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ORIGINAL RESEARCH

Molecular Evaluation of High Fluoroquinolone Resistant Genes in Endemic Cases of Shigellosis, Northeast Part of Karnataka, India

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Abstract

OBJECTIVES Shigellosis is an acute infection of the intestine caused by bacteria in the genus *Shigella* and also an important cause of diarrhea in developing countries. This study was carried out to find the extent and nature of the emerging resistance in north part of Karnataka, India, and surrounding region with huge population, and also focused on the molecular mechanism of development of resistance against different generations of fluoroquinolones and explored the diversity of restriction endonucleases; we also tried to establish the significance of reduced minimal inhibitory concentrations (MIC) values.

METHODS A total of 32 multidrug-resistant *Shigella* species (isolated from infants' stools) were subjected to MICs of fluoroquinolone-resistant isolates done by both broth dilution and E-test method. The genes implicated in resistance to fluoroquinolone generations ciprofloxacin, ofloxacin, and gatifloxacin (gyrA, gyrB, parC, and parE) were amplified using polymerase chain reaction (PCR) method and restriction digestion analysis of PCR product were performed using PvuI and HaeII enzymes.

FINDINGS Fluoroquinolone-resistant *Shigella* species (n = 32) comprising *S dysenteriae, S flexneri,* and *S sonnei* were selected for MIC; 90.6% (29/32), 93.75% (30/32), and 93.75% (30/32) of isolates were ciprofloxacin, ofloxacin, and gatifloxacin resistant and showed the MIC range from 4-128 μg/mL. The PCR amplification results were positive for all species and asserted the presence of gyrA, gyrB, parC, and pare and sizes of the amplified products. The restriction banding patterns of amplified resistant genes were employed to detect differences among the *Shigella* species.

CONCLUSIONS The present study found that the genetic basis and its characterization of fluoroquinolone resistance in *Shigella* isolates was considered for the common resistant genes, namely, gyrA, gyrB, parC, and pare, and had mutations at position 83 of gyrA and at position 80 of parC of the quinolone-resistant determining regions and associated molecular mechanism. Our study beneficial in identification of the causative agents of the infections, careful control and cautions use of antibiotics must be promoted, particularly to monitor the emergence of isolates that are fully resistant to fluoroquinolones.

KEY WORDS *Shigella* species, minimal inhibitory concentration (MICs), fluoroquinolone genes, QRDRs, gyrA, gyrB, parC, and parE, genomic DNA, PCR amplification, restriction digestion and shigellosis

This Medical Biotechnology and Phage Therapy Laboratory, Department of Biotechnology approved ethical clearance by Institutional Clearance Certificate (IECC) for in vitro and in vivo studies.
The authors declare no conflict of interest.
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INTRODUCTION

Acute gastroenteritis is one of the leading causes of illness and death in infants, children, and immune-compromised and aged individuals throughout the world, Like *Campylobacter* spp and *Vibrio cholera*, *Shigella* spp have managed to survive the antibiotic era via ingenious mechanisms of resistance. Unlike diarrhea caused by *Campylobacter jejuni*, shigellosis may occur in epidemic form, causing considerable morbidity and mortality, especially in developing nations such as India. Asia, Africa, and Latin America had an estimated 2.5 million deaths each year in children younger than 5 years of age.[1-4] Shigellosis is primarily a disease of resource-poor, crowded communities that do not have adequate sanitation or safe water and where disease rates may be high. Among the enteric pathogens, *Shigella* species are of particular concern as causes of food poisoning, abdominal tenderness, and gastroenteritis.[5] Although more prevalent in developing countries, shigellosis is a worldwide problem.[6,7] *Shigella sonnei* is predominating in Europe and the United States, and *S dysenteriae* and *S flexneri* are more prevalent in Asian and African countries.[8]

The traditional antishigellosis drugs chloramphenicol, ampicillin, and sulfamethoxazole have become outdated. In recent years, fluoroquinolones, especially ciprofloxacin, have been very successful in combating shigellosis, but unfortunately, resistant strains have emerged. The emergence of high-level ciprofloxacin resistance in *Shigella* spp has also been reported in India.[9,10] Among the *Shigella* species, a major therapeutic challenge required to control this disease. One of the reasons for emergence of multidrug-resistant *Shigella* spp is the unique capability of the pathogen to acquire resistance factors (transmissible genes) from the environment or from other bacteria. Antimicrobial resistance is usually conferred by certain genes. A large number of resistance-related genes have reported for each group of antimicrobials. It is impossible to study all the reported genes, so most commonly isolated predominant isolates and reported genes were selected for this study. Fluoroquinolones, especially ciprofloxacin, are the most commonly used drugs for shigellosis treatment. Reduced susceptibility to the fluoroquinolone group of antibiotics is usually linked with point mutations in the bacterial target genes gyrA, gyrB encoding DNA gyrase and parC, parE encoding DNA topoisomerase IV.

