Indian J Med Res 141, January 2015, pp 81-89

# Enhanced resistance to fluoroquinolones in laboratory-grown mutants & clinical isolates of *Shigella* due to synergism between efflux pump expression & mutations in quinolone resistance determining region

Neelam Taneja, Arti Mishra, Ajay Kumar, Garima Verma & Meera Sharma

*Department of Medical Microbiology, Postgraduate Institute of Medical Education & Research, Chandigarh, India*

Received January 1, 2013

***Background & objectives*: There is a worldwide emergence of fluoroquinolone resistance in *Shigella* species. To understand the molecular mechanisms associated with fluoroquinolone resistance, naturally occurring fluoroquinolone-resistant strains and laboratory-induced spontaneous mutants of *Shigella* spp. were used and the relative contributions of *acrAB-tolC* efflux pumps, gyrase and topoisomerase target gene mutations towards fluoroquinolone resistance were determined.**

***Methods*: Eight *Shigella flexneri* and six *S. dysenteriae* clinical isolates were studied. Three consecutive mutants resistant to ciprofloxacin for *S. flexneri* SFM1 (≥0.25 µg/ml), SFM2 (≥4 µg/ml) and SFM3 (≥32 µg/ml) were selected in 15 steps from susceptible isolates by serial exposure to increasing concentrations of nalidixic acid and ciprofloxacin. Similarly, two mutants for *S. dysenteriae* SDM1 (≥0.25 µg/ml) and SDM2 (≥4 µg/ml) were selected in eight steps. After PCR amplification sequence analyses of gyrase and topoisomerase target genes were performed. Expression of efflux genes *acrA, acrB, acrR* and *tolC* was measured using real-time PCR.**

***Results*: Mutations were observed in *gyrA* Ser$^{83}$→Leu, Asp$^{87}$→Asn/Gly, Val$^{196}$→Ala and in *parC* Phe$^{93}$→Val, Ser$^{80}$→Ile, Asp$^{101}$→Glu and Asp$^{110}$→Glu. Overall, *acrA* and *acrB* overexpression was associated with fluoroquinolone resistance ($P$<0.05); while *tolC* and *acrR* expression levels did not.**

***Interpretation & conclusions*: Fluoroquinolone resistance in *Shigella* spp. is the end product of either a single or a combination of mutations in QRDRs and/ or efflux activity. Novel polymorphisms were observed at Val$^{196}$→Ala in *gyrA* in clinical isolates and Phe$^{93}$→Val, Asp$^{101}$→Glu, Asp$^{110}$→Glu and in *parC* in majority of laboratory-grown mutants.**

**Key words** Efflux pumps - fluoroquinolone resistance- quinolone resistance determining regions (QRDRs) - *Shigella*

Resistance to fluoroquinolones is usually the result of a synergistic action of activation of *acrAB-tolC* efflux pumps and/or mutations in bacterial quinolone resistance determining regions (QRDRs), and/or plasmid-encoded mechanisms[1-5]. The mutations in bacterial targets *gyrA* and *gyrB* encoding for DNA gyrase and topoisomerase IV cause a change either in the target structure or its binding strength, resulting in less susceptibility[6]. The plasmid-mediated fluroquinolone resistance is either due to protection of DNA gyrase by a pentapeptide repeat family protein called *qnr* or ciprofloxacin-modification by an enzyme (aminoglycoside acetyltransferase) encoded by gene *aac*(6´)-Ib-cr[5,7]. Further, the overexpression of efflux pumps results in overall decreased accumulation of antibiotic inside the bacterial cells further resulting in decreased susceptibility[8,9]. In Gram-negative bacteria, these drug efflux pumps contribute to intrinsic antibiotic resistance and mainly include three superfamilies namely, resistance-nodulation-cell division (RND) family, major facilitator superfamliy (MFS), and the ATP-binding cassette (ABC)[4,8]. In particular, the proton/drug antiporters belonging to RND family play an important role in clinically relevant resistance in Gram-negative bacteria, and the archetypal bacterial RND pump – *acrAB-tolC* has been studied extensively in *Escherichia coli*[10,11]. This consists of a tripartite system containing a periplasmic membrane fusion protein (MFP) encoded by *acrA*, an outer membrane protein (OMP) encoded by *tolC,* and a cytoplasmic membrane transporter (RND) encoded by *acrB*. This complex forms a channel spanning the entire membrane, governing the transportation of antibiotics from the periplasmic space and cytoplasm to the extracellular environment[10,11].

Since several mechanisms are known by which shigellae become resistant to fluroquinolones, we undertook this study to determine the relative contributions of *acrAB-tolC* efflux pumps, gyrase and topoisomerase target gene mutations towards ciprofloxacin (CIP)/fluoroquinolone (FQ) resistance in *Shigella*. We used a set of laboratory-induced mutants and naturally occurring fluoroquinolone-resistant isolates of *S. flexneri* and *S. dysenteriae* serotype 1 to study the step-wise evolution of quinolone resistance, occurrence of mutations in the QRDR region and changes in the expression of efflux pump encoding genes.

## Material & Methods

This study was conducted at the Enteric Laboratory, Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India. The study protocol was approved by the institutional ethics committee.

