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Detection of Novel *gyrB* Mutation in Fluoroquinolone-Resistant *Salmonella* and *Escherichia coli* using PCR-RFLP

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Abstract

Background Emergence of fluoroquinolone resistance in gut pathogens is a cause of concern. Resistant to quinolone is mainly due to the point mutations at the quinolone-resistance determining regions (QRDR). The aim of the study was to develop polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP) to detect QRDR mutations in *gyrA* and *gyrB* regions in enteric pathogens.

Methodology PCR-RFLP was done for *gyrA* 83 region using *HinfI* and for gyrB 447 using *AcuI* for fluoroquinolone resistant and susceptible gut pathogens. The products were also sequenced to confirm the presence of restriction sites.

Results In this study, a PCR-RFLP technique was developed to detect *gyrA* 83 mutations in *Salmonella* typhi and *Escherichia coli*. A first of its kind PCR-RFLP was also developed to detect *gyrB* 447 mutation using a restriction enzyme *AcuI*. Restriction digestion of *gyrA* using *Hin*fI resulted in three bands for resistant *S*. typhi isolates due to the presence of mutation at *gyrA* 83 and four bands were seen for sensitive *S*. typhi isolates, while two bands for resistant and three bands were seen in sensitive *E. coli* isolates. Similarly, restriction digestion of *gyrB* using *Acu*I resulted in no digestion for resistant *S*. typhi isolates and two bands for resistant *E. coli* isolates. This suggest that there is mutation at *gyrB* 447 region of*E. coli*, while no mutation was found in *S*. typhi isolates.

Conclusion The PCR-RFLP developed in the present study could successfully detect *gyrA* 83 and *gyrB* 447 mutations in fluoroquinolone-resistant *S*. typhi and *E. coli*. The technique can be efficiently used in epidemiological studies instead of a cost-intensive sequencing method to detect the status of multiple point mutations in gut pathogens.

Keywords
- *Escherichia coli*
- fluoroquinolone resistance
- QRDR mutations
- *Salmonella* typhi

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Introduction

The term antibiotic is arguably the most common and powerful term in today's medical microbiology. Antibiotics when used suitably can save lives. Combined with their high productivity and low secondary effect, usage of antibiotics has been highly exploited leading to the emergence of antibiotic resistance. Antibiotic resistance is increasing in high levels globally, bringing about higher medical costs and fatality rate. *Salmonella* and *Escherichia coli* are the gram-negative human enteric pathogens capable of causing mild-to-severe infections.[1–3] It is chiefly contracted by ingestion of contaminated food or water. Fluoroquinolones are the drug of choice since several decades for the infection caused by these enteric pathogens.[4,5] The most frequently used fluoroquinolones are ciprofloxacin, levofloxacin, and moxifloxacin. Fluoroquinolones impair DNA replication when administered at a lower concentration or induce cell death when given at a lethal concentration by targeting DNA gyrase and topoisomerase IV, hence inhibiting their influence of supercoiling within the cell. The potency differs in different bacteria. Binding of fluoroquinolones to DNA gyrase or topoisomerase IV prevents religation of the DNA substrate. This DNA, enzyme, and drug complex formation is an important step in the fluoroquinolone killing pathway. However, as a result of their extensive use, the emergence of fluoroquinolone resistance has become increasingly prevalent in recent times.[5,6] Resistance to fluoroquinolones in Enterobacteriaceae can be mainly attributed to the development of spontaneous mutations in the topoisomerase II or topoisomerase IV regions, presence of plasmid mediated quinolone resistance genes, or overexpression of efflux pumps.[7,8] However, point mutations in the QRDRs (quinolone resistance determining regions) such as *gyrA*, *parC*, *gyrB,* and *parE* regions are the most commonly encountered mechanisms in fluoroquinolone resistance in bacterial pathogens.[7,9] QRDR mutations modify structure of the target protein, thereby altering the binding affinity of fluoroquinolone to enzyme, leading to drug resistance.[10] These mutations can be precisely detected by sequencing and polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP) or MAMA-PCR.[11] In this study, we have developed a rapid PCR-RFLP technique for the first time to detect *gyrA* (Ser 83) and *gyrB* (Ser 447) mutations in fluoroquinolone resistant *Salmonella* typhi and *E. coli*.