The aim of the present study was to find the extent and nature of the emerging resistance in Gulbarga (north part of Karnataka), India, and the surrounding region, with a huge population; we also focused on molecular mechanisms of development of resistance against different generations of fluoroquinolones—ciprofloxacin, ofloxacin, and gatifloxacin—and explored the diversity of restriction endonucleases to determine if restriction endonuclease production is useful for epidemiological studies and correlates the occurrence of restriction endonucleases with serotype antibiotic resistance in local collected clinical isolates of *Shigella* species, with priority on predictive value of fluoroquinolone's resistance. We also tried to establish the significance of reduced minimal inhibitory concentrations (MIC) values.

MATERIALS AND METHODS

Bacterial Isolation. The clinical isolates used in the present study were isolated from infants' stools. They were identified up to species level on the basis of colonial and Gram stain morphology, carbohydrate fermentation, and indole test, and final confirmation was performed with specific polyvalent antisera (Deben Diagnostics Ltd, Ipswich, Suffolk, UK).

Among 43 previously isolated *Shigella* isolates,[10] 32 multidrug-resistant isolates were considered for assessment in the present study. For each study, an overnight culture was diluted in fresh tryptic soy broth and further incubated to ensure exponential growth conditions.

Antibiotic Susceptibility Test. Resistance level and synergistic activity of previously isolated *Shigella* isolates to various antibiotics (Hi-Media Private Ltd., Mumbai, India) were determined by the microdilution method as described by the Clinical and Laboratory Standards Institute 2010. *Shigella dysenteriae* 13313 and *Shigella flexneri* 12022 obtained from American type culture collection were used as the reference and standard control organism in all the susceptibility procedures.

Minimal Inhibitory Concentration. Minimum inhibitory concentration was determined to evaluate the phenotypic antimicrobial resistance of a strain to a certain antibiotic. MIC was defined as the lowest antibiotic concentration that resulted in no visible growth. The antibiotic (ciprofloxacin, ofloxacin, and gatifloxacin) phenotype was determined for all 32 *Shigella* isolates.

E-strip/Hi comb test. Antibiotic E-strips, having a gradient concentration of 0.001-64 μg/mL, were used in accordance with the protocols from the manufacturer (Himedia, HiComb MIC test,

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Table 1. Primers Used for Identification of Specific Genes in *Shigella* Species

Gene Encoding Virulence Factor	Primer Pair	Oligonucleotide Sequence (5^1 to 3^1)	Location with Gene	Antimicrobial Agent/Genetic Element	Size of the Amplified Product (bp)	Accession Number
gyrA	gyrA-F	5^1-TAC ACC GGT CAA CAT TGA GG-3^1	nt 24-43	Fluoroquinolone	648	AY648051
	gyrA-R	5^1-TTA ATG ATT GCC GCC GTC GG-3^1	nt652-671			
gyrB	gyrB-F	5^1-TGA AAT GAC CCG CCG TAA AGG-3^1	nt1170-1190	Fluoroquinolone	309	AY648052
	gyrB-R	5^1-GCT GTG ATA ACG CAG TTT GTC CGGG-3^1	nt1455-1479			
parC	parC-F	5^1-GTC TGA ACT GGG CCT GAA TGC-3^1	nt 147-167	Fluoroquinolone	248	AY648053
	parCR	5^1-AGC AGC TCG GAA TAT TTC GAC AA-3^1	nt373-395			
parE	parE-F	5^1-ATG CGT GCG GCT AAA AAA GTG-3^1	nt1066-1086	Fluoroquinolone	290	AY648054
	ParE-R	5^1-TCG TCG CTG TCA GGA TCG ATA C-3^1	nt1334-1355			

Mumbai, India). Briefly, after overnight growth on tryptic soy broth, the organisms were suspended in tryptone broth with an optical density of 0.1 at 600 nm. The suspension was used to inoculate on Muller-Hinton agar plates by swabbing them with a cotton swab. After drying for 15 minutes, the E-test strips were placed on the plates and the plates were incubated for 18 hours. The MIC was interpreted as the point of intersection of the inhibition ellipse with the E-test strip edge.