Eight *S. flexneri* (618, 497, 44, 488, 804, 710, 592, 993) and six *S. dysenteriae* (1169, 677, 553, 115, 622, 53) isolates collected from stool specimens of patients suffering from dysentery between 2001-2007 were included in the present study. These isolates were confirmed by biochemical tests and serotyping (Denka-Seiken, Japan). The antibiotic susceptibility for nalidixic acid (NA, 30 µg) and ciprofloxacin (5 µg) (Oxoid Limited, Hampshire, UK) was determined and minimum inhibitory concentrations (MICs) by an E-test (bioMérieux SA, Marcy l'Etoile, France), according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2010[12].

*Selection of nalidixic acid and ciprofloxacin-resistant mutants in vitro*: Wild-type spontaneous mutants of *Shigella* exhibiting increased resistance to nalidixic acid and ciprofloxacin were selected from nalidixic acid and ciprofloxacin-susceptible *S. dysenteriae* 1169 and *S. flexneri* 497 isolates as described previously for *E. coli*[13]. The mutants were selected by *in vitro* step-wise exposure to increasing concentrations of nalidixic acid (Sigma-Aldrich, St. Louis, MO, USA) and subsequently to ciprofloxacin (Sigma-Aldrich). Briefly, nalidixic acid and ciprofloxacin-susceptible *S. flexneri* 497 and *S. dysenteriae* 1169 were grown in increasing concentration of nalidixic acid (0.5,1,2,4,16,32,64,128 µg/ml) on subsequent days in brain heart infusion broth (BHI; BD Difco™; Becton, Dickinson and Co., Detroit, MI, USA) at 37°C overnight. A 0.5 McFarland of overnight culture prepared in normal saline was spread on Mueller-Hinton agar (MHA) (BD Difco™) supplemented with similar concentrations of nalidixic acid (0.5,1,2,4,16,32,64, 128 µg/ml) and was incubated for 24 to 48h at 37°C. Single colonies grown at the respective concentrations were selected and subcultured into BHI broth supplemented with the next higher concentration of antibiotic used in MHA plate. The overnight culture was spread again onto MHA plates supplemented with the next higher concentration of nalidixic acid. *S. flexneri* (SFM) and *S. dysenteriae* (SDM) mutants selected from plates with 128 µg/ml of nalidixic acid were subsequently exposed to ciprofloxacin at concentrations ranging from 0.0325 to 44 µg/ml. *In vitro* selection procedures for ciprofloxacin were similar to those described for the nalidixic acid resistance

selections. The mutants were stocked in stock media (BHI with 20% glycerol) supplemented with desired concentration of antibiotic.

*PCR analyses for quinolone resistance genes*: The *gyrA*[14], *gyrB*[15], *parC*[16], *qnrA*[17] and *aac(6')Ib-cr*[5] were amplified using primers and conditions as described previously. The nucleotide sequencing was performed for representative amplicons and submitted to GenBank (NCBI, Bethesda, MD, USA).

*Real time PCR assay for expression of efflux pump genes*: The bacterial total RNA was extracted[18] and cDNA was synthesized from 1.0µg of RNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences, USA) using random hexamer primers and stored at -80°C. Expression for *acrA, acrB, tolC* and *acrR* genes for spontaneous mutants and the clinical isolates was studied. The gene specific primers were designed using Primer 3 software and oligonucleotide sequences are listed in Table I. The cDNA levels of target genes were determined by relative quantitative real time PCR in 10.0 µl reaction mixture containing: 1.0 µl synthesized cDNA solution, 7.0 µl 1X SYBR master mix (Fermentas Life Sciences, USA), 100 nmol/µl of each forward and reverse primer. The amplification was performed under the following reaction conditions: enzyme activation -95°C for 10 min, followed by 35 cycles of denaturation 95°C for 15 sec, annealing 68°C for 20 sec, extension 72°C for 30 sec using Light Cycler 480 (Roche Diagnostics, USA). Threshold cycle ($C_T$) values were calculated from the amplification plots, and the gene expression levels were compared by normalization with endogenous control 16SrRNA gene. The cDNA was diluted $10^{-5}$ time for 16SrRNA amplification. The expression for target genes *acrA, acrB, tolC,* and *acrR* was measured by $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T = (C_{Ttarget} - C_{Treference})S - (C_{Ttarget} - C_{Treference})T$[19], where S was sensitive strain and T was resistant test strain.

**Table I.** Oligonucleotide sequences used in the study

| Target gene | Primer | Sequence (5' to 3') |
|---|---|---|
| *TolC* | *tolcF* | TGCCGCCGATCGTGATGCTGCCT |
| | *tolcR* | TGCCGTTCGCGTCGCGGTAGC |
| *AcrA* | *acrAF* | ACGCGGCGTACGGGTTACGCC |
| | *acrAR* | AGTTCCCGCAGGACGGTACGCTGG |
| *AcrB* | *acrBF* | TGCGTTCTGCGCGCCGGAACC |
| | *acrBR* | GCGACGCTTGATGCGGTGCGG |
| *AcrR* | *acrRF* | GGGGTATCATCCACCTCGCTGGGCG |
| | *acrRR* | TGCCAGTAGATTGCACCGCGCGT |
| *16SrRNA* | *16S F* | TTGACGGGGGCCCGCACAAGC |
| | *16SR* | AGTTCCCGGCCGGACCGCTGG |

*Synergy test*: Synergy test was performed for ciprofloxacin using CIP agar method. The efflux pump inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added to MHA plate at a concentration of 100 µM[2,20]. Susceptibility testing for ciprofloxacin by E-test was performed, both in presence and absence of CCCP.