Materials and Methods

Bacterial Isolates

A total of 10 *S.* typhi isolates and 30 *E. coli* isolates were revived from the institutional repository. Ten microliters of the samples were used to inoculate into 5 mL LB (Luria Bertani) Broth (HiMedia Laboratories Pvt. Ltd., India) and incubated at 37°C with shaking until light-to-moderate turbidity was obtained. The turbidity was compared with that of standard 0.5 McFarland unit. These freshly revived overnight grown cultures were then subjected to DNA extraction for further studies.

Antimicrobial Susceptibility Assay

Antimicrobial susceptibility test was performed for all the isolates using antibiotics such as nalidixic acid (30 µg) and ciprofloxacin (5 µg) on Mueller Hinton (MH) agar (HiMedia, Laboratories Pvt. Ltd., India) using disc diffusion method as described by standard guidelines.[12] Bacterial culture lawns were prepared on well-dried MH agar plates by swabbing from cultures grown for 18 to 24 hours with 0.5 McFarland unit in 5 mL LB broth. Antibiotic discs were aseptically placed on the medium surface containing the lawn of bacteria and were incubated at 37°C for 16 to 18 hours. The isolates were then designated as sensitive, intermediate, or resistant based on the zone of inhibition by comparing with the interpretive chart of Kirby-Bauer. *E. coli* ATCC 25922 and *S.* typhimurium (ST14028) were used as quality control strains.

PCR Assay

The DNA from all the bacterial isolates was extracted and subjected to PCR for checking the presence of genus-specific gene *invA* for *Salmonella* and *uidA* gene for *E. coli* (►**Table 1**).[13,14] The PCR was performed in a thermal cycler. The 30 µL volumes of master mix consist 3 µL of 10X buffer, 50 µM concentrations each of dNTPs, 10 pmol of forward and backward primers and 1.0 U of *Taq* DNA polymerase (HiMedia Laboratories Pvt Ltd., India), with 2.0 µL of template DNA. The reaction was done at an annealing temperature of 64°C. The PCR amplified products were subjected to electrophoresis on ethidium bromide stained 1.5% agarose gel, and bands were visualized using Gel Documentation system (BioRad, California, United States).

Table 1 Oligonucleotide primers used in the study

Gene name	Oligonucleotides (5′-3′)	Product size (bp)	Annealing temperature
invA	F - GTGAAATTATCGCCACGTTCGGGCAA R - TCATCGCACCGTCAAAGGAACC	284 bp	64°C
uidA	F - AAAACGGCAAGAAAAAGCAG R - ACGCGTGGTTACAGTCTTGCG	146 bp	63°C
gyrA	F - GACCTTGCGAGAGAAATTACAC R - GATGTTGGTTGCCATACCTACG	540 bp	57°C
gyrB	F - CAAACTGGCGGACTGTCAGG R - TTCCGGCATCTGACGATAGA	346 bp	55°C

PCR- RFLP for the Detection of *gyrA* and *gyrB* Point Mutations

Fluoroquinolone-resistant isolates were chosen for PCR-RFLP to detect the presence of mutation in the QRDR regions. PCR-PFLP was targeted toward restriction sites at *gyrA* 83 and *gyrB* 447 positions using two different restriction enzymes *Hin*fI and *Acu*I, respectively. PCR was performed as described earlier using forward and reverse primers of *gyrA* and *gyrB*. The primers used are listed in ►**Table 1**. PCR reactions were performed using a thermal cycler (Bio-Rad, California, United States) with an annealing temperature of 55°C for 30 seconds. The obtained PCR products were digested using respective enzymes. Enzyme digestion was performed in a 20 µL mixture containing 16 µL (0.1–0.5 mg) of the PCR product, 0.5 µL (2 IU) of enzyme, 2 µL of 10X buffer, and 1.5 µL of sterile ultrapure water at 37°C for 2 hours followed by termination of enzyme activity at 65°C for 10 minutes using a dry bath. The digested PCR products were electrophoresed and visualized using a Gel Documentation system (Bio-Rad, California, United States).

