Molecular analysis of high-level ciprofloxacin resistance in
Salmonella enterica serovar Typhi and S. Paratyphi A: need to
expand the QRDR region?

M. R. CAPOOR1, D. NAIR1*, N. S. WALIA1, R. S. ROUTELA2, S. S. GROVER2,
M. DEB1, P. AGGARWAL1, P. K. PILLAI3 AND P. J. BIFANI4

1 Department of Microbiology, Vardhman Mahaveer Medical College and Safdarjung Hospital, New Delhi, India
2 Department of Biotechnology, National Institute of Communicable Diseases, New Delhi, India
3 Department of Microbiology, Majeedia Hospital, New Delhi, India
4 Department of Molecular Biology, Pasteur Institute, Brussels, Belgium

(Accepted 18 June 2008; first published online 8 August 2008)

SUMMARY

Fourteen strains of S. Typhi (n = 13) and S. Paratyphi A (n = 1) resistant to ciprofloxacin were compared with 30 ciprofloxacin decreased-susceptibility strains on the basis of qnr plasmid analysis, and nucleotide substitutions at gyrA, gyrB, parC and parE. In ciprofloxacin-resistant strains, five S. Typhi and a single S. Paratyphi A showed triple mutations in gyrA (Ser83→Phe, Asp87→Asn, Glu133→Gly) and a novel mutation outside the quinolone resistance determining region (QRDR) (Met52→Leu). Novel mutations were also discovered in an isolate (minimum inhibitory concentration 8 μg/ml) in gyrA gene Asp76→Asn and outside the QRDR Leu44→Ile.

Out of 30 isolates with reduced susceptibility, single mutation was found in 12 strains only. Genes encoding qnr plasmid (qnr A, qnr B, AAC1-F) were not detected in ciprofloxacin-resistant or decreased-susceptibility strains. Antimicrobial surveillance coupled with molecular analysis of fluoroquinolone resistance is warranted for reconfirming novel and established molecular patterns of resistance, which is quintessential for reappraisal of enteric fever therapeutics.

Key words: Ciprofloxacin resistance, molecular mechanisms, S. Paratyphi, S. Typhi.

INTRODUCTION

The emergence of multidrug-resistant enteric fever led to use of fluoroquinolones as the first-line of therapy. Unfortunately, broad-spectrum antibacterial activity, affordability and easy availability led to their indiscriminate use in human medicine. Furthermore, nalidixic acid-resistant S. Typhi (NARST) with reduced susceptibility to ciprofloxacin (0.125–1 μg/ml) causing clinical failure emerged worldwide and became endemic in the Indian subcontinent [1, 2].

At the molecular level, this was mediated by a single-nucleotide polymorphism (SNP) in the quinolone resistance-determining region (QRDR) of gyrA at Ser83 or Asp87. Resistant isolates harbour ≥2 mutations in gyrA, gyrB, and topoisomerase (parC and parE). Other mechanisms demonstrated are multi-antibiotic resistance associated efflux pumps (MAR locus, outer membrane proteins), qnr plasmid and up-/down-regulation of operon genes [3–5]. Experimental evidence from in vitro selection studies suggests that single mutations are associated with low-level fluorquinolone resistance and high-level resistance is built-up by sequential accumulation or perhaps a mixture of target and efflux-related mutations [6]. These are well documented in non-enteric fever
salmonellae and other organisms [7–9], as each target gene mutation reduces the susceptibility by four- to eightfold [10]. Nonetheless, a recent report observed that for S. Typhi nalidixic acid resistance does not completely predict decreased ciprofloxacin susceptibility [11]. Moreover, the emergence of plasmid-mediated quinolone resistance (PMQR) mediated by QNR, aminoglycoside acetyltransferase (AAC) and Qep A in family Enterobacteriaceae has complicated the understanding of molecular mechanisms of quinolone resistance [12]. Recent literature cites isolated reports of high-level ciprofloxacin resistance in enteric fever, from India and elsewhere [13–18]. Studies on molecular analysis of fluoroquinolone resistance in clinical isolates of Salmonella enterica serotype Typhi and Paratyphi A from India are limited [2, 4, 13, 18].

Keeping in mind the prime therapeutic role of ciprofloxacin in enteric fever, an understanding of the mechanisms involved is crucial. Therefore the current study was undertaken to characterize the molecular basis of ciprofloxacin resistance in enteric fever.

