Infection and Drug Resistance

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ORIGINAL RESEARCH

Molecular analysis of multidrug resistance in clinical isolates of *Shigella* spp. from 2001–2010 in Kolkata, India: role of integrons, plasmids, and topoisomerase mutations

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Abstract: To understand the genetic basis of high drug resistance in *Shigella*, 95 clinical isolates of *Shigella* spp. (2001–2010) were obtained from the Infectious Diseases Hospital, Kolkata, India. Ninety-three isolates were resistant to three or more antibiotics. Resistance to nalidixic acid, trimethoprim, streptomycin, and co-trimoxazole was most common in this population. Dendrogram analysis showed that *S. sonnei* strains were more clonally related when compared to the other *Shigella* species. The role of mobile genetic elements and chromosome-borne resistance factors was analyzed in detail. Integron analysis indicated the preponderance of class 2 and atypical class 1 integrons in that population. Typical class 1 integron was present in only one *S. sonnei* isolate and harbored trimethoprim resistance-encoding gene *dfrV*, while atypical class 1 integrons harbored *dfrA1–aadA* or bla_{OXA}-*aadA* gene cassettes responsible for resistance to trimethoprim, aminoglycosides, and β-lactams. Class 2 integrons harbored either *dfrA1-sat-aadA* or *dfrA1-sat* gene cassettes. Most importantly, a novel gene cassette array *InsE-InsO-dfrA1-sat* was found in class 2 integron of *S. sonnei* NK4846. Many of the resistance traits for antibiotics such as trimethoprim, co-trimoxazole, kanamycin, ampicillin, and tetracycline were transferred from parent *Shigella* isolates to recipient *Escherichia coli* during conjugation, establishing the role of plasmids in horizontal transfer of resistance genes. Multiple mutations such as S_{80}→I, S_{83}→L, and D_{87}→G/N/Y in quinolone resistance determining regions of topoisomerases from the representative quinolone-resistant isolates could explain the spectrum of minimal inhibitory concentration values for various quinolones. To the best of our knowledge, this is the first comprehensive report that describes the contribution of mobile (plasmids, integrons, and quinolone resistance genes named qnr) and innate genetic elements (mutations in topoisomerases) in determining the resistance phenotype of all the four species of *Shigella* over a span of ten years.

Keywords: mobile genetic element, conjugation, atypical class 1 integron, quinolone resistance, efflux pumps

Introduction

In February 2017, WHO published a global priority list of antibiotic-resistant pathogens that pose greatest threat to human health and for which new antibiotics are needed.[1] Based on the urgency for development of new antibiotics, pathogens have been divided into critical, high, and medium category.[1] Fluoroquinolone-resistant *Shigella* has been included as a medium priority pathogen in this list. This has reiterated that multidrug resistant (MDR) *Shigella* poses a threat to human health and underlines the need to unravel and understand the molecular basis of MDR in *Shigella*. Shigellosis caused

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http://dx.doi.org/10.2147/IDR.S148726

by *Shigella flexneri*, *S. sonnei*, *S. dysenteriae*, and *S. boydii* mostly occurs in resource-poor communities that do not have proper sanitation.[2] Annually, the number of *Shigella* episodes is estimated to be 80–165 million and ~74,000–600,000 deaths worldwide.[3] Antibiotics are used for the treatment of shigellosis, but with increasing MDR pathogens, treatment of this disease has become complicated. There have been many reports around the world indicating the rising problem of MDR in shigellosis.[4–12] Increase in resistance to fluoroquinolones, third-generation cephalosporins, and azithromycin has been reported globally.[13]

Resistance to antibiotics in bacteria has been attributed to chromosome-borne inherent and mobile genetic factors. For examples of chromosome-borne factors, an organism may lack the target of antibiotics (mutations in target genes) or export antibacterial agents before they exert their effect (efflux pumps) or may restrict an antibiotic from entering into the bacteria (porins). Mobile genetic elements (MGEs) include plasmids, integrons, and integrating conjugative elements (ICEs), which are potent vectors for acquisition and dissemination of antibiotic resistance genes among the bacterial populations.[14–19] Plasmids evolved as an essential part of the bacterial genome, providing resistance genes for carbapenem resistance, extended spectrum beta-lactamase and quinolone resistance proteins that can be easily exchanged among bacteria by horizontal gene transfer.[18] Integrons are gene-capture elements and till date five classes of integrons have been well characterized based on their integrase sequences.[17] A typical class 1 integron consists of two conserved segments (CS) at their 5′ and 3′ ends, separated by a variable region that usually comprises of one or more extraneous gene cassettes. The 5′ CS region contains the integrase gene, the integration site, and a promoter region that allows the expression of any number of gene cassettes inserted at the *attI1* site in a suitable orientation.[17] The 3′ CS region usually comprises of *qacE*Δ1 encoding resistance to quaternary ammonium compounds and *sul1* encoding resistance to sulphonamides.[17] While the atypical class 1 integron consists of 5′ CS of class 1 integron and a variable region, it consists of an IS1 element at 3′ end in place of *qacE*Δ1 and *sul1*.[16] Atypical class 1 integron was first found on the pathogenicity island carrying *Shigella* resistance locus, on chromosome of *S. flexneri* 2a strain YSH6000 where it harbored two resistance determinants for chloramphenicol and tetracycline.[15,16] The presence of atypical class 1 integron in *Shigella* isolates has commonly been reported from Asia and has also now been reported from Africa.[20–23] SXT elements are the best studied examples of ICE, which harbor the sulphonamide, trimethoprim, streptomycin, and chloramphenicol resistance genes.[14]

Shigellosis is endemic in India and is one of the major causes of diarrhea in this country [2,13] Due to varied subgroups, varied distribution of different subgroups in various geographical locations, and different location-specific antibiograms in *Shigella* infections, the problem of treatment of *Shigella* infection is multidimensional. Therefore, detailed analysis of the species/serogroup-specific antibiotic resistance patterns and the genetic factors governing these resistance phenotypes is important to understand these bacterial infections. For this purpose, 95 clinical isolates of *Shigella* were analyzed for their antibiotic resistance profiles. Various genetic factors borne on chromosomes or plasmids or integrons were deciphered as reasons for the observed MDR phenotypes.

Materials and methods

Bacterial strains and DNA isolation

Patients who took any antibiotic were not enrolled in this study. Confirmed isolate from each patient was included for further analysis. Only one isolate was taken from each patient. Stool specimens collected from patients with dysentery were analyzed for common enteric pathogens using standard microbiological technique.[24] Subsequently, the identity of the *Shigella* isolates was confirmed by biochemical tests[24] and they were serotyped with an antiserum kit (Denka Seiken Co. Ltd, Tokyo, Japan). These *Shigella* isolates were representation of a large number of *Shigella* cases that were treated at the hospital during the ten-year period. As performing detailed molecular analysis for such a large number of isolates was not feasible, only representative population was taken without any selection bias. Therefore, 95 *Shigella* isolates (*S. flexneri* [n=42], *S. sonnei* [n=42], *S. dysenteriae* [n=5], and *S. boydii* [n=6]) were collected from patients with bloody diarrhea admitted to the Infectious Diseases Hospital, Kolkata, India, from 2001 to 2010. The patients provided written informed consent for participating in the study, and in the case of children, written informed consent was obtained from their parents. The consent procedure was approved by the Institutional Ethical Clearance Committee of NICED. The study was approved by the Institutional Biosafety Committee of Indian Institute of Advanced Research, Gandhinagar, and the Review Committee on Genetic Manipulation governed by the guidelines laid down by the Department of Biotechnology, Government of India. Genomic and plasmid DNA were prepared as described previously.[25] Plasmid DNA was prepared using alkaline lysis method.[25,26] The plasmid DNA samples were electrophoresed in 1% agarose gel prepared in 1× Tris-Acetate-EDTA (TAE) using 1× TAE running buffer at 6–7

V/cm. 1 kb DNA ladder and λ *Hind* III marker were used to determine the molecular size of plasmids.

Pulsed field gel electrophoresis (PFGE) analysis

Genomic DNA for PFGE was prepared in agarose plugs using the method described earlier.[27] PFGE of *Not*I- or *Xba*I-digested genomic DNA was performed using a CHEF-Mapper (Bio-Rad Laboratories) according to the PulseNet standardized protocol for subtyping of *Shigella* species. PFGE images were captured using a gel documentation system (Vilber Lourmat, Marne-la-Vallée, France). PFGE profiles were analyzed using the BioNumerics version 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium). The tagged image file formats were normalized by using the universal *Salmonella enterica* serotype Braenderup (H9812) size standard on each gel against the reference in the database. In the dendrogram analysis, the PFGE profiles were matched using the Dice coefficient and unweighted pair group method using arithmetic averages. Clustering of PFGE profiles was made using 1.5% band position tolerance window and 1.5% optimization.