DNA Sequencing

The *gyrA* and *gyrB* PCR products of fluoroquinolone-resistant *E. coli* and *S.* typhi were sent for sequencing (Biokart India Pvt Ltd) and the obtained sequences were analyzed using NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequences of corresponding genes were obtained from the Expasy translate tool (https://web.expasy.org/translate/) and the sequences were submitted to GenBank.

Results

Antibiogram Analysis

The *S.* typhi isolates were positive for *invA* and *E. coli* isolates were positive for *uidA* gene. The strains were also resistant to both nalidixic acid and ciprofloxacin.

PCR-RFLP

PCR-RFLP was done for all the isolates for the detection of QRDR mutation in *gyrA* and *gyrB* regions. Restriction digestion by *Hin*fI for *gyrA* resulted in three bands (337 bp, 149bp, and 54 bp) for resistant *S.* typhi isolates (S16 & S20) due to the presence of mutation at *gyrA* 83 position and four bands (238 bp, 149 bp, 99 bp, and 54 bp) for sensitive *S.* typhi isolates while two bands (337 bp and 203 bp) for resistant (J4) and three bands (239 bp, 202 bp, and 99 bp) were seen in sensitive *E. coli* isolates. Similarly, restriction digestion by the restriction enzyme *Acu*I for *gyrB* resulted in no digestion for resistant *S.* typhi isolates and two bands (142 bp and 204 bp) for nine resistant *E. coli* isolates. These suggest that there is mutation at *gyrB* 447 region of *E. coli,* while no mutation was found in *S.* typhi isolates (►**Fig. 1**).

DNA Sequencing

The resistant isolates (S16, S20, J4) that showed mutation at *gyrA* 83 region were selected for sequencing for further confirmation (►**Figs. 2** and **3**). The sample S16 showed *gyrA* mutation at position 83 with an amino acid substitution for serine with tyrosine (TCG to TAC). The *gyrA* region of fluoroquinolone sensitive *S.* typhi contains 3 restriction sites for the enzyme *Hin*fI. In the case of resistant *S.* typhi (S16), there is an abolishment of one of the sites at the 83rd position resulting in only three bands of 337 bp, 149 bp, and 54 bp upon digestion. However, in the case of *E. coli* the sample J4 showed mutation at position 83 with an amino acid substitution for serine with leucine (TCG to TTG). The *gyrA* region of fluoroquinolone sensitive *E. coli* contains two restriction sites for the enzyme *HinfI*. In the case of resistant *E. coli* (J4), there is an abolishment of one of the sites at the 83rd position resulting in only two bands of 337 bp and 203 bp upon digestion. Similar mutations were seen in other *E. coli* isolates (CP092819, CP055022, CP091169) and in *S.* typhi (CP030749, CP082409, CP074335) for *gyrA.*

