Point mutation in *gyrA* (codon) residue S83	Point mutation in *gyrA* (codon) residue D87	Point mutation in *parC* (codon)
23522/06Wild	0.094	NP	NP	NP
23522/06M-1	1	NP	NP	NP
23522/06M-2	4	S83 F	NP	NP
23522/06M-4	8	S83→F	D87→G	S80→I
23522/06M-4	12	S83→F	D87→G	S80→I
23522/06M-5	16	S83→F	D87→G	S80→I
23522/06M-6	>32	S83→F	D87→G	S80→I
9425/01 Wild	0.25	NP	NP	NP
9425/01M-1	1	S83→F	NP	NP
9425/01M-3	4	S83→F	D87→G	S80→I
9425/01M-4	8	S83→F	D87→N	S80→I
9425/01M-5	12	S83→F	D87→N	S80→I
9425/01M-6	16	S83→F	D87→N	S80→I
3269/99 Wild	0.19	NP	NP	NP
3269/99M-1	1	S83→F	NP	NP
3269/99M-3	4	S83→F	D87→N	S80→I
3269/99M-4	8	S83→F	D87→N	S80→I
3269/99M-5	16	S83→F	D87→N	S80→I
3269/99M-6	>32	S83→F	D87→N	S80→I
6509/93 Wild	0.016	NP	NP	NP
6509/93M-1	1	S83→Y	NP	NP
6509/93M-2	4	S83→F	D87→G	S80→I
6509/93M-3	8	S83→F	D87→G	S80→I
6509/93M-5	16	S83→Y	D87→N	S80→I
6509/93M-6	>32	S83→Y	D87→N	S80→I
23460/06 Wild	>32	S83→F	D87→N	S80→I
23460/06M-1	>32	S83→F	D87→N	S80→I

S, serine; Y, tyrosine; F, phenylalanine; D, aspartic acid; N, asparagine; I, isoleucine; G, glycine
NP, not present. [*]The number following each mutant (M) indicates the number of induction steps

isolates with high-level resistance to fluoroquinolones (MIC range, 4 to >32 µg/ml) induced by *in vitro* selection with ciprofloxacin had double mutation at both Ser83 and Asp87 in the *gyrA* gene, they did not report any mutation in *parC* gene. Another important factor noticeable in the mutation experiment was that there was 2-4 fold rise of MIC of the mutant isolates on sequential exposure to ciprofloxacin without any new mutation after 8 µg/ml. Thus, these mutated isolates become more tolerant to ciprofloxacin on exposure and could be another important factor for clinical failure. As the increase in MIC to ciprofloxacin did not directly lead to increase in mutations, there could be other mechanisms responsible for raised MICs such as efflux pumps associated with multi-antibiotic resistance (outer membrane proteins, MAR locus), up/downregulation of operon genes. Plasmid which are responsible for quinolone resistance are qnr (qnr A, qnr B), aminoglycoside acetyltransferase (AAC)[13,20,21]. The mixture of mutations in target enzymes and efflux pump perhaps builds up high-level resistance in enteric fever[22].

Of the 20 isolates included in the second part of the study, triple mutations were found in six isolates, which means there is accumulation of mutations and clonal spread of highly resistant *S.* Typhi clone. This also has been reported by Gaind *et al*[23] from India. Upon exposure to the selective pressure of the ciprofloxacin, the increase in the level of resistance could be due to an increase in the rate of mutations or clonal expansion of mutant with single mutation on to which the second mutation can add up[24,25]. This step-wise evolution is consistent with the conclusions that the acquisition of mutations during treatment is one of the most important factors contributing to therapy failure[26].

There is no new antibiotic molecule developed for GNB (Gram-negative bacteria) in the recent years[27], which again emphasizes the need for strict and rational use of ciprofloxacin in human and veterinary therapeutics. The judicious use by optimal dosage intervals taking Pharmacodynamics and pharmacokinetics parameters that prevent emergence of pre-existing or newly formed mutants should now be guiding the treatment in such infections[28]. Thus, the molecular analysis of fluoroquinolone resistance with continuous antimicrobial surveillance is warranted in future.

Acknowledgment

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