METHODS

The present study was conducted in Vardhman Mahaveer Medical College and Safdarjung Hospital, a 1700-bed tertiary-care hospital, and Majeedia Hospital, a referral centre in New Delhi over a period of 1 year and 10 months (December 2004–September 2006). One hundred ninety-eight isolates of S. Typhi and S. Paratyphi A from suspected enteric fever patients were identified by standard biochemical reactions [19] and serotyping with specific antisera (Central Research Institute, Kasauli, India). Socio-demographic (age, gender) and clinical information (antimicrobial management and in-hospital mortality) of the patients in study were noted. The antimicrobial susceptibility of the isolates was determined by disk diffusion method according to CLSI guidelines [20]. The agar dilution method to determine MICs was repeated three times and the mean was taken as the final value. Interpretive criteria for sensitive, intermediate and resistant strains for ciprofloxacin were \( \leq 1 \mu g/ml \), \( =2 \mu g/ml \) and \( \geq 4 \mu g/ml \) and for nalidixic acid \( \leq 1 \mu g/ml \), \( =2 \mu g/ml \) and \( \geq 4 \mu g/ml \), respectively, in accordance with CLSI guidelines [20]. Decreased susceptibility to ciprofloxacin was defined as isolates having a MIC of \( \geq 0-125 \mu g/ml \) but \( \leq 1 \mu g/ml \). The control strain used was E. coli ATCC 25922. Antimicrobial disks and powders used in the study were obtained from Hi Media, Sigma Laboratories (India).

Representative isolates resistant to ciprofloxacin were compared by molecular methods [plasmid analysis (qnr allele), nucleotide substitutions at gyrA, gyrB, parC and parE] with decreased- susceptibility strains at Pasteur Institute, Brussels, Belgium and the National Institute of Communicable Diseases, New Delhi. DNA was extracted according to the protocol for the isolation of genomic DNA from Gram-negative bacteria (Qiagen; Qiamp DNA mini kit) under sterile conditions. Samples (200 \( \mu l \)) were processed in parallel. The species identification of a few strains was carried out by sequencing the 16S rRNA. DNA amplification was performed by polymerase chain reaction (PCR). PCR mixture consisted of 5 \( \mu l \) of extracted DNA in 45 \( \mu l \) PCR mixture composed of 1x buffer (Ozyme, New England Biolabs, Beverly, MA, USA), 0.5M MgCl_2, 0.24 mm dNTP, 25 pmol of each primer and 1 U Taq polymerase (Ozyme, New England Biolabs). Internal controls were included in all PCR assays consisting of PCR inhibition control as performed for biological extract, positive controls to validate the amplification conditions and negative controls to ensure that there was no PCR/sample cross-contamination. The thermal cycling DNA amplification conditions consisted of 15 min at 95 °C for activation of the polymerase, 2 min at 92 °C followed by 35 cycles of denaturation at 93 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 1 min and elongation at 72 °C for 8 min. Sequencing was performed on PCR-amplified amplicons by the dideoxy chain-termination method with the Big Dye Terminator Cycle sequencing kit (PerkinElmer, Applied Biosystems, Foster City, CA, USA) and run on a DNA analysis system model 373 (PerkinElmer, Applied Biosystems). PCR amplification and direct DNA sequencing of QRDR regions (gyrA, gyrB, parC, parE genes) was performed according to Giraud et al. [21], with an ABI prism dye terminator.
Ciprofloxacin resistance in *S. enterica* serovar Typhi and *S. Paratyphi A*

(PerkinElmer, Applied Biosystems) on an ABI 3730 automated sequencer.

The known sequence genes were used for designing primers. Oligonucleotide primers used for PCR assay were:

- **gyrA (F):** 5'-CCAGATGT(A/C/T)CG(A/C/T)GATGG-3' (F)
- **gyrA (R):** 5'-ACGAATACAC(G/C)GT(C/T)CTTTTTTC-3' (R)
- **gyrB5 (F):** 5'-AAGCGAGTGCGCAAAAGG-3' (F)
- **gyrB5 (R):** 5'-AACGGTCTGCTATCACGAAAGG-3' (R)

The nucleotide and deduced amino acids were analysed, using Sequence Navigator Software followed by BLAST at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast).