Broth Dilution Method. MIC was determined by broth dilution technique by following the reference standard established by CLSI 2010. Serial 2-fold dilutions of the antibacterial agents were inoculated with an overnight culture at a final concentration of 10^{4-6} colony forming units (CFU/mL).

Extraction of DNA. Total genomic DNA from the *Shigella* isolates was extracted from overnight cultures in tryptic soy broth by the conventional phenol-chloroform method according to Sambrook 1989.[11] DNA integrity was checked on 1% agarose gel, purity was determined by A260/A280 ratio, and quantification was done with a Nanodrop spectrophotometer (ND-1000 Version 3.5, Wilmington, USA).

Genotypic Detection for Antibacterial Genes. Isolates of *Shigella* spp resistant to the fluoroquinolone are often recovered from the infant's stool samples. Amplification of fluoroquinolone-resistant genes from genomic DNA was carried out by polymerase chain reaction (PCR) and agarose electrophoresis. The PCR assays were achieved pointing 4 genes correlate to the fluoroquinolone drugs listed in the Table 1. Selection of the gene was done on the basis of highest reported prevalence worldwide, including India. The amplification condition was carried out using 20-µL reaction cocktail in MJ Research PTC 100 thermal cycler (Watertown, NY); the PCR program was standardized for 30 cycles and performed using modified conditions as in Table 2.[12] The amplified product was run to 1% (W/V) agarose gel electrophoresis with 1 kb DNA as a standard molecular weight marker; the electrophoretic profile was cited using photo gel documentation system (Vilber Lourmat, Marne La Valleé, France) and a photograph was printed using a thermal printer.

Designing of Primers for PCR. The sequences of the PCR primers that were evaluated in this study to detect the fluoroquinolone resistance genes in *Shigella* isolates. The PCR primer set designed for

Table 2. Polymerase Chain Reaction Amplification Program Conditions for Fluoroquinolone-Resistant Genes in *Shigella* Species [12]

Process	Temperature (°C)	Time	Cycles
Initial denaturation	94	2 min	1
Denaturation	92	1 min	
Annealing	64	1 min	30
Extension	74	2 min	
Final extension	74	10 min	1
Storage	4	—	

Table 3. The Components of Polymerase Chain Reaction Mixture

Constituents	Quantity (μL)	Final Concentrations
Template DNA	2.0	25 ng
10× Taq DNA assay buffer	2.0	1×
dNTPs	0.4	200 μM each dNTPs
Primer	2.0	20 picomoles
Taq DNA polymerase	0.3	1 unit
Sterile water	13.3	20 μL

the study was developed either based on a sequence available from GenBank using the primer select software program or were obtained from published primer sequences or previously reported primers, as shown in Table 3, that are resistant to fluoroquinolone drugs.[13]

Gene Sequencing and Nucleotide Accession Numbers. For determining the nucleotide sequences of fluoroquinolone resistant determinants, the PCR amplified products were purified using a PCR purification kit and the sequence was commercially sequenced at Medauxin Pvt Ltd, Bangalore, India, and its subsequent analysis was done. The antibiotic-resistant genes identified in the *Shigella* isolates reported in this study have been deposited in the International Nucleotide Sequence Database—that is, in the National Center for Biotechnology Information; the accession numbers are listed in result section.

Restriction Digestion Analysis. Restriction digestion of PCR product was digested using the restriction enzymes PvuI and Hae II. The reaction mixture was composed of 1-3 μg of total DNA as a template. The digestion procedure was executed following the instructions from the manufacturer (Sigma Aldrich, Bangalore, India) for each enzyme. The PCR product was digested with 20 U of the reaction enzyme in the presence of appropriate 1× restriction enzyme buffer. The total volume of the reaction mixture was made up to 50 μL. The contents of the reaction were mixed thoroughly and incubated overnight at 37°C. After incubation, the reaction was terminated by heating the reaction mixture at 65°C for 10 minutes. The digested DNA was analyzed by agarose gel (0.8%) electrophoresis.

RESULTS

Fluoroquinolone-resistant *Shigella* spp (n = 32) comprising *S dysenteriae* (n = 16), *S flexneri* (n = 9), and *S sonnei* (n = 7), previously isolated from infants' stool samples,[10] were considered for evaluation in the present study.