*Ciprofloxacin accumulation assay*: Accumulation of ciprofloxacin was studied as described by Charvalos *et al.*[21] with some modifications. Cells were grown up to an $OD_{600}$ of 0.4, harvested and washed with 50 mM phosphate buffer, *p*H 7.2. Cells were resuspended in the same buffer maintaining an $OD_{600}$ of 0.20 and energized with 0.2 per cent glucose for 20 min at 30°C. Ciprofloxacin was added at a concentration of 10 µg/ml. Aliquots of 0.5 ml were withdrawn at different time intervals (0, 10 and 20 min) and dispensed into 1 ml glycine hydrochloride, incubated for 60 min at 37°C and fluorescence was measured in a spectrofluorimeter (ciprofloxacin: excitation wavelength 275 nm, emission wavelength 440 nm). CCCP was added (100 µM/ml) to each aliquot at 20 minutes and fluorescence was measured again as described above.

## Results

*In vitro selection of Shigella mutants resistant to ciprofloxacin*: Three consecutive mutants resistant to ciprofloxacin for *S. flexneri* SFM1 (≥0.25 µg/ml), SFM2 (≥4 µg/ml) and SFM3 (≥32 µg/ml) were selected in 15 steps. Similarly, two mutants for *S. dysenteriae* SDM1 (≥0.25 µg/ml) and SDM2 (≥4 µg/ml) were selected in eight steps. Table II shows the mutation frequencies for different steps varying between 8.06-1.2 x $10^{-6}$.

*Antibiotic susceptibility for clinical isolates*: Three resistance patterns were identified in *S. flexneri*: group I consisted of two susceptible isolates (618, 497) to nalidixic acid ($NA^S$) and ciprofloxacin ($CIP^S$); group II consisted of two isolates (44 and 488) resistant to $NA^R$ and susceptible to $CIP^S$; and group III consisted of four isolates (710, 592, 804 and 993) resistant to both $NA^R$ and $CIP^R$. For *S. dysenteriae*: group I consisted of $NA^S$ and $CIP^S$ (1169, 677) and group II $NA^R$, $CIP^{MS}$ (moderately sensitive to CIP) consisted of 533 and 622, while group III $NA^R$ and $CIP^R$ consisted of isolates 52 and 115 (Table III).

**Table II.** Step-wise selection of ciprofloxacin (CIP) resistant spontaneous mutants of *S. flexneri* (SFM) and *S. dysenteriae* (SDM)

| Step | CIP (µg/ml) in medium | No. of colonies for SFM | Mutation frequency# | No. of colonies for SDM | Mutation frequency# |
|---|---|---|---|---|---|
| 1. | 0.0325 | 121 | $8.06 \times 10^{-6}$ | 81 | $5.4 \times 10^{-6}$ |
| 2. | 0.0625 | 112 | $7.4 \times 10^{-6}$ | 99 | $6.6 \times 10^{-6}$ |
| 3. | 0.125 | 68 | $4.5 \times 10^{-6}$ | 97 | $6.4 \times 10^{-6}$ |
| 4. | 0.25 | 109 | $7.2 \times 10^{-6}$ | 62 | $4.3 \times 10^{-6}$ |
| 5. | 0.5 | 105 | $7.0 \times 10^{-6}$ | 45 | $3.0 \times 10^{-6}$ |
| 6. | 1 | 44 | $2.9 \times 10^{-6}$ | 44 | $2.9 \times 10^{-6}$ |
| 7. | 2 | 56 | $3.7 \times 10^{-6}$ | 27 | $1.8 \times 10^{-6}$ |
| 8. | 4 | 68 | $4.5 \times 10^{-6}$ | 23 | $1.5 \times 10^{-6}$ |
| 9. | 8 | 25 | $1.6 \times 10^{-6}$ | 0 | $<3.0 \times 10^{-8}$ |
| 10. | 10 | 99 | $6.6 \times 10^{-6}$ | - | |
| 11. | 16 | 41 | $2.7 \times 10^{-6}$ | - | |
| 12. | 20 | 32 | $2.1 \times 10^{-6}$ | - | |
| 13. | 24 | 27 | $1.8 \times 10^{-6}$ | - | |
| 14. | 28 | 19 | $1.2 \times 10^{-6}$ | - | |
| 15. | 32 | 21 | $1.4 \times 10^{-6}$ | - | |
| 16. | 44 | 0 | $<3.0 \times 10^{-8}$ | - | |

Nalidixic acid resistant mutants selected on medium containing nalidixic acid. Total of $3 \times 10^8$ cells were inoculated in each step during step-wise selection of mutants. # Mutation frequency was calculated by dividing the total number of cfu obtained on each agar plate by total cells ($3 \times 10^8$) inoculated per milliliter

*Identification of mutations in clinical isolates and mutants in gyrA, gyrB and parC*: On the sequence analyses, mutations were detected in the sequences of *gyrA* and *parC* genes. Mutations observed in *gyrA* were $Ser^{83}$→Leu (618, 497, 44, 804, 710, 592, 993, 1169, 533, 622 and 115), $Asp^{87}$→Asn/Gly (497, 804, 710, 592, 993, 1169, 533, 622 and 115) and $Val^{196}$→Ala (44, 804, 993 and 622). In *parC* mutations detected were $Phe^{93}$→Val (497, 804, 592, 622 and 993) and $Ser^{80}$→Ile (497, 1169, 533, 115 and 622). *S. flexneri* mutants SFM1, SFM2, SFM3 exhibited same genetic profile as wild type 497 strain for *gyrA*, while substitution in *parC*, $Asp^{101}$→Glu was observed in SFM3. *S. dysenteriae* mutants SDM1 and SDM2 exhibited same genetic profiles for *gyrA* to wild type 1169 strain, while SDM2 showed substitutions in *parC*, $Phe^{93}$→Val and $Asp^{110}$→Glu (Table III). No mutation was observed in *gyrB* either in isolates or mutants. The *qnrA* and *aac(6') Ib-cr* genes, which are plasmid mediated quinolone resistance gene, were found absent in all isolates.