Antimicrobial susceptibility tests and minimal inhibitory concentration (MIC) assays

Shigella isolates were tested for their susceptibility to ampicillin (10 µg), azithromycin (15 µg), ceftriaxone (30 µg), cefuroxime (30 µg), chloramphenicol (30 µg), co-trimoxazole (1.25 µg trimethoprim/23.75 mg sulfamethoxazole), ciprofloxacin (CIP; 5 mg), gentamicin (10 µg), streptomycin (10 µg), trimethoprim (5 µg), tetracycline (30 µg), nalidixic acid (NAL; 30 µg), norfloxacin (NOR; 10 µg), kanamycin (30 µg), and ofloxacin (OFX; 5 µg) by disk diffusion method using commercial disks (HiMedia, Mumbai, India) in accordance with the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) standards.[28] For the interpretation of antibiotic susceptibility, CLSI chart for enterobacteriaceae was used. MDR was defined as resistance acquired to at least one drug in three or more antimicrobial categories.[29]

MIC assays were performed for quinolones using commercial strips (EzyMIC strip; HiMedia) as per the manufacturer's protocol. *Escherichia coli* ATCC 25922 was used for quality control in both the experiments. Experiments were performed at least three times.

Ascertaining the role of efflux pumps in drug resistance

To ascertain the role of efflux pumps in imparting resistance to drugs, synergy test was carried out as described earlier.[30–32] The test was performed using various antibiotics, i.e., ampicillin, azithromycin, chloramphenicol, and CIP to which a particular clinical isolate was resistant. The efflux pump inhibitor carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) was added on Mueller-Hinton Agar at 4 mg/L concentration. Susceptibility testing for antibiotics by MIC strip was performed as described in earlier section, both in the presence and absence of CCCP. A decrease in the MIC of the isolates in the presence of CCCP indicated the role of efflux pumps in reducing the concentration of that drug inside the cell by throwing it out.

Polymerase chain reaction (PCR)

Presence of factors such as integrons, SXT elements, quinolone resistance genes, and topoisomerases in *Shigella* isolates was established by PCR using primers specific for each element. Genomic DNA (200 ng) were used as templates in PCR with the primers and conditions mentioned in Table 1 for the screening of class 1, class 2, and class 3 integrons and SXT integrase. PCR was carried out with an initial denaturation at 95°C for 4 min. Subsequent to this, 25–30 amplification cycles were performed, each consisting of an initial denaturation at 95°C for 0.5 min followed by annealing and extension steps (Table 1). The final polymerization was carried out at 72°C for 10 min. Recombinant Taq polymerase (Thermo Scientific) was used along with appropriate buffers containing ammonium sulfate (1×) and magnesium chloride (2 mM).

All the quinolone-resistant isolates (n=85) were analyzed for plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrD*, *qnrB*, *qnrS*, *oqxA*, *aac-(6′)-Ib-cr*, and *qnrC*) by multiplex PCR using the conditions described earlier (Table 1).[38] The positive controls for PMQRs were taken from the bacterial lysates derived from *Enterobacter cloacae* (*qnrA*), *Klebsiella pneumoniae*, and *S. enterica* Newport (*qnrB*), *S. enterica* Saintpaul (*qnrS*), *S. enterica* Typhimurium (*aac(6′)Ib-cr)*, *E. coli* transformant pHSII (*qnrC*), and *E. coli* transformant p2007057 (*qnrD*).[39–44] These bacterial lysates were procured from Technical University of Denmark (courtesy Dr Lina Cavaco). *K. pneumoniae* MTCC 7028 for *oqxA* was obtained from Microbial Type Culture Collection, Chandigarh, India.

Based on their quinolone resistance profiles, two or more representative isolates from each of the species were used to study the effect of topoisomerase mutations in quinolone resistance. A quinolone-sensitive isolate from each of the *Shigella* species was included as a control. The quinolone resistance determining regions (QRDRs) of topoisomerases (*gyrA*, *gyrB*, *parC*, *parE*) were amplified using the primers and conditions described in Table 1.[37] PCR amplicons were

Table 1 Primers used in the present study

Serial no.	Primer name	Description (to detect the genes)	Primer sequence 5′→3′	Annealing temp (°C)/ time	Extension temp (°C)/ time	Amplicon length (bp)	References
1	L2 L3	5′ CS of Class 1 integron	GACGATGCGTGGAGACC CTTGCTGCTTGGATGCC	55/30 s	72/1 min	300	33
2	qac EΔ1 Sul1B	3′ CS of Class 1 integron	ATCGCAATAGTTGGCGAAGT GCAAGGCGGAAACCCGCC	58/2 min	72/1 min	798	33
3	In F In B	Variable region of Class 1 integron	GGCATCCAAGCAGCAAGC AAGCAGACTTGACCTGAT	60/1 min	72/1 min	Variable	33
4	Int1CA F IS1 CA R	Variable region of atypical Class 1 integron	CGTAGAAGAACAGCAAGG AGTGAGAGCAGAGATAGC	52/1 min	72/3 min	Variable	16
5	Int 2 F Int 2 R	Integrase of Class 2 integron	GTAGCAAACGAGTGACGAAATG CACGGATATGCGACAAAAAGGT	66.2/1 min	72/1 min	789	This study
6	Int 2 VA F Int 2 VA R	Variable region of Class 2 integron	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	55/1 min	72/3 min	Variable	34
7	Int3 F Int3 R	Integrase of Class 3 integron	AGTGGGTGGCGAATGAGTG TGTTCTTGTATCGGCAGGTG	59/1 min	72/1 min	600	35
8	SXT F SXT R	Integrase from SXT element	ATGGCGTTATGAGTTAGCTC GCGAAGATCATGCATAGAC	57/30 s	72/1 min	1000	36
9	S- gyrA F S- gyrA R	QRDR region of gyrA	TACACCGGTCAACATTGAGG TTAATGATTGCCGCCGTCGG	64/30 s	72/1 min	648	37
10	S- gyrB F S- gyrB R	QRDR region of gyrB	TGAAATGACCCGCCGTAAAGG GCTGTGATAACGCAGTTTGTCCGGG	60.7/30 s	72/1 min	310	37
11	S-parC F S-parC R	QRDR region of parC	GTCTGAACTGGGCCTGAATGC AGCAGCTCGGAATATTTCGACAA	68.6/30 s	72/1 min	249	37
12	S-parE F S-parE R	QRDR region of pare	ATGCGTGCGGCTAAAAAAGTG TCGTCGCTGTCAGGATCGATAC	63/30 s	72/1 min	290	37
13	qnrA F qnrA R	qnrA protein	CAGCAAGAGGATTTCTCACG AATCCGGCAGCACTATTACTC	63/1 min	72/1.5 min	630	38
14	qnrD F qnrD R	qnrD protein	CGAGATCAATTTACGGGGAATA AACAAGCTGAAGCGCCTG	63/1 min	72/1.5 min	581	38
15	qnrB F qnrB R qnrB R(D)	qnrB protein	GGCTGTCAGTTCTATGATCG GAGCAACGATGCCTGGTAG SAKCAACGATGCCTGGTAG	63/1 min	72/1.5 min	488	38
16	qnrS F qnrS R	qnrS protein	GCAAGTTCATTGAACAGGGT GTCAGGAWAAACAACAATACC	63/1 min	72/1.5 min	518	This study
17	oqxA F oqxA R	oqxA efflux pump	CCGCACCGATAAATTAGTCC GGCGAGGTTTTGATAGTGGA	63/1 min	72/1.5 min	313	38
18	aac(6′)-Ib-cr F aac(6′)-Ib-cr R	aac(6′)-Ib-cr enzyme	TTGGAAGCGGGGACGGAM ACACGGCTGGACCATA	63/1 min	72/1.5 min	260	38
19	qnrC F qnrC R	qnrC protein	GCAGAATTCAGGGGTGTGAT AACTGCTCCAAAAGCTGCTC	63/1 min	72/1.5 min	118	38

Abbreviations: CS, conserved segments; QRDR, quinolone resistance determining regions.

sequenced and these sequences were compared with the standard or control *Shigella* topoisomerase sequences to detect the mutations.