The primers used to detect qnr plasmids (5) were:

- **5'-GGG TAT GTA TAT TGA TAAG-3' for qnr A**
- **5'-CTAT CTA ATG CAGG CAG CAC TAT TAT-3' for qnr B**
- **5'-GTGAATTATTGCGGAA**

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**RESULTS**

The most common age group in the study was 20–30 years. The majority were males (64%). Twenty-nine percent of patients were hospitalized and 71% were from the out-patient department. Ciprofloxacin was the drug of choice in out-patients and the therapy was changed to ceftriaxone on therapeutic failure (ciprofloxacin resistance or decreased susceptibility). None of the patients died in hospital. A total of 198 isolates, comprising of 158 (79.8%) *S. Typhi* and 40 (20.2%) *S. Paratyphi A* were analysed. Of the 198 isolates studied 25 *S. Typhi* and five *S. Paratyphi A* were observed to be resistant to ciprofloxacin. The disk diffusion antimicrobial testing of these 30 isolates revealed the following resistance pattern: ampicillin, 2 (6.7%); chloramphenicol, 5 (16.7%); trimethoprim/sulfamethoxazole, 18 (60%); tetracycline, 18 (60%); nalidixic acid, 30 (100%); ceftriaxone, 1 (3.3%); cefixime, 0 (0%); and cefepime, 1 (3.3%). Multidrug resistance (ACCo) was seen in two (6.7%) of the isolates.

Out of a total of 198 isolates reduced susceptibility (0.125–1 µg/ml) was seen in 168 isolates. High-level ciprofloxacin resistance was seen in 25 *S. Typhi* and five *S. Paratyphi A* (MICs 8 to >512 µg/ml). MIC 90 for ciprofloxacin and ceftriaxone was 1 µg/ml and 0.25 µg/ml, respectively and MIC 50 for ciprofloxacin and ceftriaxone was 0.5 µg/ml and 0.125 µg/ml, respectively. The MIC 50 and MIC 90 for ofloxacin were identical to that of ciprofloxacin. All ciprofloxacin-resistant and decreased-susceptibility strains had a nalidixic acid MIC breakpoint of >256 µg/ml, with the exception of a single outlier [a nalidixic acid-sensitive *S. Typhi* (NASST) isolate with decreased susceptibility at 0.25 µg/ml].

Thirteen *S. Typhi* isolates and a single *S. Paratyphi A* isolate resistant to ciprofloxacin were selected randomly and compared by molecular methods with 30 decreased-susceptibility strains. Relevant clinical, microbiological (MICs), molecular data of these patients with ciprofloxacin resistance is depicted in Table 1. Eight (57%) patients had history of incomplete fluoroquinolone therapy and six (43%) presented fever with no prior antimicrobial therapy on days 2–4.

Amongst the ciprofloxacin-resistant isolates, five *S. Typhi* and a single *S. Paratyphi A* showed triple mutations in *gyrA* (Ser83→Phe, Asp87→Asn, Glu183→Gly) and a novel mutation outside the QRDR region (Met82→Leu). Novel mutations were also discovered in an isolate (8 µg/ml) in the *gyrA* gene Asp76→Asn and outside the QRDR Leu44→Ile. Of note are single replacements at Asp76→Asn (four isolates) and Phe72→Tyr (three isolates) of *S. Typhi*. Out of 30 isolates with reduced susceptibility, single mutation was seen in 15 strains, six had mutations in *gyrA* at Asp87→Asn, five at Phe72→Tyr, two at Ser83→Phe and one each at Asp76→Asn and...
Table 1. Presence of mutations in DNA gyrase, topoisomerase IV and qnrA, qnrB, AAC in S. Typhi \((n = 13)\) and S. Paratyphi A \((n = 1)\) isolates with resistance to ciprofloxacin