*Expression of efflux pump genes*: Relative quantification of *acrA, acrB, acrR* and *tolC* genes showed highly variable expression in clinical isolates (Table IV). Up- and downregulation of these genes was investigated after culture under the same conditions for all isolates. The normalization of the expression values was done against the sensitive isolates *viz.*, 497 for *S. flexneri* and 1169 for *S. dysenteriae* using 16SrRNA as an endogenous control gene. The expression for *S. flexneri* isolates varied between 1.02-3.4 for *acrA*, 0.84-2.84 for *acrB*, 0.41-1.73 for *tolC*, 1.01-1.59 for *acrR* (Table IV). Overall a higher expression level of *acrA* and *acrB* mRNA in the ciprofloxacin resistant isolates (group III) was seen as compared with susceptible isolates (group I). In case of *tolC* and *acrR*, there was no difference in expression among the three groups. The mRNA expression for *S. dysenteriae* isolates varied between 1.0-3.6 for *acrA*, 0.77-5.6 for *acrB*, 0.17-1.05 for *tolC* and 0.24-0.895 for *acrR*.

The spontaneous bacterial mutant colonies were passed in BHI broth in absence and presence of designated concentration of ciprofloxacin and after overnight growth the mRNA expression levels for *acrA, acrB, tolC* and *acrR* were measured (Table V).

**Table III.** Antibiotic susceptibilities, mutation analyses and synergy testing of study isolates

| Strain | No. | Year | Disk diffusion | | MIC (μg/ml) | | | Mutation analyses | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *gyrA* | | | | *ParC* | | |
| | | | NA | CIP | NA | CIP | CIP + CCCP | S-L 83 | D-N 87 | V-A 196 | S-1 80 | F-V 93 | D-E 101 | D-E 110 |
| *S. flexneri* Rough | 618 | 2001 | S | S | 2 | 0.094 | 0.064 | L | D | V | S | F | D | D |
| *S. flexneri* serotype 2a | 497 | 2009 | MS | MS | 6 | 2 | 0.256 | L | G | V | I | V | D | D |
| *S. flexneri* serotype 6 | 44 | 2002 | R | S | 128 | 0.006 | <0.002 | L | D | A | S | F | D | D |
| *S. flexneri* Y variant | 488 | 2007 | R | S | 128 | 0.25 | 0.125 | - | - | - | - | - | - | - |
| *S. flexneri* serotype 3a | 804 | 2007 | R | R | 128 | >32 | >32 | L | N | A | S | V | D | D |
| *S. flexneri* serotype 2a | 710 | 2004 | R | R | 128 | >32 | 0.5 | L | N | V | S | F | D | D |
| *S. flexneri* | 592 | 2006 | R | R | 128 | >32 | 0.256 | L | N | V | S | V | D | D |
| *S. flexneri* X variant | 993 | 2009 | R | R | 128 | >32 | >32 | L | N | A | S | V | D | D |
| *S. dysenteriae* serotype 1 | 1169 | 2002 | S | S | 2 | 0.047 | 0.031 | L | G | V | I | F | D | D |
| *S. dysenteriae* serotype 1 | 677 | 2007 | S | S | 2 | 0.016 | <0.002 | - | - | - | - | - | - | - |
| *S. dysenteriae* serotype 1 | 533 | 2002 | R | MS | 128 | 2 | 0.19 | L | G | V | I | F | D | D |
| *S. dysenteriae* serotype 1 | 115 | 2003 | R | R | 128 | 8 | 0.125 | L | N | V | I | F | D | D |
| *S. dysenteriae* serotype 1 | 622 | 2006 | R | MS | 128 | 4 | 2 | L | N | A | I | V | D | D |
| *S. dysenteriae* serotype 1 | 52 | 2005 | R | R | 128 | >32 | 0.125 | - | - | - | - | - | - | - |
| SFM1(0.25) | - | - | R | S | 128 | >0.25 | 0.08 | L | G | V | I | V | D | D |
| SFM2(4) | - | - | R | MS | 128 | >4 | 0.064 | L | G | V | I | V | D | D |
| SFM3(32) | - | - | R | R | 128 | >32 | 0.19 | L | G | V | I | V | E | D |
| SDM1(0.25) | - | - | R | S | 128 | 0.25 | 0.094 | L | G | V | I | F | D | D |
| SDM2(4) | - | - | R | MS | 128 | >4 | 0.25 | L | G | V | I | V | D | E |

Each value shows average of two replicates. R, Resistant; S, Sensitive; MS, moderately sensitive; CIP, ciprofloxacin; NA, nalidixic acid; I, isoleucine; S, serine; L, leucine; D, aspartic acid; G, Glycine; N, asparagine; V, valine; A, alanine; E, glutamic acid; F, phenylalanine. Values in parentheses represent MIC (μg/ml)