Conjugation

Based on antibiograms, agarose gel analysis of plasmid preparations, and integron analysis, representative isolates of *Shigella* were selected to study transferability of their resistance traits to the recipient by conjugation.[36,45] *E. coli* XL1-BLUE (resistant to tetracycline and NAL) or *E. coli* J53 KACC 16628 (resistant to sodium azide) was used as a recipient. Briefly, the recipient and donor were mixed in a ratio of 1:1 on a sterile 0.45 μm nylon membrane (Nytran N, Whatman) and incubated overnight for mating on Luria-Bertani agar at 37°C. Transconjugants were selected on MacConkey agar containing appropriate antibiotics and

tested for acquired resistance traits by determination of their antibiotic susceptibility profiles.

Sequence analysis

DNA sequencing was performed by Sanger's chain termination method using DNA sequencer (Applied Biosystems; 3730/3730xl DNA analyzer). All the amplicons except topoisomerases were cloned in pDrive (Qiagen) or pBAD (Invitrogen) TA cloning vectors. The amplicons (>1.0 kb) were sequenced by primer walking, and the sequences were assembled and analyzed by Basic Local Alignment Search Tool (BLAST). The ORF (open reading frame) finder tool at the NCBI website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to predict all the possible ORFs in these sequences. Sixty seven assembled sequences were submitted to GenBank (accession numbers KX583660-KX583675, KX817766-KX817795, KX768278-KX768283, KX536824-KX536827, KX463270-KX463271, KX781232-KX781233, KX777251-KX777252, KX792556, and KX863674-KX863676).

Results

Bacterial isolates and their resistance profiles

Ninety five clinical isolates of *Shigella* included 42 *S. flexneri*, 42 *S. sonnei*, 6 *S. boydii*, and 5 *S. dysenteriae* isolates. Among *S. flexneri* isolates, serotype 2a was most common (22/42), followed by serotype 3a (11/42). Rest of the nine isolates consisted of serotypes 1a (2/42), 1b (3/42), 2b (2/42), and one each of serotypes 4a and 6. *S. boydii* population had representations from serotypes 1, 2, 4, 11 (2/6), and 12. *S. dysenteriae* consisted of serotypes 3 (2/5), 9, 5, and 12.

Out of the 95 isolates, 93 were resistant to three or more antibiotics. The percentage of *Shigella* isolates resistant to each of the antibiotics is shown in Table 2. Intermediate resistance and complete resistance were together considered to be a resistance trait. More than 90% of *S. flexneri* isolates showed resistance to streptomycin, trimethoprim, and tetracycline, and 69–85% showed resistance to NAL, chloramphenicol, co-trimoxazole, and CIP (Table 2). Above 80% of *S. sonnei* isolates showed resistance to NAL, kanamycin, trimethoprim, co-trimoxazole, streptomycin, and azithromycin. *S. dysenteriae* isolates chiefly showed resistance to co-trimoxazole, trimethoprim, tetracycline, NAL, and streptomycin. In *S. boydii* isolates, resistance to trimethoprim and streptomycin was prominent, i.e., above 80%.

Year-wise analysis of the aforementioned antibiotic susceptibility profiles (Figure 1) revealed that the MDR status of these isolates persisted through all these years (Figures 2 and 3). Most importantly, during ten years, resistance to fluoroquinolone drugs such as CIP, NOR, and OFX was remarkably increased as compared to NAL (Figure 1). Resistance to either of the four quinolone drugs was 89.5%, while resistance to all the four quinolone drugs together was 47.4%. An interesting pattern was observed for resistance to cephalosporins. A bell-shaped pattern showed the maximal resistance to them (13% for ceftriaxone and 21.7% for cefuroxime) from the years 2005 to 2007. This resistance decreased in later years.

Table 2 Percentage of drug resistance in *Shigella* isolates

Antibiotics	*Shigella flexneri* (n=42)	*Shigella sonnei* (n=42)	*Shigella dysenteriae* (n=5)	*Shigella boydii* (n=6)	Total (n=95)
	Percentage of drug resistance				
Ampicillin	66.6	7.14	40.0	0	34.72
Azithromycin	23.7	80.94	60.0	50	52.62
Ceftriaxone	2.3	9.52	0	0	5.25
Chloramphenicol	83.33	2.3	20	16.6	39.99
Ciprofloxacin	69.04	59.52	60	0	59.99
Co-trimoxazole	78.57	95.23	100	66.66	86.31
Cefuroxime	19.04	9.52	0	0	12.63
Gentamicin	2.3	57.13	20	16.6	28.42
Kanamycin	64.28	100	40	50	77.89
Nalidixic acid	85.71	100	80	50	89.46
Norfloxacin	64.28	42.85	0	0	47.36
Ofloxacin	64.27	42.85	0	0	47.36
Streptomycin	92.85	90.47	80	83.3	90.52
Tetracycline	90.39	45.23	80	33.33	66.31
Trimethoprim	90.47	95.23	100	83.3	92.63

Notes: Complete resistant and intermediate resistant traits were considered to be resistant traits.

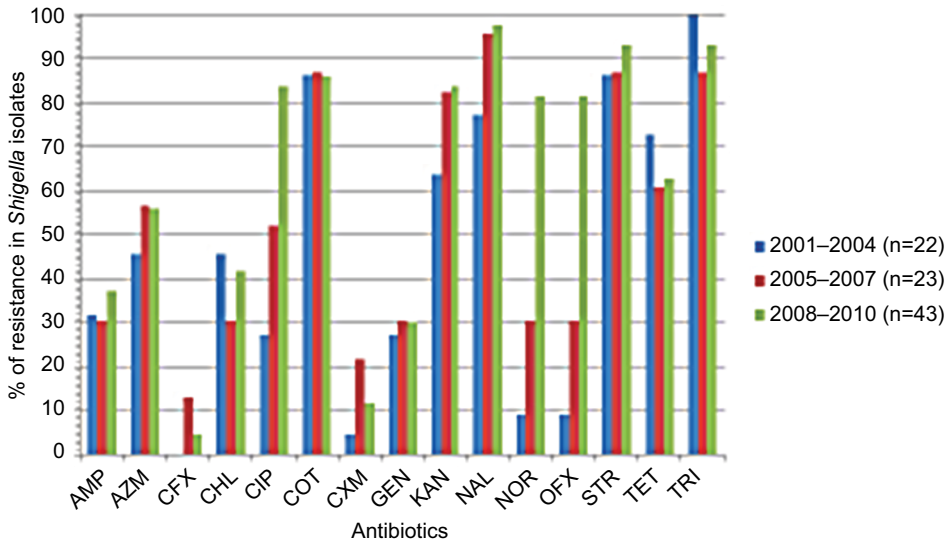
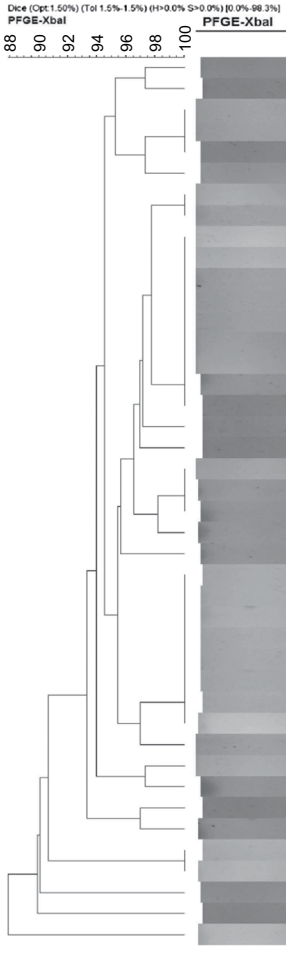
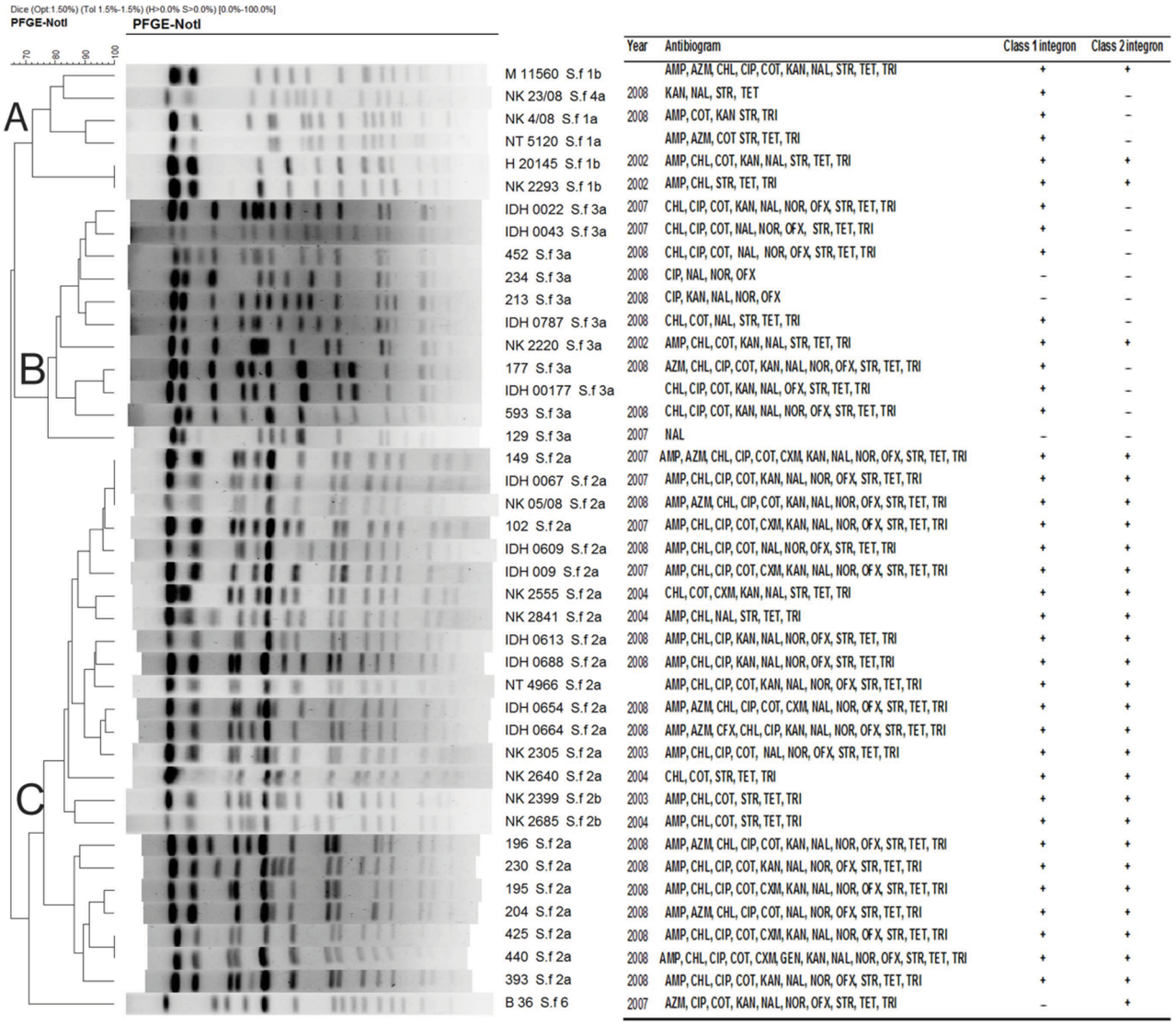


