4. Rsults and Discussion

Role of various mobile genetic elements in multidrug resistant *Shigella* isolates

4.1. Introduction

Shigella has been one of the antibiotic resistant priority pathogens in the list published by WHO in February 2017 [WHO, 2017]. 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 is caused by *Shigella flexneri*, *S. sonnei*, *S. dysenteriae* and *S. boydii,* and mostly occurs in resource-poor communities that do not have proper sanitation [Niyogi, 2005]. Annually, the number of *Shigella* episodes is estimated to be 80-165 million and around 74,000-6,00,000 deaths occur worldwide [Bowen, 2016]. Antibiotics are the mainstay of treatment for shigellosis but with increasing multidrug resistance (MDR), treatment of this disease has become complicated. There have been many reports around the world indicating the rising problem of MDR in shigellosis [Bhattacharya et al., 2015; WHO, 2014; Nogrady et al., 2013; Mandal et al., 2012; Ghosh et al., 2011; Chang et al., 2011; Khurana et al., 2008; Pazhani et al., 2008; Pazhani et al., 2005]. Increase in resistance to fluoroquinolones, third generation cephalosporins and azithromycin has been reported globally [Taneza and Mewara, 2016].

Resistance to antibiotics in bacteria has been attributed to inherent as well as mobile genetic factors. For example, inherently, an organism may lack the target of antibiotics (mutations in target genes) or can export antibacterial agents before they exert their effect (efflux pumps) or may restrict the antibiotic entry into the bacteria (porins) or the bug may inactivate/modify the antibiotics (β-lactams/ aminoglycosides). Mobile genetic elements (MGEs) include plasmids, integrons and integrative conjugative elements (ICEs) which are potent vectors for acquisition and dissemination of antibiotic resistance genes among the bacterial populations [Bhardwaj et al., 2014; Carattoli, 2013; Cambray et al., 2010; Pan et al., 2006; Luck et al., 2001; Waldor et al., 1996]. Plasmids evolved as an essential part of the bacterial genome, providing a large array of resistance genes such as carbapenem resistance, extended spectrum beta-lactamase and quinolone resistance genes that can be easily

exchanged among bacteria by horizontal gene transfer [Carattoli, 2013]. Integrons are gene capture elements and till date, five classes of integrons have been well characterized based on their integrase sequences [Cambray et al., 2010]. 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 expression of any number of gene cassettes inserted at the *attI*1 site in a suitable orientation [Cambray et al., 2010]. The 3′CS region usually comprises of *qacE*Δ1 encoding resistance to quaternary ammonium compounds and *sul*1 encoding resistance to sulphonamides [Cambray et al., 2010]. 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 *sul*1 [Pan et al., 2006]. Atypical class 1 integron was first found on the pathogenicity island carrying *Shigella* resistance locus, on the chromosome of *S. flexneri* 2a strain YSH6000 where it harboured two resistance determinants for chloramphenicol and tetracycline [Pan et al., 2006; Luck et al., 2001]. SXT elements are the best-studied examples of ICE which harbour the sulphonamide, trimethoprim, streptomycin and chloramphenicol resistance genes [Waldor, 1996]. This element was first found in *Vibrio cholerae* O139 strain MO10 isolated from Madras, India in 1992 [Waldor et al., 1996].

Shigellosis is endemic in India and one of the major cause of diarrhoea in this country [Taneza and Mewara, 2016; Niyogi, 2005]. 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 is important to understand these bacterial infections. The present study was executed with this aim. Ninety-five clinical isolates of *Shigella* were analysed for their antibiotic resistance profiles. Various genetic factors borne on chromosomes or plasmids or integrons were deciphered as reasons for the observed MDR phenotypes.