Minimal Inhibitory Concentration. All 32 multidrug-resistant *Shigella* spp were selected for MIC on the basis of resistance pattern to fluoroquinolone antibiotics (ciprofloxacin, ofloxacin, and gatifloxacin) tested among the isolates, as shown in Table 4 and Figures 1 and 2.

Ciprofloxacin. Out of 32 *Shigella* isolates, MICs of 29 (90.6%) isolates were determined by broth as well as agar dilution method, from the total 29 isolates; 12 were *S dysenteriae* (41.4%), 10 *S flexneri* (34.5%), and 10 *S sonnei* (34.5%). All 3 types of *Shigella* isolates had an MIC range from 4-128 μg/mL.

Ofloxacin. MICs of 30 of 32 (93.75%) *Shigella* isolates were determined by E-strip method comprising 17 *S dysenteriae* (56.6%), 6 *S flexneri* (20%), and 7 *S sonnei* (23.3%), and all the isolates had an MIC range from 4-64 μg/mL.

Gatifloxacin. Out of 32, 30 (93.75%) *Shigella* isolates were resolved by E-strip method, including 14 *S dysenteriae* (46.6%), 9 *S flexneri* (30%), and 7 *S sonnei* (23.3%), and isolates had an MIC range from 8-64 μg/mL.

Extraction of DNA. The selected multidrug-resistant *Shigella* isolates were used for the isolation of genomic DNA and characterization. The molecular weight of genomic DNA was found to be approximately >10 kb (Fig. 3); isolated DNA of all effective isolates were quantified in solution by

Table 4. Minimal Inhibitory Concentration of Fluoroquinolone Antibiotic Resistance Against Different *Shigella* Species

Shigella Species	No. of Isolates for Each Antibiotic and MIC Value μg/mL		
	Ciprofloxacin (4-128 μg/mL)	Ofloxacin (4-64 μg/mL)	Gatifloxacin (8-64 μg/mL)
Shigella dysenteriae	12	17	14
Shigella flexneri	10	6	9
Shigella sonnei	7	7	7
Total	29 (90.62%)	30 (93.75%)	30 (93.75%)

E-test strip MIC range 0.016-256 μg/mL; no zone observed and MIC considered > the highest value on the strip.
MIC, minimal inhibitory concentration.

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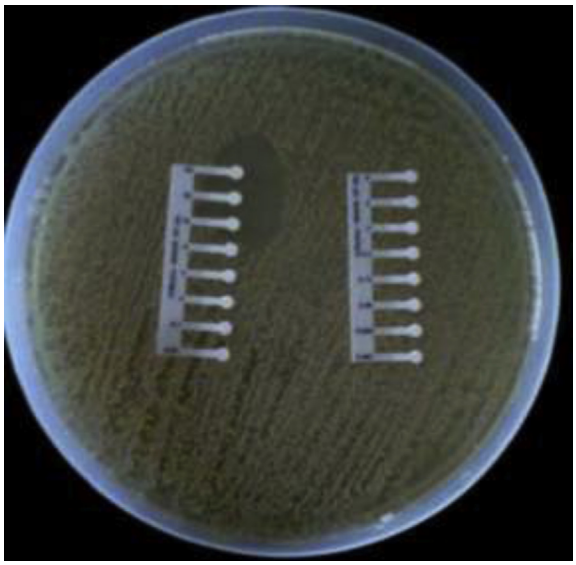

Figure 1. Minimal Inhibitory Concentration determination (MICs) by E-strip method: the point where eclipse converge the MIC value scale (in μg/ml) is the MIC. In this case it is 16 μg/ml.

measuring the absorbance of light (260 nm), an absorbance at A260 of the DNA concentration of 150 ng/μL, 159 ng/μL, 152 ng/μL, and 180 ng/μL, respectively. The absorbance ratio of DNA preparation was 1.8 to 2.0, which determines the purity of the DNA samples, which are considered to be free from protein contamination.

Genotypic Detection for Antibacterial Drugs. To verify the presence of fluoroquinolone-resistant genes for *Shigella* isolates (covering all 4 different species) were selected based on their MIC level, and highest drug-resistant pattern,[10] they were further evaluated by PCR amplification on agarose gel electrophoresis. The amplification results were positive for all species and asserted the presence of gyrA, gyrB, parC, and parE genes and sizes of the amplified products, as shown in Figure 4.