Figure 1 Year-wise percentage of drug resistance in the clinical isolates of *Shigella*. AMP, ampicillin (10 mg); AZM, azithromycin (15 mg); CFX, ceftriaxone (30 mg); CHL, chloramphenicol (30 mg); CIP, ciprofloxacin (5 mg); COT, co-trimoxazole (1.25 mg trimethoprim/23.75 mg sulfamethoxazole); CXM, cefuroxime (30 mg); GEN, gentamicin (10 mg); KAN, kanamycin (30 mg); NAL, nalidixic acid (30 mg); NOR, norfloxacin (10 mg); OFX, ofloxacin (5 mg); STR, streptomycin (10 mg); TET, tetracycline (30 mg); TRI, trimethoprim (5 mg).



	Year	Antibiogram	Class 1 integron	Class 2 integron
NK 4010	2005	AZM, COT, GEN, KAN, NAL, STR, TRI	–	+
NK 4795	2005	AZM, COT, KAN, NAL, TRI	–	+
2969	2010	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
IDH 2969	2010	CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
NK 4766	2005	COT, KAN, NAL, STR, TRI	–	+
K 18323	2005	AMP, AZM, COT, CXM, GEN, KAN, NAL, STR, TET, TRI	–	+
NK 1825	2001	AZM, COT, KAN, NAL, STR, TET ,TRI	–	+
S 22		COT, CXM, KAN, NAL, STR, TRI	–	+
1328	2009	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
1490	2009	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
IDH 1694	2009	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TRI	–	+
IDH 2343	2009	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
IDH 2347	2009	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
NK 2070	2002	AZM, CIP, COT, GEN, KAN, NAL, STR, TET, TRI	–	+
NK 2251	2002	AZM, CIP, COT, KAN, NAL, STR, TRI	–	+
NK 2537	2004	AZM, COT, GEN, KAN, NAL, TRI	–	+
NK 4199	2006	AZM, CIP, GEN, KAN, NAL, STR	–	–
NK 4971	2006	CIP, COT, KAN, NAL, STR, TET ,TRI	–	+
NK 4219	2006	AZM, KAN, COT, NAL, TRI	–	+
IDH 1541	2009	AZM, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
NK 2437	2004	AZM, COT, GEN, KAN, NAL, STR, TRI	–	+
NK 4040	2004	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
NK 2497	2004	AZM, COT, KAN, NAL, STR, TRI	–	+
NK 4174	2004	AZM, COT, GEN, KAN, NAL, STR, TRI	–	+
445	2008	AZM, COT, KAN, NAL, STR, TRI	–	+
IDH 0734	2008	AZM, CIP, COT, KAN, NAL, NOR, OFX, STR, TRI	+	+
IDH 0741	2008	AMP, AZM, CIP, COT, CXM, GEN, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
IDH 0796	2008	AZM, CIP, CFX, COT, GEN, KAN, NAL, NOR, OFX, STR, TRI	–	+
IDH 0988	2008	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TRI	–	+
IDH 1170	2008	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TRI	–	+
IDH 1219	2008	CIP, COT, KAN, NAL, NOR, OFX, STR, TRI	–	+
NK 4675	2007	AZM, CFX, COT, GEN, KAN, NAL, STR, TET, TRI	–	+
IDH 1690	2009	AZM, CIP, COT, KAN, NAL, NOR, OFX, TRI	–	+
2340	2009	AZM, CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TRI	–	+
NK 4224	2004	AZM, COT, GEN, KAN, NAL, STR, TRI	–	+
L 1137	2006	AMP, AZM, CHL, COT, CXM, KAN, NAL, STR, TRI	–	+
NK 4290	2004	CIP, COT, KAN, NAL, STR, TET, TRI	–	+
968	2008	CIP, COT, GEN, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+
NK 4888	2007	AZM, CFX, COT, GEN, KAN, NAL, STR, TET, TRI	–	+
NK 4005	2005	AZM, CIP, COT, GEN, KAN, NAL, STR, TET, TRI	–	+
NK 4465	2006	COT, KAN, NAL, STR, TRI	–	+
NK 4846	2007	AZM, CFX, CIP, GEN, KAN, NAL, STR, TET	–	+

Figure 2 Dendrogram of *Xba*I-digested pulsed-field gel electrophoresis profiles of the clinical isolates of *Shigella sonnei*. Scale bar indicates degree of similarity.
Abbreviations: AMP, ampicillin; AZM, azithromycin; CFX, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; COT, co-trimoxazole; CXM, cefuroxime; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; STR, streptomycin; TET, tetracycline; TRI, trimethoprim.



Year	Antibiogram	Class 1 integron	Class 2 integron
	AMP, AZM, CHL, CIP, COT, KAN, NAL, STR, TET, TRI	+	+
2008	KAN, NAL, STR, TET	+	–
2008	AMP, COT, KAN STR, TRI	+	–
	AMP, AZM, COT STR, TET, TRI	+	–
2002	AMP, CHL, COT, KAN, NAL, STR, TET, TRI	+	+
2002	AMP, CHL, STR, TET, TRI	+	+
2007	CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	+	–
2007	CHL, CIP, COT, NAL, NOR, OFX, STR, TET, TRI	+	–
2008	CHL, CIP, COT, NAL, NOR, OFX, STR, TET, TRI	+	–
2008	CIP, NAL, NOR, OFX	–	–
2008	CIP, KAN, NAL, NOR, OFX	–	–
2008	CHL, COT, NAL, STR, TET, TRI	+	–
2002	AMP, CHL, COT, KAN, NAL, STR, TET, TRI	+	+
2008	AZM, CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	+	–
	CHL, CIP, COT, KAN, NAL, OFX, STR, TET, TRI	+	–
2008	CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	+	–
2007	NAL	–	–
2007	AMP, AZM, CHL, CIP, COT, CXM, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2007	AMP, CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, AZM, CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2007	AMP, CHL, CIP, COT, CXM, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, CHL, CIP, COT, NAL, NOR, OFX, STR, TET, TRI	+	+
2007	AMP, CHL, CIP, COT, CXM, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2004	CHL, COT, CXM, KAN, NAL, STR, TET, TRI	+	+
2004	AMP, CHL, NAL, STR, TET, TRI	+	+
2008	AMP, CHL, CIP, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, CHL, CIP, KAN, NAL, NOR, OFX, STR, TET,TRI	+	+
	AMP, CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, AZM, CHL, CIP, COT, CXM, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, AZM, CFX, CHL, CIP, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2003	AMP, CHL, CIP, COT, NAL, NOR, OFX, STR, TET, TRI	+	+
2004	CHL, COT, STR, TET, TRI	+	+
2003	AMP, CHL, COT, STR, TET, TRI	+	+
2004	AMP, CHL, COT, STR, TET, TRI	+	+
2008	AMP, AZM, CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, CHL, CIP, COT, CXM, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, AZM, CHL, CIP, COT, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, CHL, CIP, COT, CXM, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, CHL, CIP, COT, CXM, GEN, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2008	AMP, CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	+	+
2007	AZM, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	–	+

Figure 3 Dendrogram of *Not*I-digested pulsed-field gel electrophoresis profiles of the clinical isolates of *Shigella flexneri.* Clustering identified three clades (**A**–**C**). Scale bar indicates degree of similarity.