4.2. Results

4.2.1. Bacterial isolates and their resistance profiles

The work was carried out with 95 clinical isolates of *Shigella* spp. procured from NICED, Kolkata. These ninety-five clinical isolates of *Shigella* consisted of 42 *Shigella flexneri*, 42 *S. sonnei*, 6 *S. boydii* and 5 *S. dysenteriae* isolates. Among *S. flexneri* isolates, serotype 2a was the most common (22/42), followed by serotype 3a (11/42). Rest of the 9 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.

Resistance profile of these isolates was determined using antibiotic susceptibility tests in accordance with the CLSI (Clinical Laboratory Standard Institute) criteria. Antibiotic susceptibility test was performed at least three times for all the *Shigella* isolates. *E. coli* ATCC25922 was taken as standard in this experiment for quality control. Results revealed that out of 95 isolates, 93 were resistant to three or more antibiotics (Figure 4.1 for *S. flexneri* and *S. sonnei,* and Table 4.1 for *S. dysenteriae* and *S. boydii*). Isolates showing intermediate resistance or complete resistance were considered as a resistance trait. More than 90% *S. flexneri* isolates showed resistance to streptomycin, trimethoprim and tetracycline, and 69-85% were resistant to nalidixic acid, chloramphenicol, co-trimoxazole and ciprofloxacin (Figure 4.1a and Appendix-11). Above 80% of *S. sonnei* isolates showed resistance to nalidixic acid, kanamycin, trimethoprim, co-trimoxazole, streptomycin and azithromycin (Figure 4.1b and Appendix-12). All the isolates of *S. dysenteriae* and *S.boydii* showed multiple drug resistance except *S. boydii* NK19108, which was found to be sensitive to all the drugs tested (Table 4.1).