**Table IV.** mRNA expression levels for *acrA, acrB, tolC* and *acrR* genes for *S. flexneri* and *S. dysenteriae* clinical isolates

| Isolate number | Group | Fold expression* | | | |
|---|---|---|---|---|---|
| *S. flexneri* | | *acrA* | *acrB* | *tolC* | *acrR* |
| 618 | I | 1.02 | 1.101 | 1.03 | 1.01 |
| 497 | I | 1.17 | 0.86 | 1.34 | 1.08 |
| 44 | II | 2.25 | 0.84 | 1.45 | 1.13 |
| 488 | II | 1.5 | 1.12 | 1.37 | 1.5 |
| 804 | III | 2.73 | 1.68 | 0.41 | 1.28 |
| 710 | III | 3.4 | 2.84 | 1.73 | 1.19 |
| 592 | III | 3.35 | 2.78 | 1.38 | 1.59 |
| 993 | III | 2.9 | 2.18 | 1.08 | 1.2 |
| *S. dysenteriae* | | | | | |
| 1169 | I | 1 | 1.09 | 1.05 | 0.707 |
| 677 | I | 1.69 | 0.77 | 0.4 | 0.872 |
| 533 | II | 1.58 | 3.81 | 0.5 | 0.895 |
| 622 | II | 1.53 | 1.11 | 0.84 | 0.68 |
| 115 | III | 1.7 | 2.08 | 0.7 | 0.54 |
| 52 | III | 3.6 | 5.6 | 0.17 | 0.246 |

*mRNA expression levels for *S. flexneri* and *S. dysenteriae* were measured relative to that for 618 and 1169 isolates, respectively. Each isolate was measured in duplicate and average values are given.

It was observed that the mRNA expression levels for various efflux pump genes in mutants in absence of ciprofloxacin were comparable to the expression observed in resistant clinical isolates. An up-regulation was observed in the expression for *acrA, acrB* and *tolC* for mutants in presence of ciprofloxacin while expression for *acrR* was comparable either in absence or presence of ciprofloxacin.

*Synergy testing and ciprofloxacin accumulation*: To confirm the role of efflux pump towards ciprofloxacin resistance, the susceptibilities of various clinical isolates and spontaneous mutants were checked against ciprofloxacin in absence and presence of CCCP. Overall, a change in susceptibility was observed with 1.5 to 256-fold difference in MIC values on addition of CCCP when compared to those obtained in CCCP-free medium (Table III). The clinical isolates and mutants susceptible to ciprofloxacin (618, 44, 488, 1169, 677, SFM1 and SDM1) showed almost comparable MIC values (only 2.5-7.5-fold difference), showing that there was not much difference in efflux pump activity in these susceptible isolates. Some resistant clinical isolates (804 and 993) showed similar MIC values (>32) both in presence and absence of CCCP showing that resistance towards ciprofloxacin in these clinical isolates seemed to be independent of efflux pump activity. A few resistant clinical isolates (710, 592, 115 and 52) and spontaneous mutants (SFM2, SFM3 and SDM2) showed 64 to 256-fold change in the MIC values in presence and absence of CCCP. In these isolates, after addition of CCCP, the MICs reverted to levels <1 µg/ml in the susceptible range, thus, showing efflux pump activity to be the main mechanism of ciprofloxacin resistance in these isolates.

Ciprofloxacin accumulation was further carried out to validate efflux activity (Table VI). The efflux pump activity could be demonstrated for strain numbers 44,592,488,52,677, SFM2 and SFM3 whereas no increase in ciprofloxacin accumulation after CCCP uncoupling could be demonstrated for strain numbers 61

**Table V.** Expression of *acrA, acrB, acrR* and *tolC* in spontaneous mutants of *S. flexneri* 497 and *S. dysenteriae* 1169

| Mutant* | Fold expression† | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *acrA* | | *acrB* | | *tolC* | | *acrR* | |
| | -¥ | +# | - | + | - | + | - | + |
| SFM1 | 1.73 | 5.3 | 0.87 | 1.658 | 1.33 | 3.58 | 1.72 | 2.87 |
| SFM2 | 2.3 | 4.4 | 1.1 | 4.403 | 1.39 | 3.93 | 1.81 | 3.11 |
| SFM3 | 2.5 | 8.3 | 0.87 | 8.836 | 2.16 | 5.31 | 1.85 | 2.64 |
| SDM1 | 1.48 | 5.2 | 2.71 | 8.93 | 0.7 | 4.79 | 0.68 | 2.86 |
| SDM2 | 1.87 | 4.5 | 1.89 | 9.317 | 0.3 | 6.11 | 0.378 | 2.81 |

*Mutants included spontaneous mutants of *S. flexneri* 497 - SFM1-0.25 µg/ml; SFM2-4.0 µg/ml and SFM3-32.0 µg/ml and *S. dysenteriae* 1169- SDM1-0.25 µg/ml and SDM2-4µg/ml.¥ Mutants were grown overnight in BHI in absence of ciprofloxacin. # Mutants were grown in BHI in presence of ciprofloxacin. † mRNA expression levels for *S. flexneri* and *S. dysenteriae* were measured relative to that for 497 and 1169 isolates respectively. Each isolate was measured in duplicate and average values are given.