Abbreviations: AMP, ampicillin; AZM, azithromycin; CFX, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; COT, co-trimoxazole; CXM, cefuroxime; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; STR, streptomycin; TET, tetracycline; TRI, trimethoprim.

Clonality in *Shigella* isolates

The genotypes obtained by PFGE were analyzed separately according to *Shigella* species: *S. sonnei* (Figure 2), *S. flexneri* (Figure 3), *S. boydii* (Figure S1), and *S. dysenteriae* (Figure S2). PFGE analysis of *Xba*I-digested chromosomal DNA of the 42 *S. sonnei* strains yielded 21 reproducible PFGE patterns. As shown in Figure 2, two PFGE patterns were shared by nine isolates (21%) from the years 2002, 2004, 2006, and 2009, and eight isolates (19%) from the years 2007 and 2008. The similarity index for all *S. sonnei* isolates was 92–100%, suggesting that they were clonally related to each other. PFGE analysis of *S. flexneri* after digestion with *Not*I revealed serotype-specific clusters, with ~80–100% similarity within each serotype. *S. flexneri* exhibited three distinct clades (Figure 3). Clade A and B belonged to *S. flexneri* 1a, 1b, and 4a and *S. flexneri* 3a isolates, respectively. Clade C consisted of *S. flexneri* 2a and 2b. PFGE analysis revealed that *S. boydii* isolates belonging to different serotypes had ~80% similarity and *S. dysenteriae* isolates were also observed to be 80% clonally related. As could be observed from Figures 2 and 3, where antibiogram profiles and PFGE profiles are aligned, there was no correlation between clonality and MDR status of the isolates belonging to the same clade.

Presence of integrons

Out of 95, 42 isolates were found to be positive for 5′ CS of class 1 integrase by PCR. When analyzed for the presence

of 3′ CS, only one *S. sonnei* isolate IDH0734 yielded the expected 0.8 kb amplicon indicating that rest of the 41 isolates harbored atypical class 1 integron. Since IDH0734 harbored atypical class 1 integron with 5′ CS and 3′ CS, it was analyzed for its variable region where it yielded ~750 bp amplicon. Atypical class1 integrons were found in 38 out of 42 *S. flexneri*, two out of five *S. dysenteriae*, and one out of six *S. boydii* isolates (Table 3). For analysis of atypical class 1 integrons, their variable regions were amplified with the primer pair intI1CA F/IS1CA R in all the 41 isolates. Gel analysis of PCR products showed a major band with mobility of 2.4 kb in *S. flexneri* (2a, 1a, 1b, and 2b) and *S. dysenteriae*, while 2.0 kb band was observed in *S. flexneri* 3a and *S. boydii*.

In PCR, 83.2% isolates (79/95) were found to be positive for class 2 integrase (Table 3). Integrase 2-positive isolates were subsequently used to amplify variable regions using the primers specific for 5′ and 3′ conserved sequences (Int2VA F/Int2VA R primers) of class 2 integrons. Amplicons with varying sizes (1.4 kb or 2.4 kb or 2.2 kb) were obtained. Of the class 2 integrase–positive *S. flexneri* isolates, 28 out of 29 harbored 2.2 kb variable region and H20145 harbored 1.4 kb variable region. Of the *S. sonnei* isolates, 41 out of 42 were positive for class 2 integrase and all of them except two carried 1.4 kb variable region. One of these two harbored 2.4 kb variable region, while the other one did not show any amplification. All the five *S. dysenteriae* and four out of six *S. boydii* isolates were positive for class 2 integrase, and these harbored either 1.4 kb or 2.2 kb variable regions. Class 3 integron and SXT element were absent in these isolates.

Presence of both atypical class 1 and class 2 integrons in 28 out of 42 *S. flexneri* and two out of five *S. dysenteriae* isolates (Table 3) underlined the important role of integrons in imparting drug resistance to these isolates, also later proved by sequence analysis. In the *S. sonnei* population, only IDH0734 carried both typical class 1 and class 2 integrons (Table 3).

Sequence analysis of integrons

Sequences corresponding to 5′ CS and 3′ CS described in the earlier section were analyzed and submitted to GenBank (KX768278-KX768283 and KX777252). As mentioned earlier, these *Shigella* isolates carried variable regions with a major band of 750 bp for typical class 1 integron: bands of 2.4 kb and 2.0 kb for atypical class 1 integrons. Variable regions of 1.4 kb, 2.4 kb, and 2.2 kb were obtained in class 2 integrons. For each species, a sample representative of each band size was used for the sequence analysis. For example,

Table 3 Presence of integrons in *Shigella* isolates and the gene cassettes found in the variable regions of integrons

Isolates	Total no. of isolates	Presence of class 1 integron	Band size (kb)	Gene cassettes found on class 1 integron	Presence of atypical class 1 integron	Band size (kb)	Gene cassettes found on atypical class 1 integron	Presence of class 2 integron	Band size (kb)	Gene cassettes found on class 2 integron	Presence of both the integrons	Isolates without any integrons
Shigella flexneri	42	0	0	0	38	2.4, 2.0	bla_{OXA}-*aadA*, *aadA*, *dfrA1*-*aadA*	29	2.2, 1.4	*dfrA1*-*sat*-*aadA*, *dfrA1*-*sat*	28	3
Shigella sonnei	42	1	0.75	*dfrV*	0	0	0	41	1.4, 2.4	*dfrA1*-*sat*, *InsE*-*InsO*-*dfrA1*-*sat*	1	1
Shigella boydii	6	0	0	0	1	2.0	*dfrA1*-*aadA*	4	2.2, 1.4	*dfrA1*-*sat*-*aadA*, *dfrA1*-*sat*	0	1
Shigella dysenteriae	5	0	0	0	2	2.4	bla_{OXA}-*aadA*	5	2.2, 1.4	*dfrA1*-*sat*-*aadA*, *dfrA1*-*sat*	2	0
Total	95	1	–	–	41	–	–	79	–	–	31	**5**

two band sizes were found in variable regions of class 2 integrons in *S. flexneri* isolates, so representative 1.4 kb and 2.2 kb variable regions were sequenced.

Sequence of the variable region of typical class 1 integron from *S. sonnei* IDH0734 revealed that it encoded *dfrV* (KX777251) responsible for trimethoprim resistance. The 2.0 kb variable region of atypical class 1 integron in *S. flexneri* 593 and *S. boydii* NK1919 (Table 3) showed the cassettes *dfrA1-aadA* (KX817770 and KX817771). The 2.4 kb variable region of atypical class 1 integron from *S. flexneri* 102 and *S. dysenteriae* 1244 carried bla_{oxa}-*aadA* (KX817769 and KX951422, respectively) gene cassettes (Table 3). Therefore, atypical class 1 integrons carried resistance traits for trimethoprim (*dfrA1*), beta-lactams (bla_{oxa}), and aminoglycosides (*aadA*).