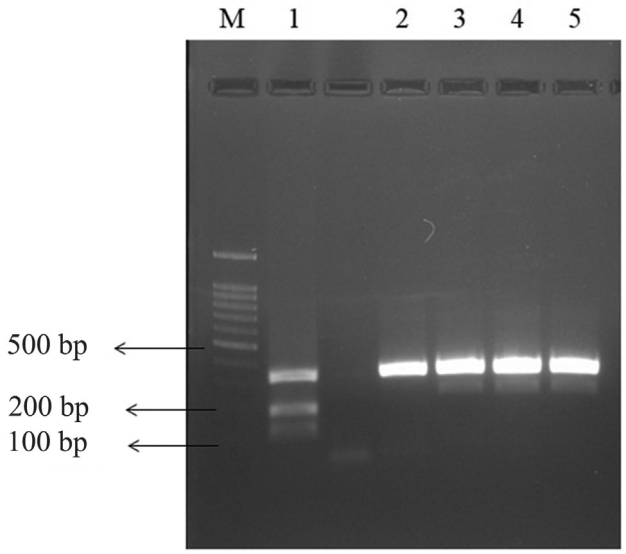

Fig. 1 Agarose gel electrophoresis of polymerase chain reaction-restriction fragment length polymorphism assay products of *gyrB* at 447th position with mutation. Lane M: 100bp marker, Lane 1: fluoroquinolone-resistant *Escherichia coli* (J4), Lane 2–5: fluoroquinolone-resistant *Salmonella* typhi isolates.

The samples S16, S20, and J4 were also sequenced to detect mutation in *gyrB* region (►**Figs. 4** and **5**). Both the *S.* typhi (S16 and S20) strains showed silent *gyrB* mutation at position 447 as the codon AAG is changed to AAA, wherein both codes for the amino acid Lysine. The enzyme could not digest the *gyrB* of *S.* typhi (S16 and S20) with AAA due to abolishment of restriction site for the enzyme *Acu*I. However, in the case of *E. coli* (J4) the isolate did not have any mutation at 447th position of *gyrB,* thereby allowing *AcuI* enzyme to digest the region. All the sequences are submitted to GenBank (Accession numbers: MZ826340, MZ826341, MZ826342, MZ826343). Similar mutations were seen in *gyrB* at 447th position in other *E. coli* isolates (CP095856, CP095446, CP095454) and in other bacterial pathogens such as *Klebsiella pneumoniae* (CP091152) and *S.* typhi (CP034233, CP023470, LS483465).

Discussion

Gastrointestinal bacteria like *Salmonella* and *E. coli* are important human pathogens capable of causing severe

Fig. 2 DNA sequence analysis of representative fluoroquinolone-resistant *Salmonella* typhi (S16) showing mutation at *gyrA* gene at amino acid position 83 with restriction sites for the enzyme *Hin*fI.

Fig. 3 DNA sequence analysis of representative fluoroquinolone-resistant *Escherichia coli* isolate (J4) showing mutation at *gyrA* gene at amino acid position 83 with restriction sites for the enzyme *Hinfl.*