<table>
<thead>
<tr>
<th>SNO</th>
<th>Clinical history</th>
<th>CP MIC</th>
<th>gyrA</th>
<th>gyrB/parC/parE/qnrA, AAC</th>
<th>Outside QRDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-yr-old boy with fever for 3 days, no h/o fluoroquinolones, S. Typhi isolated; ciprofloxacin resistant; successfully treated with ceftriaxone</td>
<td>&gt;512</td>
<td>Ser89→Phe</td>
<td>Met (^{42} \rightarrow) Leu</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10-yr-old boy with fever for 3 days, no h/o fluoroquinolones, S. Typhi isolated; ciprofloxacin resistant; left against medical advice on 5th day before culture report</td>
<td>32</td>
<td>Ser89→Phe</td>
<td>Met (^{42} \rightarrow) Leu</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13-yr-old boy with fever for 4 days, no h/o fluoroquinolones, S. Typhi isolated; ciprofloxacin resistant; successfully treated with ceftriaxone</td>
<td>32</td>
<td>Ser89→Phe</td>
<td>Met (^{42} \rightarrow) Leu</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6-yr-old boy with fever for 2 days, no h/o fluoroquinolones/ antimicrobials, S. Typhi isolated; ciprofloxacin resistant; successfully treated with ceftriaxone</td>
<td>64</td>
<td>Ser89→Phe</td>
<td>Met (^{42} \rightarrow) Leu</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>16-yr-old girl with fever for 4 days, no h/o fluoroquinolones, S. Typhi isolated; ciprofloxacin resistant; successfully treated with ceftriaxone</td>
<td>256</td>
<td>Ser89→Phe</td>
<td>Met (^{42} \rightarrow) Leu</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6-yr-old boy with fever for 3 days, no h/o fluoroquinolones/ antimicrobials, S. Typhi isolated; ciprofloxacin resistant; successfully treated with ceftriaxone</td>
<td>8</td>
<td>Phe72→Tyr</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>25-yr-old male, doctor, with fever; treated with levofloxacin for 2 days before blood culture was sent; S. Typhi isolated; admitted and treated with ceftriaxone, discharged on cefixime</td>
<td>8</td>
<td>Asp76→Asn</td>
<td>Leu(^{44} \rightarrow) Ile</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Adult male patient; no response to ciprofloxacin treatment; culture grew S. Typhi; lost to follow up</td>
<td>64</td>
<td>Asp76→Asn</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>11-yr-old girl with PUO for 1 month; no response to ciprofloxacin treatment for 5 days; left against medical advice on 6th day before culture report of S. Typhi communicated</td>
<td>16</td>
<td>Asp76→Asn</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>22-yr-old female with fever of 10 days’ duration; took oflox 2 days; no response; admitted elsewhere due to deterioration; was treated with ciprofloxacin, ceftriaxone; left against medical advise; admitted at our hospital; S. Typhi isolated, responded to ceftriaxone. History of enteric fever in her brother with strain of similar resistotype 20 days previously</td>
<td>64</td>
<td>Asp76→Asn</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>25-yr-old male, a case of PUO × 6 months; took intermittent treatment with ciprofloxacin and other broad spectrum antibiotics, remained afebrile for few weeks; fever re-emerged; prescribed ofloxacin for 5 days; no response; blood culture yielded S. Typhi; admitted; successfully treated with ceftriaxone</td>
<td>128</td>
<td>Asp76→Asn</td>
<td>NP</td>
<td></td>
</tr>
</tbody>
</table>
Phe\textsuperscript{31}→Tyr (outside QRDR), substitutions, respectively. No mutations could be detected in 15 isolates. Genes encoding \textit{qnr} plasmid (\textit{qnr} A, \textit{QNR} B, \textit{AAC}1-F) were not detected in ciprofloxacin-resistant or decreased-susceptibility strains.

**DISCUSSION**

In the present study, multi-drug resistance (ACC\textit{O}) was observed in 6.6\% of ciprofloxacin strains. A re-emergence of sensitivity to the classical first-line
agents has been observed due to their restricted use in the 1990s. The use of quinolone led to a concomitant decrease in susceptibility to ciprofloxacin and nalidixic acid from this region [2]. All of the NARST isolates had a decreased susceptibility to ciprofloxacin (MIC $\geq 0.125 \mu g/ml$). Several workers have corroborated this finding abroad [2, 13, 22]. The MIC 50 and MIC 90 for ofloxacin was identical to that of ciprofloxacin. A single outlier isolate was observed (a NASST isolate with decreased susceptibility to ciprofloxacin at 0.25 $\mu g/ml$). This observation has been reported in a recent study [11].

Single Ser$^{83}$→Phe, Asp$^{76}$→Asn, Phe$^{72}$→Tyr substitutions are commonly associated with NARST [2, 5, 9]. Nonetheless, the single mutations seen in NARST isolates at Asp$^{76}$→Asn in gyrA and Phe$^{83}$→Tyr outside the QRDR of gyrA are hitherto unknown [3, 13, 21]. However, substitution at Asp$^{76}$→Asn has been reported from a ciprofloxacin intermediate strain from a molecular study (2001–2003) from this hospital [2]. As mutations were not found in 15 NARST strains, other possibilities of mutations present outside the QRDR region or other mechanisms of quinolone resistance possibly exist.