Sequences of class 2 integrases from each species were analyzed by ORF finder tool and submitted to GenBank (KX536824-KX536827, KX463270-KX463271). Results revealed that these were non-functional class 2 integrase genes carrying an internal stop codon TAA. This was in accordance with earlier observation that the gene encoding class 2 integrase contains a nonsense mutation in codon 179 (ochre 179) and thereby it yields a non-functional protein which can be recovered by a single mutation.[46] Sequences of variable regions of class 2 integrons were analyzed from the representative isolates of each species (Table 3). The 1.4 kb band from *S. flexneri* H20145, *S. boydii* 442, and *S. sonnei* L1137 harbored *dfrA1-sat* (KX817766, KX817768, and KX781233, respectively) cassettes, while 2.2 kb band from *S. flexneri* 102 and *S. dysenteriae* 1244 carried *dfrA1-sat-aadA* gene cassettes (KX817767 and KX792556, respectively). *S. sonnei* NK4846 harbored a new cassette array *InsE-InsO-dfrA1-sat* (KX781232) of 2.4 kb band size on class 2 integron (Table 3). Therefore, class 2 integrons in this population of *Shigella* isolates carried the resistance traits for trimethoprim (*dfrA1*), streptothricin (*sat*), and aminoglycosides (*aadA*). In addition, the genes for transposases or insertion sequences (*InsE, InsO*) were also observed in one of the cassettes.

Quinolone resistance

For deciphering quinolone resistance due to mutations, sequences from the QRDRs of topoisomerases were analyzed from the quinolone resistant/sensitive isolates derived from each *Shigella* species as described in section "Materials and methods". These sequences from the four topoisomerases, i.e., GyrA, GyrB, ParC, and ParE, were submitted to GenBank (KX583660-KX583675 and KX817772-KX817795). These isolates were also analyzed for their MICs to four quinolones (NAL, CIP, OFX, and NOR) in order to correlate the effect of mutations on their quinolone resistance phenotypes. A variety of mutations were detected in these isolates (Table 4). *S. flexneri* NK2640 (sensitive to all the tested quinolones) carried mutation in GyrA at $V_{196}\rightarrow A$ outside the QRDR region. *S. flexneri* 2a isolate 102 highly resistant to

Table 4 Topoisomerase mutations in quinolone resistance determining regions (QRDR) of representative *Shigella* isolates

Strain	Strain ID no.	Quinolone resistance	MIC (decreasing order of MIC per species)				Mutations in QRDR regions of topoisomerase (in the order of decreasing number of mutations)	
			CIP	NAL	NOR	OFX	GyrA	ParC
Shigella flexneri 2a	102	CIP, NAL, NOR, OFX	>256	>256	>256	>32	$S_{83}\rightarrow L$ $D_{87}\rightarrow N$ $H_{211}\rightarrow Y$	$S_{80}\rightarrow I$ $E_{84}\rightarrow G$
Shigella flexneri 2a	NK2640	Sensitive to all quinolones	0.032	1	0.125	0.047	$V_{196}\rightarrow A$	No mutation
Shigella sonnei	IDH1694	CIP, NAL, NOR, OFX	32	>256	48	8	$S_{83}\rightarrow L$ $D_{87}\rightarrow G$ $V_{196}\rightarrow A$	$S_{80}\rightarrow I$
Shigella sonnei	IDH0734	CIP, NAL, NOR, OFX	24	>256	24	12	$S_{83}\rightarrow L$ $D_{87}\rightarrow G$	$S_{80}\rightarrow I$
Shigella sonnei	NK4846	NAL	0.125	>256	0.25	0.25	$S_{83}\rightarrow L$	No mutation
Shigella sonnei	NK4219	NAL	0.094	>256	0.38	0.19	$D_{87}\rightarrow Y$ $V_{196}\rightarrow A$	No mutation
Shigella dysenteriae 3	1244	NAL	0.19	>256	0.38	0.50	$S_{83}\rightarrow L$	No mutation
Shigella dysenteriae 9	NK4036	Sensitive to all quinolones	0.047	1	0.064	0.064	No mutation	No mutation
Shigella boydii 11	442	NAL	0.19	48	0.38	0.19	$S_{83}\rightarrow L$	No mutation
Shigella boydii 2	NK1919	Sensitive to all quinolones	0.047	1.5	0.125	0.047	No mutation	No mutation

Abbreviations: CIP, ciprofloxacin; NAL, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin.

all the four quinolones carried multiple mutations in GyrA (S_{83}→L, D_{87}→N, and H_{211}→Y) and ParC (S_{80}→I and E_{84}→G). This was also evident in their high MIC values: >256 µg/mL for CIP, NAL, and NOR and >32 µg/mL for OFX. Two mutations D_{87}→Y (inside the QRDR) and V_{196}→A (outside the QRDR) were found in GyrA of NAL-resistant *S. sonnei* NK4219, while three mutations S_{83}→L, D_{87}→G, and V_{196}→A were observed in GyrA of *S. sonnei* IDH1694 (resistant to all the quinolones). *S. sonnei* IDH1694 also harbored mutation S_{80}→I in ParC region. *S. sonnei* IDH0734 which carried two mutations in GyrA (S_{83}→L, D_{87}→G) and one mutation in ParC (S_{80}→I) showed much higher MIC values (48–192 folds) and resistance to all four quinolones as compared to *S. sonnei* NK4846 (Table 4). *S. dysenteriae* 1244 and *S. boydii* 442 harbored a mutation S_{83}→L in GyrA. These mutations were not observed in control isolates *S. dysenteriae* NK4036 and *S. boydii* NK1919. No mutations were observed in GyrB and ParE from all these isolates. Therefore, as clearly depicted in Table 4 and discussed earlier, the increase in the number of mutations directly correlated with increase in resistance to spectrum of quinolones.

Results of a multiplex PCR for seven PMQR genes revealed that they were absent in these isolates except *S. flexneri* isolate M11560 that harbored a *qnrS* gene. The monoplex PCR for each PMQR gene was standardized using the controls described in section "Materials and methods". Subsequently, multiplex PCR for all the seven genes was performed with *Shigella* isolates.

Transfer of resistance by conjugation

Shigella isolates harbored multiple plasmids ranging from 1.0 kb to 23 kb as observed by agarose gel (Figure S3). Based on antibiotic susceptibility, plasmid profile, and presence of integrons, six isolates were selected from the present population to examine the resistance traits transferable through conjugation (Table 5). The choice of recipient *E. coli* was based on the resistance profile of donor isolates. Transconjugants of *S. boydii* 442 were selected on trimethoprim and tetracycline, while transconjugants from rest of the *Shigella* isolates were selected either on trimethoprim+sodium azide or streptomycin+sodium azide. The transconjugants were obtained successfully with conjugation efficiencies ranging from 10^{-6} to 10^{-7} transconjugants per recipient cell (Table 5). These experiments showed the transferability of resistance traits such as ampicillin, azithromycin, chloramphenicol, CIP, co-trimoxazole, kanamycin, NAL, OFX, streptomycin, tetracycline, and trimethoprim to recipient *E. coli*, establishing the role of plasmids in horizontal gene transfer (Table 5).

Table 5 Transferable resistance traits of transconjugants from *Shigella* isolates

Conjugation		Conjugation efficiency	Plasmid profile of donor (kb)	Resistance phenotype		Plasmid profile of transconjugants (kb)	Resistance traits transferred to the transconjugants
Donor	Recipient			Donor	Recipient		
Shigella boydii 442	*E. coli* XL1Blue	13.75×10^{-7}	14, 5.5, 4.5	AZM, COT, GEN, KAN, NAL, STR, TRI, sodium azide	NAL, TET	14, 5.5, 4.5	AZM, COT, KAN, STR, TRI
Shigella dysenteriae 1244	*E. coli* J53	8.6×10^{-6}	5.5, 4.5, 2.1, 1.6, 1.3, 1.1	AMP, AZM, CHL, CIP, COT, GEN, KAN, NAL, STR, TET, TRI	Sodium azide	5.5, 4.5, 2.1, 1.6, 1.3, 1.1, **0.9**	AMP, CHL, COT, NAL, KAN, TET, TRI
Shigella flexneri NK2220	*E. coli* J53	3.0×10^{-6}	5.5, 4.0, 2.4, 2.3, **2.0**, 1.3	AMP, CHL, COT, KAN, NAL, STR, TET, TRI	Sodium azide	5.5, 4.0, 2.4, 2.3, 1.3	AMP, CHL, COT, NAL, TET, TRI
Shigella flexneri 102	*E. coli* J53	3.9×10^{-7}	6.0, 4.2, 4.0, 2.5, 2.0	AMP, CHL, CIP, COT, CXM, KAN, NAL, NOR, OFX, STR, TET, TRI	Sodium azide	6.0, 4.2, 4.0, 2.5, 2.0	AMP,CHL, CIP,COT, KAN, NAL, NOR, OFX, STR, TET, TRI
Shigella sonnei IDH0734	*E. coli* J53	4.8×10^{-6}	**4.2**, 3.6, 2.0, 1.3	AZM, CIP, COT, KAN, NAL, NOR, OFX, STR, TRI	Sodium azide	3.6, 2.0, 1.3	CIP, COT, KAN, NAL, OFX, STR, TRI
Shigella sonnei NK4846	*E. coli* J53	0	10, 8, 3.5, 3.0, 2.7, 2.0, 1.7, 1.3	AZM, CFX, CIP, GEN, KAN, NAL, STR, TET	Sodium azide	–	Non-conjugable strain

Notes: Conjugation efficiency is the number of transconjugants per recipient cells. Bold values indicate plasmids that are not common between donor and transconjugants.
Abbreviations: AMP, ampicillin, AZM, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; COT, co-trimoxazole; CXM, cefuroxime; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; STR, streptomycin; TET, tetracycline; TRI, trimethoprim

Most commonly, the resistance traits for co-trimoxazole and trimethoprim were transferred in all the cases, confirming the carriage of trimethoprim resistance gene on plasmids.