**Table VI.** Ciprofloxacin (CIP) accumulation before and after carbonyl cyanide chlorophenyl hydrazone (CCCP) treatment

| Strain number | Group | Before CCCP (min) | | | After CCCP (min) | | |
|---|---|---|---|---|---|---|---|
| *S. flexneri* | | 0 | 10 | 20 | 0 | 10 | 20 |
| 618 | I | 0.462 | 0.39 | 0.476 | 0.482 | 0.382 | 0.405 |
| 497 | I | 0.754 | 0.478 | 0.517 | 0.698 | 0.51 | 0.235 |
| 44 | II | 0.68 | 0.666 | 0.497 | 0.673 | 0.6 | 0.744 |
| 488 | II | 0.551 | 0.558 | 0.328 | 0.741 | 0.756 | 0.544 |
| 804 | III | 0.551 | 0.473 | 0.69 | 0.844 | 0.843 | 0.814 |
| 710 | III | 0.788 | 0.587 | 0.672 | 0.757 | 0.485 | 0.556 |
| 592 | III | 0.19 | 0.383 | 0.562 | 0.368 | 0.628 | 0.474 |
| 993 | III | 0.558 | 0.498 | 0.744 | 0.616 | 0.481 | 0.418 |
| 497 (0.25) | SFM1 | 0.41 | 0.43 | 0.47 | 0.5 | 0.51 | 0.52 |
| 497 (4) | SFM2 | 0.453 | 0.617 | 0.465 | 0.421 | 0.818 | 0.696 |
| 497(32) | SFM3 | 0.605 | 0.337 | 0.379 | 0.5 | 0.59 | 0.571 |
| *S. dysenteriae* | | | | | | | |
| 677 | I | 0.395 | 0.589 | 0.552 | 0.592 | 1.048 | 0.779 |
| 622 | II | 0.686 | 0.69 | 0.428 | 0.671 | 0.569 | 0.53 |
| 115 | III | 0.168 | 0.531 | 0.337 | 0.625 | 0.540 | 0.464 |
| 52 | III | 0.395 | 0.679 | 0.789 | 0.538 | 0.694 | 0.847 |
| 1169(0.25) | SDM1 | 0.591 | 0.41 | 0.434 | 0.47 | 0.442 | 0.43 |
| 1169 (4) | SDM2 | 0.416 | 0.552 | 0.535 | 0.82 | 0.482 | 0.49 |

Steady-state accumulation values in the absence and presence of the CCCP were calculated at 0, 10, and 20 min. Values in parentheses represent MIC (μg/ml).

8,497,804,710,993,622,115,SDM1 and SDM2. Overall, without CCCP, the pump was active and ciprofloxacin was extruded from cells as measured by decreased accumulation inside the cells. Generally, the pump reached its peak activity at 10 min. After inhibition by CCCP, ciprofloxacin started accumulating. An initial increase in ciprofloxacin level reaching a peak between 10 to 20 min was observed (Table VI).

## Discussion

The fluoroquinolone resistance in *Shigella* spp. is on rise in our region[22-24]. To understand the molecular mechanisms associated with rising fluoroquinolone resistance in *Shigella* spp., laboratory-induced spontaneous mutants and clinical isolates of *S. flexneri* and *S. dysenteriae* exhibiting varied susceptibilities against fluoroquinolones were used to determine the relative contributions of *acrAB-tolC* efflux pumps and mutations in QRDRs towards ciprofloxacin resistance. The partial sequences of *gyrA, gyrB* and *parC* in clinical isolates and mutants were analysed. It is known that atleast two mutations in *gyrA* are required to reach CLSI breakpoint. Substitution of the highly conserved residue Serine-83 (to Leucine) in *gyrA* is the commonest mutation. The alteration of the residue Aspartic acid-87 (to Asparagine) further increases the effect of Serine-83 mutation for resistance increase[13]. All isolates in our study exhibited mutation in *gyrA* at position 83 ($Ser^{83}$→Leu) and a few isolates at position 87 ($Asp^{87}$→Asn/Gly) also. These mutations had already been reported from India[5], Bangladesh and Nepal[25].

Additionally, a novel alteration in *gyrA* at position 196 ($Val^{196}$→Ala) was observed in a few isolates. A mutation in *parC* at position 80 ($Ser^{80}$→Ile) was also detected as previously reported from Kolkata[26]. A new mutation was observed in *parC* at position 93 ($Phe^{93}$→Val). Two other novel substitutions were observed $Asp^{101}$→Glu in SFM3 and $Asp^{110}$→Glu in SDM2. Further studies are needed to establish

association of these polymorphisms with ciprofloxacin resistance. But it is evident that due to the selection pressure exerted by the overuse of fluoroquinolones, new mutations are expected.

Using real-time PCR based relative quantification, expression of *acrA*, *acrB*, *tolC* and *acrR* genes in *Shigella* clinical isolates and spontaneous mutants was studied; *acrA* and *acrB* overexpression was found to be associated with fluoroquinolone resistance. Therefore, *acrAB* overexpression could be an indicator of fluoroquinolone resistance. The *acrAB* overexpression has already been reported from fluoroquinolone resistant clinical isolates of *S. flexneri*[27]. Overall, the level of *acrA* and *acrB* mRNA expression in the ciprofloxacin-resistant isolates was significantly higher than that in the susceptible isolates, which supported that overexpression was associated with resistance in certain clinical isolates of *S. flexneri* and *S. dysenteriae*. Expression of RND efflux pump target genes in laboratory-grown mutants in absence of ciprofloxacin was comparable to ciprofloxacin-resistant clinical isolates (group III). However, when mutants were grown in the presence of ciprofloxacin, *tolC* was also upregulated significantly in addition to *acrA* and *acrB*. This observation suggested that *acrAB-tolC* efflux pumps were further overexpressed in presence of ciprofloxacin, leading to decreased accumulation of antibiotic, and reduced susceptibility.