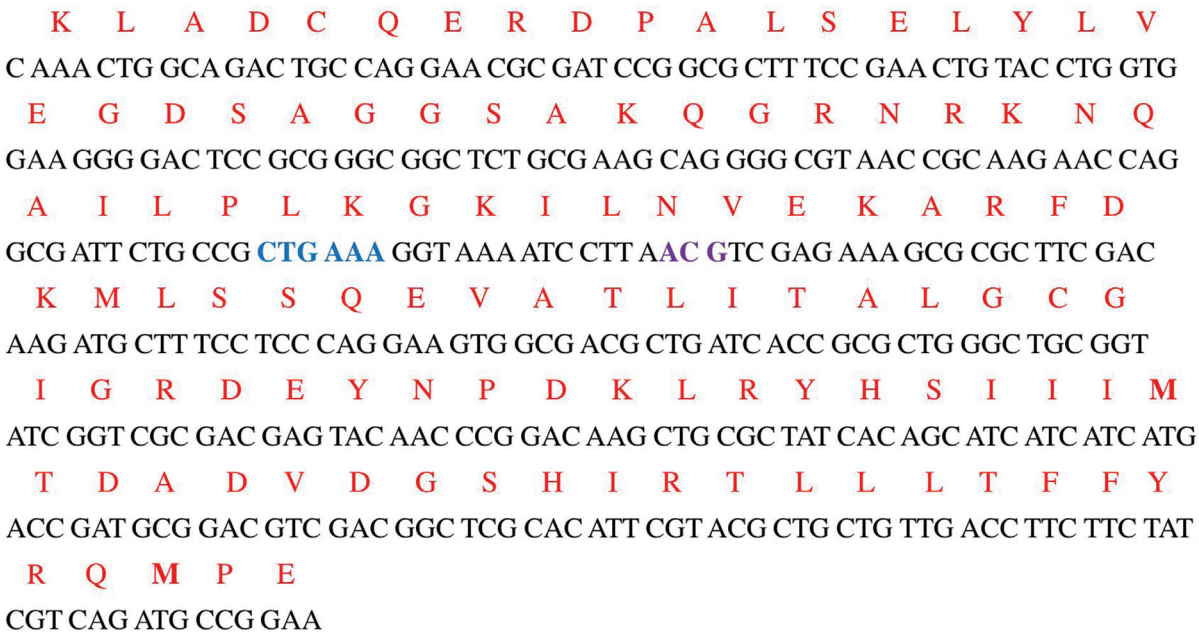

Fig. 4 DNA sequence analysis of representative fluoroquinolone-resistant *Salmonella* typhi (S16) showing mutation at *gyrB* gene at amino acid position 447 with restriction site for the enzyme *AcuI*.

Fig. 5 DNA sequence analysis of representative fluoroquinolone-resistant *Escherichia coli* isolate (J4) showing mutation at *gyrB* gene at amino acid position 447 with restriction site for the enzyme *AcuI*.

infections worldwide including urinary tract infections and gastroenteritis. These infections are usually treated with β-lactams or fluoroquinolones. However, resistance to these antibiotics is a major problem throughout the world. In the present study, a PCR-RFLP technique was developed to detect QRDR mutations in fluoroquinolone-resistant *E. coli* and *Salmonella* isolates. Majority of *S.* typhi isolates used in the study showed resistance to nalidixic acid. This is in agreement with several studies that showed most of the *S.* typhi clinical isolates of maximum resistance toward nalidixic acid.[15] Though multiple point mutations are observed in the QRDR regions of fluoroquinolone resistant strains, the *gyA* 83 and *parC* 80 mutations are most commonly encountered changes.[11,16–18] The *gyrA* 83 PCR-RFLP developed in this study could successfully detect a point mutation at *gyrA* 83 region of *S.* typhi and *E. coli*. This was further confirmed by sequencing of gyrase A region. This is in similar with earlier studies, wherein 94% of the isolates showed *gyrA* 83 mutation in PCR-RFLP.[11,19,20] Since fluoroquinolone resistant is attributed to multiple mutations, it was out interest to look for novel point mutations in other regions such as *gyrB*. The most common mutations in the *gyrB* region are *gyrB* 426, *gyrB* 447, *gyrB* 464, and *gyrB* 466.[21] However, all these mutations cannot be detected by PCR-RFLP. Nevertheless, the first of its kind *gyrB* 447 PCR-RFLP developed in this study could detect a point mutation at *gyrB* 447 region. However, upon sequencing the mutation was found to be silent with little or no effect on the activity of the enzyme. Further

studies are essential to confirm the relevance of *gyrB* 447 mutation in fluoroquinolone-resistant *S*. typhi and *E. coli*.

Conclusion

The PCR-RFLP developed in the present study could detect *gyrA* 83 and for the first time *gyrB* 447 mutations in fluoroquinolone-resistant *S*. typhi and *E. coli*. Though in the present study none of isolates showed other mutations, it is important to screen these regions to understand the status of QRDR mutations among fluoroquinolone resistant isolates. In addition, the PCR-RFLP developed in the study was found to be an ideal technique to screen large number of drug-resistant bacterial pathogens during epidemiological investigations.

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Conflict of interest
None declared.

Compliance with Ethical Standards
The isolates used in the study were obtained from the institutional repository. However, all the ethical standards have been included in the study before performing the experiments.

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