Agarose gel analysis of the transconjugants showed that multiple plasmids were transferred during conjugation (Table 5). Therefore, associating the resistance traits to any particular plasmid was not possible. In addition, the comparison of plasmid profiles from the parent isolates and their transconjugants showed that most of the plasmids visualized on agarose gel were transferred during conjugation except for the ones shown in bold (Table 5).

Role of efflux pumps in drug resistance phenotype

The role of efflux pumps in the antibiotic resistance phenotype of the isolates used in the aforementioned studies was assessed using synergy test. MIC for different group of antibiotics was evaluated with or without efflux pump inhibitor CCCP and change in MIC was observed. The test was carried out with the antibiotics for which these *Shigella* isolates were resistant. *S. flexneri* 129 that was sensitive to all the antibiotics tested was included as a control. A reduction of 0.65- to 2.0-fold in MIC was observed on addition of CCCP as compared to the CCCP-free control in all the tested *Shigella* isolates (Table 6). This fold reduction in MIC was also observed in the control strain *S. flexneri* 129 that was sensitive to all these drugs. In the same assays, the other control *Vibrio fluvialis* isolates showed 1.33- to 5.33-fold changes in MIC. Therefore, these results revealed that there was no significant change in MIC in the presence of CCCP, indicating that efflux pumps did not play a major role in the resistance to these drugs (Table 6). It was also observed that the *S. flexneri* 2a isolate 102 had very high MICs in all the drugs tested.

Discussion

Antibiotic resistance within wide range of pathogenic bacteria is a growing public health concern globally. It hampers the effective prevention and treatment of infectious agents. Therefore, to mitigate the problem of MDR, it becomes pertinent to decipher various factors/mechanisms involved in antibiotic resistance of these infectious bacteria. This study was carried out to determine the patterns of antimicrobial resistance in 95 clinical isolates of *Shigella* (from 2001 to 2010) from Kolkata, India, and to unravel the possible genetic factors responsible for the observed resistance phenotypes. In this population of *Shigella* isolates, there was a predominance of *S. flexneri* and *S. sonnei*. Except one or two, all the isolates were resistant to three or more antibiotics out of the 15 antibiotics tested. This enormity of MDR was in accordance with the WHO reports published recently.[1,11] These reports cautioned that the danger of increasing MDR was resulting in treatment failure that could lead to mortality even in the case of minor injuries and common infections in

Table 6 Evaluation of efflux pump activity in *Shigella* isolates by synergy test

Antibiotics	Antibiotics+ CCCP (4 mg/L)	*Shigella flexneri* 102	*Shigella flexneri* 129	*Shigella sonnei* NK4846	*Shigella sonnei* IDH0734	*Shigella dysenteriae* 1244	*Shigella boydii* 442	*Vibrio fluvialis* L13828	*Vibrio fluvialis* L15318
Ampicillin	–	>256	1.5	ND	ND	>256	ND	24	>256
	+	>256	1	ND	ND	>256	ND	16	48–64
Fold change		1	**1.5**	–	–	1	–	**1.5**	**>4–5.33**
Chloramphenicol	–	>256	1.5	ND	ND	>256	ND	1	12
	+	>256	1	ND	ND	>256	ND	1	8
Fold change		1	**1.5**	–	–	1	–	1	**1.5**
Ciprofloxacin	–	>256	0.125	0.19	6	0.25	ND	0.125	2
	+	>256	0.19	0.19	6	0.125	ND	0.125	1.5
Fold change		1	**0.65**	1	1	**2**	–	1	**1.33**
Trimethoprim	–	>32	0.75	ND	>32	>32	>32	1.5	>32
	+	>32	0.75	ND	>32	>32	>32	1.0	>32
Fold change		1	1	–	1	1	1	**1.5**	1
Tetracycline	–	>256	1.5	3	ND	>256	ND	1.5	48
	+	>256	1	3	ND	>256	ND	0.75	16
Fold change		1	**1.5**	1	–	1	–	**2**	**3**
Streptomycin	–	>256	4	4	128	48	>256	ND	ND
	+	>256	4	4	128	48	>256	ND	ND
Fold change		1	1	1	1	1	1	–	–

Note: Bold values indicate the antibiotics for which the efflux pumps were minimally active.
Abbreviation: ND, not done (as these isolates were sensitive to the tested antibiotics except for *S. flexneri* 129, which being sensitive to all the tested drugs was taken as a negative control).

the post-antibiotic era.[11] A WHO report published in February 2017 has included *Shigella* in the list of priority pathogens for which new antibiotics are urgently needed.[1] Our study presented here clearly shows the prevalence of MDR *Shigella*, which as specified by WHO, could be a matter of serious concern. In the present study, resistance to quinolone drugs used for treatment of diarrheal diseases was observed to increase markedly through years. Emerging fluroquinolone resistance has also been earlier reported in *Shigella* spp. from India.[5,47] Increase in fluoroquinolone resistance in these isolates could be attributed to an increase in the clinical prescription or over-the-counter sale and use of these drugs.[48,49] Interestingly, the resistance for ampicillin, azithromycin, and cephalosporins (ceftriaxone and cefuroxime) was either not remarkably increased or reduced with years, a desirable feature for drug management of the disease. There are large numbers of reports on emergence of extended spectrum beta-lactamase producers from many countries.[50] Generally, most of the shigellae remained susceptible to cephalosporins as this group of antibiotics are less used in Kolkata for the treatment of acute diarrhea/dysentery. Clonality analysis using PFGE revealed that the clonality of these isolates could not be correlated with their antibiograms.

Molecular analysis of the genetic factors that could be responsible for the observed MDR phenotypes indicated a major role of integrons, plasmids (MGEs), and topoisomerase mutations (chromosome-borne). Most strikingly, integrons of various classes, i.e., class 1 integrons, class 2 integrons, and atypical class 1 integrons were present in the majority (90/95) of the isolates, where the gene cassettes harbored by their variable regions conferred drug resistance traits on the parent isolates. Out of 95, 31 isolates harbored both class 1 and class 2 integrons. In addition to integrons, presence of multiple plasmids in all the isolates could also be the possible source of drug resistance as supported by conjugation experiments. Predominantly, the genes for trimethoprim and aminoglycoside resistance were associated with these three classes of integrons. Typical class 1 integron was present in only one *S. sonnei* isolate, while atypical class 1 integron was found in *S. flexneri*, *S. dysenteriae*, and *S. boydii*. Class 2 integron was highly prevalent in these *Shigella* isolates (83%). Class 2 integrases are non-functional proteins due to an internal stop codon at 179th position of the protein sequence.[46,51] Therefore, the majority of the cassette arrays on class 2 integrons are usually constant. *S. sonnei* NK4846 harbored a new cassette array *InsE-InsO-dfrA1-sat* on class 2 integron with insertion elements and resistance genes for trimethoprim and streptothricin. In an earlier report, *satI* gene cassette was interrupted with the IS911 element on class 2 integron of *S. sonnei* isolates.[52] In another report, a class 2 integron with an IS630 element was found in a *S. flexneri* isolate, and a third report described a class 2 integron with an IS1 in an *E. coli*.[34,53] *S. sonnei* NK4675 harboring the class 2 integrase did not show amplification with primers specific to the variable region of class 2 integron suggesting either the presence of null integron or mutations in the regions where primers anneal for amplifying the variable regions. Therefore, in this study, MGEs such as plasmids, class 1 integron, atypical class 1 integrons, and class 2 integrons seemed to play an important role in dissemination of drug resistance in these isolates.