It is known that *acrAB-tolC* overproduction results in decreased accumulation of antibiotic inside the cells and increased MICs[8]. These *acrAB-tolC* pumps are proton/drug antiporters and utilize the energy of proton-motive force to transport antibiotics out of cell[28]. Therefore, to determine the direct role of efflux pumps overexpression in ciprofloxacin resistance uncoupler CCCP (which inhibits efflux activity) was used. After the disruption of the efflux pump, the MICs for certain ciprofloxacin resistant isolates were almost comparable to ciprofloxacin susceptible isolates. Expectedly, in the ciprofloxacin accumulation assay, a significant decrease in CIP accumulation upon addition of efflux pump inhibitor (CCCP) was observed in a few ciprofloxacin resistant isolates. As evident, efflux pump when operative leads to extrusion of antibiotic and after addition of CCCP antibiotic gets accumulated in the cytosol. This suggested that efflux pump could be one of the factors responsible for the development of resistance in isolates in our region. The role of active efflux using CCCP has also been elucidated previously in *Shigella* spp.[5] and enterotoxigenic *E. coli*[29] from India.

Some of the resistant isolates did not show any decrease in MIC after disruption of efflux activity and exhibited high MIC values. Similar results were obtained with CIP accumulation assay, suggesting strain variations in efflux pump activity. This indicates that resistance in such isolates was due to mutations in QRDRs and independent of efflux activity. In addition to mutations in QRDRs, role of other plasmid-mediated quinolone resistant (*qnr*) determinants[5] and porin proteins[30] needs to be studied. Ciprofloxacin is a hydrophilic quinolone that enters the Gram-negative bacteria by outer membrane porins[13]. Though *qnr* gene was absent in the present isolates there might be other differences due to porins as described for *E.coli*[30].

The present findings suggest that *acrAB* overexpression may be used as an indicator of fluoroquinolone resistance in *S. flexneri* and *S. dysenteriae*. The expression of these efflux pump genes was found to vary in different clinical isolates with varying susceptibilites. This can be due to the presence of single/double or multiple mutations in QRDRs. Therefore, to remove the bias contributed by these mutations, we induced spontaneous mutants resistant to fluoroquinolones. These spontaneous mutants showed that the resistance was contributed mainly by enhanced expression of *acrA* and *acrB* genes, validating the expression in clinical isolates. Further, the use of CCCP abolished efflux activity and some resistant isolates showed comparable MICs to susceptible isolates proving the role of efflux activity towards fluoroquinolone resistance. Some resistant isolates showed no decrease in the MIC values, indicating that resistance was due to a mechanism other than efflux activity. It appears that the mechanism of fluoroquinolone resistance is unique to each isolate and is due to either a single or combination of mutations in QRDRs and/ or efflux activity.

## Acknowledgment

This work was financially supported by the Indian Council of Medical Research (ICMR), New Delhi, India.

## References

1. Chu C, Su LH, Chu CH, Baucheron S, Cloeckaert A, Chiu CH. Resistance to fluoroquinolones linked to *gyrA* and *parC* mutations and overexpression of *acr AB* efflux pump in *Salmonella enterica* serotype Choleraesuis. *Microb Drug Resist* 2005; *11* : 248-53.
2. Kim JY, Kim SH, Jeon SM, Park MS, Rhie HG, Lee BK. Resistance to fluoroquinolones by the combination of target site mutations and enhanced expression of genes for efflux

pumps in *Shigella flexneri* and *Shigella sonnei* strains isolated in Korea. *Clin Microbiol Infect* 2008; *14* : 760-5.