Gene Sequencing and Nucleotide Accession Numbers. For determining the nucleotide sequence of fluoroquinolone-resistant genes, unidirectional sequencing was carried out by dideoxy chain termination method using specific primers at the

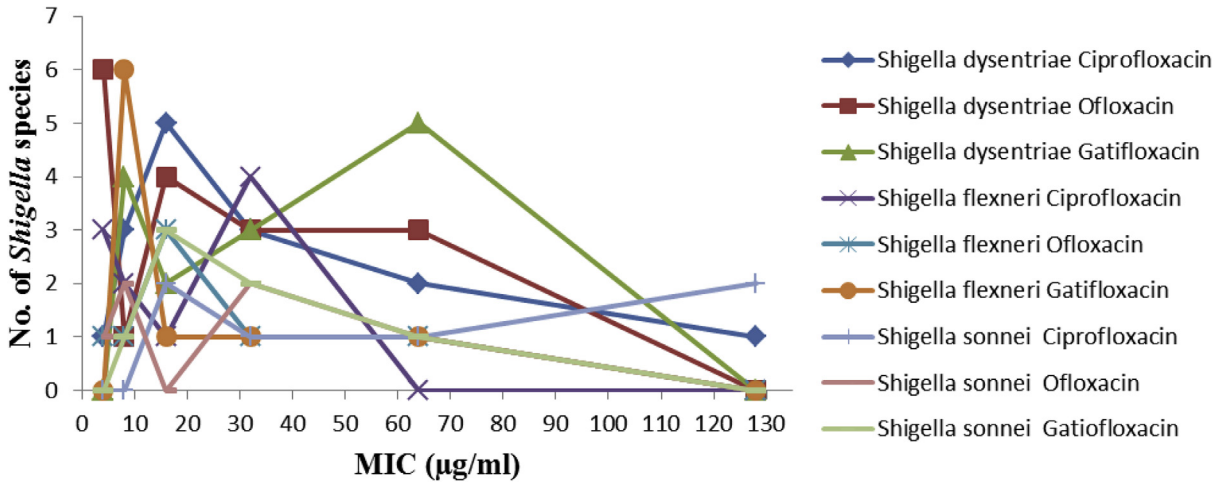

Figure 2. Minimal inhibitory concentration (MICs) of fluoroquinolone resistant antibiotics among different *Shigella* species (n = 29, 30 and 30).

sequencing facility at Medauxin Pvt Ltd. The gene sequences obtained were analyzed by using the BLAST search program. The PCR sequences of the fluoroquinolone-resistant *Shigella* isolates in this study were deposited in the GenBank database under the accession numbers KT965296 to KT965303.

Restriction Digestion Analysis. Restriction analysis of the PCR products determined that the 2 enzymes PvuI and Hae II could be employed to detect differences among the *Shigella* species. The gyrA gene digestion for *Shigella* spp (*S dysenteriae*, *S flexneri,* and *S sonnei*) using PvuI produced 1 strong band of about 405 bp and 1 weak band of 243 bp and for gyrB, parC, and parE gene digestion using Hae II produced 1 strong band of 226 bp, 222 bp, and 238 bp and 1 very weak band of about 83 bp, 26 bp, and 52 bp. The sequence of PCR products from all 4 resistant genes of different *Shigella* spp amplification indicated that PvuI and HaeII enzymes had 1 cutting site to produce 2 bands at 243 bp, 83 bp, 26 bp, and 52 bp, as shown in Figure 5.