Previously, *Shigella* spp. were susceptible to co-trimoxazole, but on emergence of resistance to this antimicrobial, treatment recommendations were shifted to quinolone group of antibiotics and azithromycin.[11] Eventually, these bacteria also developed quinolone resistance.[11,13,37,54] In this study, majority of the isolates were resistant to NAL, and resistance to other quinolone antibiotics such as CIP, NOR, and OFX was higher in *S. flexneri* as compared to *S. sonnei.* Resistance to quinolones is generally caused due to mutations in topoisomerase genes, efflux pump activity, and *qnr* and *aac(6′)-Ib-cr* genes.[19] In this study, an interesting array of mutations was observed in the QRDR regions of topoisomerases. Clearly, mutations in GyrA S_{83} or D_{87} positions were chiefly responsible for resistance to NAL. Mutations in GyrA have been shown to be a major reason for resistance to quinolone in various organisms such as *Vibrios*, *Shigella*, and *Salmonella.*[13,19,30,36,45,55] An earlier study from the Democratic Republic of the Congo revealed that these mutations in *Salmonella enterica serover Typhi* were also responsible for the decreased CIP susceptibility.[55] Wherever mutations were detected in both the topoisomerases GyrA and ParC, wider spectrum of resistance to multiple quinolone drugs was observed concomitant with high MIC values. In addition, a V_{196}→A mutation outside the QRDR of GyrA did not appear to contribute toward quinolone resistance.

In synergy test, the isolates resistant to the tested drugs did not show a significant decrease in MIC after disruption of efflux pumps with CCCP. This indicated that efflux pumps did not play a role in mediating resistance to these drugs. Some other laboratories have shown a 64–256 times decrease in MICs for some of the mutants of *Shigella*, but parent isolates showing this substantial decrease in MIC have not been reported.[30]

Present study was not a surveillance study but strictly aimed at understanding the genetic factors responsible for

emergence and dissemination of MDR. Therefore, though the number of isolates used in this study was generally small, it was sufficient to lend an insight into the genetic basis of observed drug resistance phenotypes in this geographical location. Another limitation of the study was that the patients were not followed up to get additional information on duration of shedding of MDR *Shigella* isolates.

To summarize, the study has indicated the prevalence of highly drug-resistant pathogens belonging to the genus *Shigella* from the region of Kolkata. Interplay of large number of genetic factors such as plasmids, integrons, and multiple mutations accounted for the extensive drug resistance found in these isolates.

Acknowledgments

The authors are grateful to Prof VK Chaudhary and Ms Shilpi, University of Delhi, Delhi, for sequencing of the DNA fragments. The authors are thankful to Dr Lina Cavaco, Technical University of Denmark, for sending positive controls for *qnr* genes and Korean Agricultural Culture Collection for providing the *E. coli* J53 strain. The authors also thankfully acknowledge the Puri Foundation for Education in India for providing infrastructure facilities. The technical support and advice provided by Mr Priyabrata Mohanty, Mr K Vinothkumar, Ms Aneri Shah, and Mr Shaileshkumar Bhalara are thankfully acknowledged.

The laboratory is supported by the grants from Indian Council of Medical Research, New Delhi, India (AMR/49/11-ECDI), and Gujarat State Biotechnology Mission, Department of Science and Technology, Government of Gujarat (GSBTM/MD/PROJECTS/SSA/1535/2013-14). NR is a recipient of Senior Research Fellowship from Indian Council of Medical Research, New Delhi, India.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

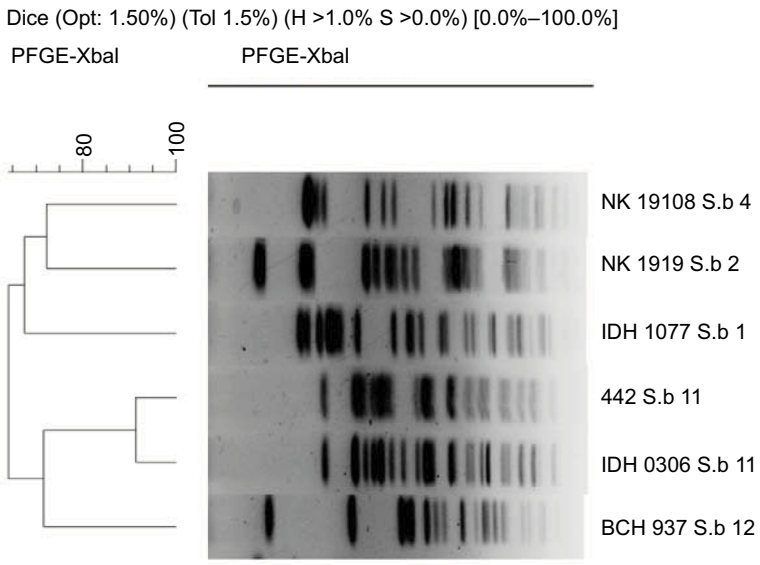

Figure S1 Dendrogram of *Xba*I-digested pulsed-field gel electrophoresis profiles of the clinical isolates of *Shigella boydii* isolates. Scale bar indicates degree of similarity.

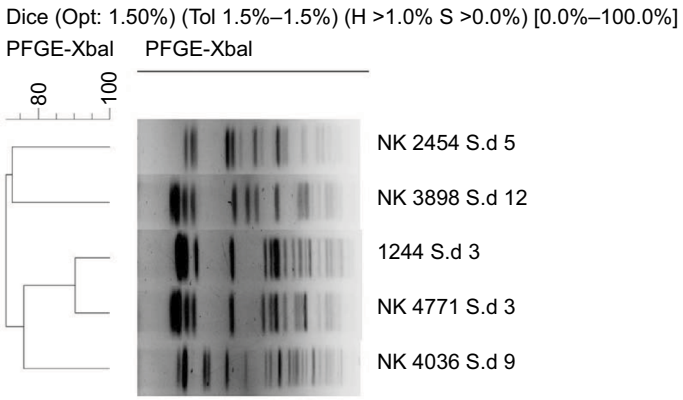

Figure S2 Dendrogram of *Xba*I-digested pulsed-field gel electrophoresis profiles of the clinical isolates of *Shigella dysenteriae* isolates. Scale bar indicates degree of similarity.

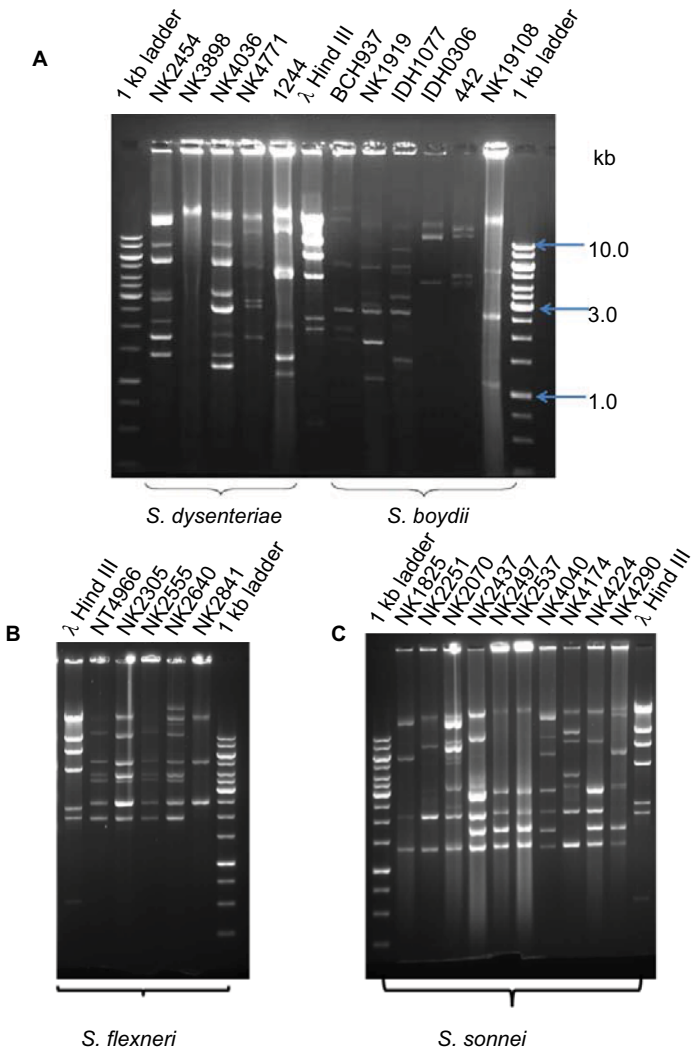

Figure S3 1% agarose gel analysis of plasmid DNA profile of *S. dysenteriae* and *S. boydii* isolates (**A**); *S. flexneri* isolates (**B**); *S. sonnei* isolates (**C**). Marker positions have been indicated on left.

Abbreviations: *S. dysenteriae*, *Shigella dysenteriae*; *S. boydii*, *Shigella boydii*, *S. flexneri*, *Shigella flexneri*; *S. sonnei*, *Shigella sonnei*.