3. Oethinger M, Kern WV, Jellen-Ritter AS, McMurry LM, Levy SB. Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in *Escherichia coli* in the absence of the *acrAB* efflux pump. *Antimicrob Agents Chemother* 2000; *44* : 10-3.
4. Poole K. Efflux-mediated multiresistance in Gram-negative bacteria. *Clin Microbiol Infect* 2004; *10* : 12-26.
5. Pazhani GP, Niyogi SK, Singh AK, Sen B, Taneja N, Kundu M, *et al*. Molecular chararcterization of multidrug resistant *Shigella* species isolated from epidemic and endemic cases of shigellosis in India. *J Med Microbiol* 2008; *57* : 856-63.
6. Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol Rev* 1997; *61* : 377-92.
7. Pu XY, Pan JC, Wang HQ, Zhang W, Huang ZC, Gu YM. Characterization of fluoroquinolone-resistant *Shigella flexneri* in Hangzhou area of China. *J Antimicrob Chemother* 2009; *63* : 917-20.
8. Saier MH Jr, Paulsen IT, Sliwinski MK, Pao SS, Skurray RA, Nikaido H. Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. *FASEB J* 1998; *12* : 265-74.
9. Van Bambeke F, Balzi E, Tulkens PM. Antibiotic efflux pumps. *Biochem Pharmacol* 2000; *60* : 457-70.
10. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. *Drugs* 2009; *69* : 1555-623.
11. Zgurskaya HI. Multicomponent drug efflux complexes: architecture and mechanism of assembly. *Future Microbiol* 2009; *4* : 919-32.
12. Clinical and Laboratory Standards Institute (CLSI). *Performance standards for antimicrobial susceptibility testing,* 20th informational supplement M100-S20. Wayne, PA, USA: CLSI; 2010.
13. Heisig P, Tschorny R. Characterization of fluoroquinolone-resistant mutants of *Escherichia coli* selected *in vitro*. *Antimicrob Agents Chemother* 1994; *38* : 1284-91.
14. Rahman M, Mauff G, Levy J, Couturier M, Pulverer G, Glasdorff N, *et al.* Detection of 4-quinolone resistance mutation in *gyrA* gene of *Shigella dysenteriae* type 1 by PCR. *Antimicrob Agents Chemother* 1994; *38* : 2488-91.
15. Gharib AA, Norhan K Abd El-Aziz. Molecular an analysis of quinolone resistance-determining regions in avian pathogenic *Escherichia coli*. *Int J Adv Res* 2013; *1* : 145-57.
16. Vila J, Ruiz J, Goni P, De Anta MT. Detection of mutations in *parC* in quinolone resistant clinical isolates of *E.coli*. *Antimicrobial Agents Chemother* 1996; *40* : 491-3.
17. Wang M, Sahm DF, Jacoby GA, Hooper DC. Emerging plasmid-mediated quinolone resistance associated with the *qnr* gene in *Klebsiella pneumoniae* clinical isolates in the United States. *Antimicrob Agents Chemother* 2004; *48* : 1295-9.
18. Chomczynski, P, Mackey K. Modification of the Trizol reagent procedure for isolation of RNA from polysaccharide and proteoglycan-rich sources. *Biotechniques* 1995; *19* : 942-5.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001; *25* : 402-8.
20. Pournaras S, Maniati M, Spanakis N, Ikonomidis A, Tassios PT, Tsakris A, *et al*. Spread of efflux pump-overexpressing, non-metallo-beta-lactamase-producing, meropenem-resistant but ceftazidime-susceptible *Pseudomonas aeruginosa* in a region with $bla_{VIM}$ endemicity. *J Antimicrob Chemother* 2005; *56* : 761-4.
21. Charvalos E, Tselentis Y, Hamzehpour MM, Köhler T, Pechere JC. Evidence for an efflux pump in multidrug-resistant *Campylobacter jejuni*. *Antimicrob Agents Chemother*1995; *39* : 2019-22.
22. Taneja N, Mewara A, Kumar A, Verma G, Sharma M. Cephalosporin-resistant *Shigella flexneri* over 9 years (2001-09) in India. *J Antimicrob Chemother* 2012; *67* : 134-53.
23. Taneja N. Changing epidemiology of Shigellosis and emergence of ciprofloxacin-resistant Shigellae in India. *J Clin Micobiol* 2007; *45* : 678-9.
24. Taneja N, Lyngdoh V, Vermani A, Mohan B, Rao P, Singh M, *et al*. Re-emergence of multi-drug resistant Shigella dysenteriae with added resistance to ciprofloxacin in north India and their plasmid profiles. *Indian J Med Res* 2005; *122* : 348-54.
25. Talukder KA, Khajanchi BK, Islam MA, Dutta DK, Islam Z, Safa A, *et al*. Genetic relatedness of ciprofloxacin res*istant Shigella dysenteriae* type 1 *s*trains isolated in South Asia. *J Antimicrob Chemother* 2004; *54* : 730-4.
26. Dutta S, Kawamura Y, Ezaki T, Nair GB, Iida K, Yoshida S. Alteration in the gyrA subunit of DNA gyrase and the parC subunit of topoisomerase IV in quinolone-resistant *Shigella dysenteriae* serotype 1 clinical isolates from Kolkata, India. *Antimicrob Agents Chemother* 2005; *49* : 1660-1.
27. Yang H, Duan G, Zhu J, Lv R, Xi Y, Zhang W, *et al*. The AcrAB-TolC pump is involved in multidrug resistance in clinical *Shigella flexneri* isolates. *Microb Drug Resist* 2008; *14* : 245-9.
28. Paulsen IT, Brown MH, Skurray RA. Proton-dependent multidrug efflux systems. *Microbiol Rev* 1996; *60* : 575-608.
29. Pazhani GP, Chakraborty S, Fujihara K, Yamasaki S, Ghosh A, Nair GB, *et al*. QRDR mutations, efflux system and antimicrobial resistance genes in enterotoxigenic *Escherichia coli* isolated from an outbreak of diarrhoea in Ahmedabad, India. *Indian J Med Res* 2011; *134* : 214-23.
30. Lou H, Chen M, Black SS, Bushell SR, Ceccarelli M, Mach T, *et al*. Altered antibiotic transport in OmpC mutants isolated from a series of clinical strains of multi-drug resistant *E. coli*. *PLoS One* 2011; *6* : e25825.

*Reprint requests*: Dr Neelam Taneja, Additional Professor, Department of Medical Microbiology, Postgraduate Institute of Medical Education & Research, Chandigarh 160 012, India
e-mail: drneelampgi@yahoo.com