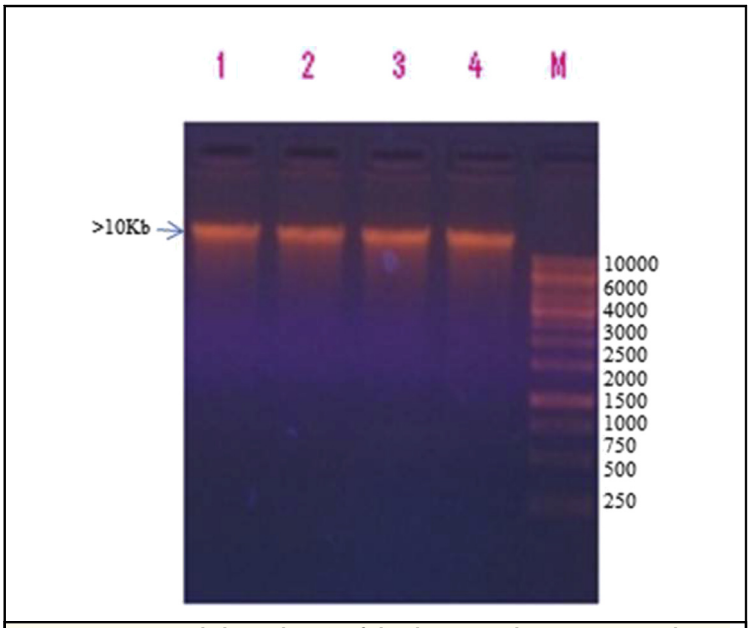

Figure 3. Agarose gel electrophoresis of the chromosomal DNA: Lane M: 1Kb DNA ladder; Lane 1-3: *Shigella dysentriae, Shigella flexneri* and *Shigella sonnei* isolates; Lane 4: MTCC culture.

DISCUSSION

The present study, covering the years 2013-2014, denotes a proportional morbidity for shigellosis of 11%-13% among children suffering from gastroenteritis in Gulbarga district, Karnataka, India, which is somewhat higher than reported elsewhere for other cities in India and other Asian countries.[10,14] In our study, the incidence of shigellosis was found to be higher among children aged <5 years. *Shigella dysenteriae* and *S flexneri* were the predominant species, with mean prevalence of 49% and 33%, respectively. Changing patterns of antimicrobial susceptibilities among *Shigella* isolates pose major difficulties in selecting an appropriate drug for the treatment of shigellosis.[15] Over the past few decades, *Shigella* spp have become resistant to most of the widely used antimicrobials.[16,17] The emergence and spread of drug resistance to newer and more potent agents used in treatment of shigellosis is a major therapeutic challenge.[18] As the regular shigellosis drugs have become outdated,

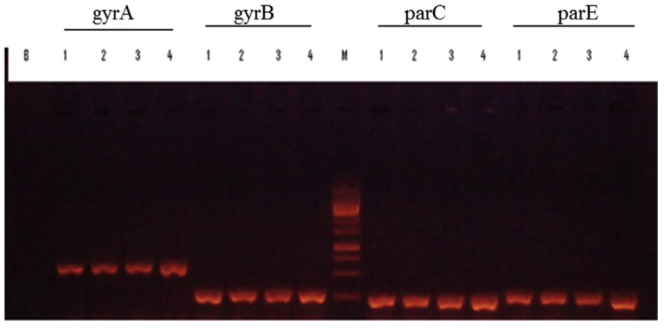

Figure 4. PCR amplification of fluroquinolones resistant genes (gyrA, gyrB, parC and parE) from multidrug resistant *Shigella* species: Lane M: 1Kb DNA Ladder; gyrA Lane 1-4: Lane 1- *Shigella dysentery* (610 bp); Lane 2- *Shigella flexxneri* (610 bp); Lane 3: *Shigella sonnei* (352 bp); Lane 4- MTCC (340 bp); gyrB Lane 1-4: Lane 1- *Shigella dysentery* (327 bp); Lane 2- *Shigella flexxneri* (256 bp); Lane 3: *Shigella sonnei* (283 bp); Lane 4- MTCC (311 bp);); parC Lane 1-4: Lane 1- *Shigella dysentery* (251 bp); Lane 2- *Shigella flexxneri* (220 bp); Lane 3: *Shigella sonnei* (251 bp); Lane 4- MTCC (265 bp); parE Lane 1-4: Lane 1- *Shigella dysentery* (308 bp); Lane 2- *Shigella flexxneri* (302 bp); Lane 3: *Shigella sonnei* (311 bp); Lane 4- MTCC (270 bp).