The mechanisms of high-level fluoroquinolone resistance in clinical isolates of S. Typhi and S. Paratyphi A are not completely understood, as there are only a few reports on resistance in the literature (Table 2). Most studies, including the present study, give evidence for sequential accumulation of target gene mutations in the QRDR region. In stark contrast to previous communications, in the present study, seven (50%) of the ciprofloxacin-resistant isolates had a single mutation in gyrA Phe$^{72}$→Tyr (three isolates), and Asp$^{76}$→Asn (four isolates), sufficient to confer high-level resistance (8–256 $\mu g/ml$). This unique observation coupled with the absence of mutations in other topoisomerases and 50% of NARST isolates reinforce the fact that other mechanisms must co-exist to mediate resistance. Moreover, even the MIC of the isolates was unlinked to the number of mutations observed. Substitution at Glu$^{110}$→Gly has not been observed previously in S. Typhi, S. Paratyphi A, other salmonellae or E. coli [3, 5, 9, 16, 21, 24]. The mutations at positions 76 and 72 are also infrequently reported; nonetheless, there are a few citations of single substitution at Asp$^{76}$→Asn and Phe$^{72}$→Tyr in S. Typhi [2] and Phe$^{72}$→Tyr in combination with Ser$^{83}$→Phe in S. Senftenberg [7].

However, the most striking observation was the co-existence of mutations outside the QRDR region (Met$^{82}$→Leu, Leu$^{44}$→Ile) in seven strains (50%). In Salmonella, QRDR spans from amino acids 54 to 171 [16]. Therefore, it is speculated that QRDR of Salmonella should be expanded to include these positions. In a review of the literature, few reports document mutations outside the QRDR domain [2, 25].

Due to paucity of sequencing data on clinical isolates of ciprofloxacin-resistant S. Typhi and S. Paratyphi A, the aforementioned novel substitutions or observations could not be compared with prior reports. Nevertheless, they may become frequent in future owing to selective pressures exerted by the irrational use of ciprofloxacin in human and veterinary therapeutics, in a population endemic with NARST strains. Meanwhile, more such studies are warranted in order to determine whether such novel mutations, when present alone, confer resistance or decreased susceptibility to ciprofloxacin in vitro or are just ‘bystanders’.

Moreover, the finding of sequential accumulations of mutations is further corroborated by the fact that eight (57%) patients had history of incomplete fluoroquinolone therapy. It is probable, as inferred from their history records that these patients were initially infected with isolates having decrease susceptibility that subsequently mutated to high-level ciprofloxacin isolates. Observation of de novo mutation is supported by the fact that in six patients (43%) prior history of fluoroquinolone was not elicited, therefore, only molecular typing will clarify these relevant issues. In the present study three groups emerged showing mutations at positions 83, 87, 133 and at 72 and 76. The phenotypic expression of level of resistance (MIC) of these groups were heterogeneous. Establishing the clonality, or the lack of it, is therefore critical at this stage in India, as there seems to be an unusually increasing occurrence of high-level ciprofloxacin-resistant S. Typhi.

In the present study genes encoding qnr plasmid (qnrA, QNR B, AAC1-F) were not detected in ciprofloxacin-resistant or decreased-susceptibility strains. These proteins protect the target enzymes (DNA gyrase and type IV topoisomerase) from quinolone inhibition and the AAC enzyme acetylates quinolones. Although these PMQR determinants confer only low-level resistance, nonetheless, they provide a background against which selection of additional chromosomal-encoded quinolone-resistance mechanisms occur. These might become important in future in S. Typhi and S. Paratyphi A as linkage
between qnr plasmid, genes encoding extended spectrum β-lactamases and AmpC type β-lactamases may reflect association between resistance to quinolones and extended-spectrum cephalosporins [12, 22].

The limitation of this work is, that epidemiological typing of ciprofloxacin-resistant strains was not performed. Furthermore, investigations for possible mutations at other loci (MAR) and OMP profile, qepA gene, etc., for other mechanisms of resistance were not elucidated. A large number of studies is warranted from this region to find novel target genes thereby aiding in new drug discoveries.

In the present study, MIC 90 and MIC 50 for ceftriaxone were 0.25 μg/ml and 0.125 μg/ml, respectively. A high MIC for the third-generation cephalosporins has also been observed in previous studies [26, 27]. The therapeutics of ciprofloxacin-resistant enteric fever narrows to third- and fourth-generation cephalosporins and azithromycins which are not affordable in nations with limited resources. Of the first-line antimicrobials ampicillin, chloramphenicol and cotrimoxazole, especially chloramphenicol, need to be utilized.

Therefore, antimicrobial surveillance coupled with molecular analysis of fluoroquinolone resistance is warranted for reconfirming novel and established molecular patterns for therapeutic reappraisal and for novel drug targets.

DECLARATION OF INTEREST
None.

REFERENCES