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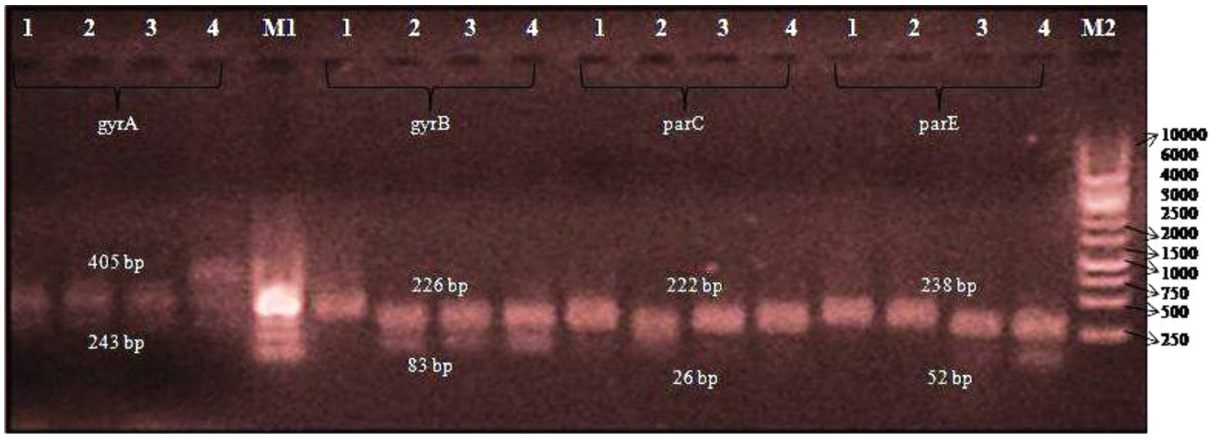

Figure 5. **Restriction enzyme analysis of fluoroquinolone resistant from multi drug resistant *Shigella* speices: Lane M1: 100bp ladder; Lane M2: 1kb ladder; gyrA Lane 1-4; digested with pvul sites produces two (243 and 405bp), gyrB Lane 1-4: digested with HaeII sites produces two (83 and 226bp); parC Lane 1-4: digested with HaeII sites produces two (26 and 222bp); parE Lane 1-4: digested with HaeII sites produces two (52 and 238bp): Lane 1-4 : *Shigella dysentriae*, *Shigella flexneri, Shigella sonnei* and *Shigella* MTCC culture.**

cephalosporins and fluoroquinolones are the main treatment for *Shigella* infection. In the early days of fluoroquinolone treatment for gastrointestinal infections, ciprofloxacin and other fluoroquinolones were found to be highly active in vitro and clinically effective in the treatment of infant diarrhea. However, a study performed during the period 2013-2014 indicated that moderate resistance is emerging against these drugs, which is distressing.[19,20]

In our recent study, fluoroquinolone-resistant *Shigella* isolates were isolated from infants. Fluoroquinolone (ciprofloxacin, ofloxacin, and gatifloxacin)—resistant *Shigella* spp were detected in 95.33% of 43 positive *Shigella* isolates from 334 infants ranging in age from 1 month to 5 years. The MICs of these 3 antibiotics was resolved for 32 clinical isolates of *Shigella* species, and analysis of *Shigella* isolates indicated resistance to all 3 antibiotics with MIC values of 4-128 μg/mL. Higher MIC values for ciprofloxacin were observed in *S dysenteriae* and *S sonnei* at 128 μg/mL, similar to that of gatifloxacin and ofloxacin, which displayed high resistance with an MIC value of 64 μg/mL (Table 4 and Fig. 2).

The genetic basis and its characterization of fluoroquinolone resistance in these isolates was considered for the common resistant genes, namely, gyrA, gyrB, parC, and pare, and had mutations at position 83 of gyrA and at position 80 of parC of the quinolone-resistant determining regions, comparable to that in isolates from other areas.[21,22] These mutations are responsible for mediating fluoroquinolone resistance. Prominently, all these tested *Shigella* isolates either had mutation at position 87 of gyrA or carried the fluoroquinolone-resistant genes.[8,23] Partial sequence homologies of PCR amplified gyrA, gyrB, parC, and parE resistant genes of *Shigella* isolates indicated that the nucleotide sequence of all 4 genes of isolates has 80%-90% homology with *Shigella* spp and most with enterotoxigenic *Escherichia coli* reported from India.[24]

In conclusion, although the increased frequency of multidrug resistance in *Shigella* isolates, several acquired genes encoding the fluoroquinolone resistant. The emergence of fluoroquinolone-resistant *Shigella* spp is a major problem in the treatment of shigellosis. Our study will aid in identification of the causative agents of the infection. Our experience with the isolates suggests that continuous surveillance for multidrug resistance will be needed to determine the distribution of resistant development in the *Shigella* isolates. Careful infection control and cautious use of antibiotics must be promoted, particularly to monitor the emergence of isolates that are fully resistant to fluoroquinolone, so as to understand the actual disease burden and provide guidance for the clinical treatment of shigellosis.

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