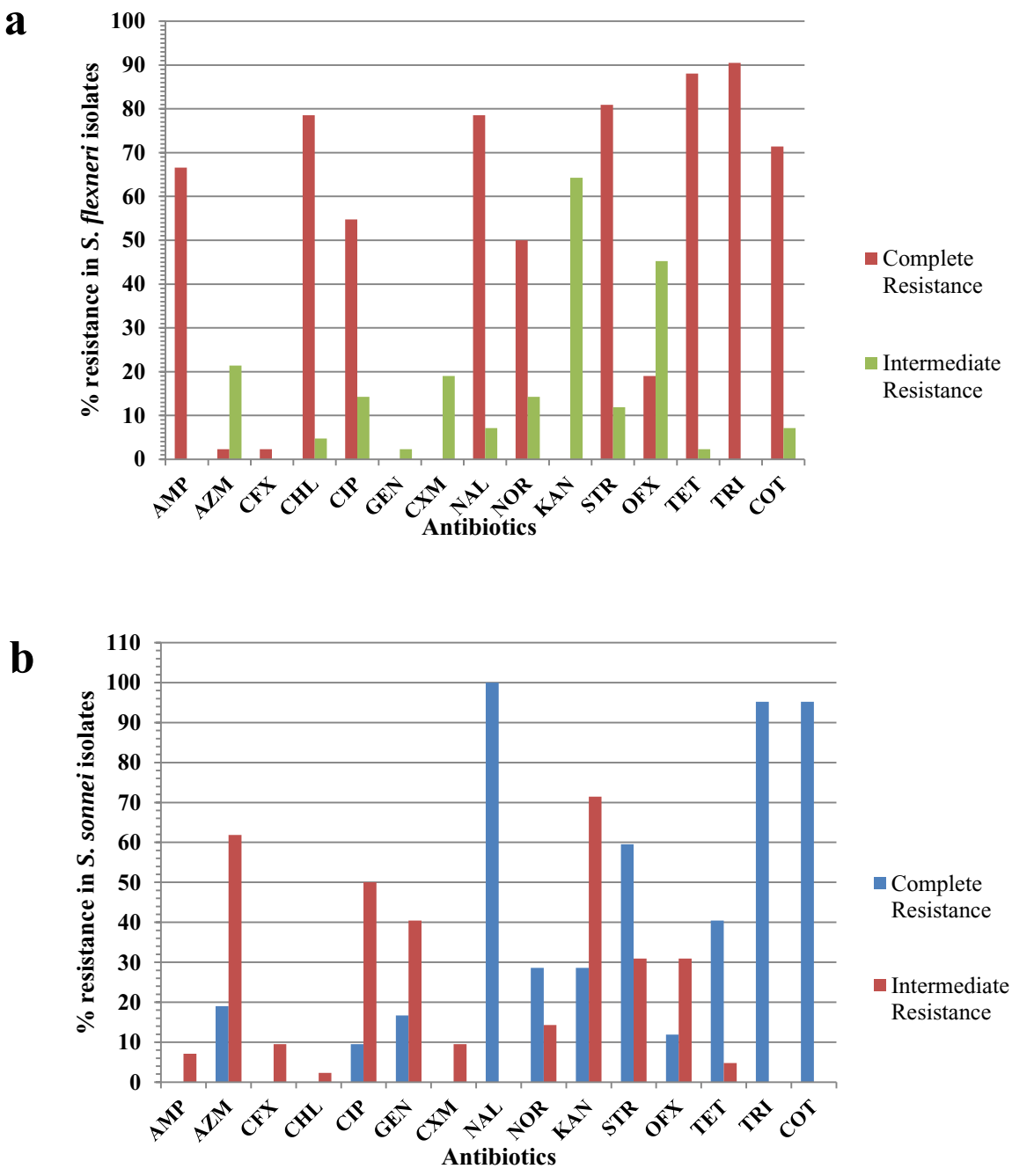


Figure 4.1. Antibiotic susceptibility profile of clinical isolates of *Shigella flexneri* (a) and *Shigella sonnei* (b). AMP, ampicillin (10 µg); AZM, azithromycin (15 µg); CFX, ceftriaxone (30 µg); CHL, chloramphenicol(30 µg); CIP, ciprofloxacin (5 µg); GEN, gentamicin (10 µg); CXM, cefuroxime (30 µg); NAL, nalidixic acid (30 µg); NOR, norfloxacin (10 µg); KAN, kanamycin (30 µg); STR, streptomycin (10 µg); OFX, ofloxacin(5 µg); TET, tetracycline (30 µg); TRI, trimethoprim (5 µg); COT, co-trimoxazole (1.25 µg trimethoprim/23.75 µg sulfamethoxazole).

Table 4.1. Antibiotic susceptibility profiles of *Shigella dysenteriae* and *Shigella boydii* isolates

Sr. No	NICED No.	Strain Description	Date of Isolation	Antibiogram		Presence of class 1 integron	Presence of class 2 integron
				Resistant	Intermediate		
1	NK2454	*S. dysenteriae* (5)	2003	NAL, STR, TET, TRI, COT	AZM, CIP, KAN	-	+
2	NK3898	*S. dysenteriae* (12)	1/12/04	NAL, TET, TRI, COT	-	-	+
3	NK 4036	*S. dysenteriae* (9)	22/03/05	STR, TRI, COT	AZM	-	+
4	NK 4771	*S. dysenteriae* (3)	04/04/07	AMP, NAL, TET, TRI.	CIP , STR, COT	+	+
5	1244	*S. dysenteriae* (3)	2008	AMP, CHL, NAL, KAN, TET, TRI, COT	AZM, CIP, GEN, STR	+	+
6	BCH 937	*S. boydii* (12)	1996	STR, TET, TRI, COT	KAN	-	+
7	NK 1919	*S. boydii* (2)	03/01/02	CHL, TET, TRI	AZM. STR	+	-
8	442	*S. boydii* (11)	2008	NAL, STR, TRI, COT	AZM, GEN, KAN	-	+
9	IDH1077	*S. boydii* (1)	27/10/08	NAL, STR, TRI, COT	AZM, KAN	-	+
10	IDH0306	*S. boydii* (11)	28/02/08	NAL, STR, TRI, COT	-	-	+
11	NK19108	*S. boydii* 4	Not known	-	-	-	-

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

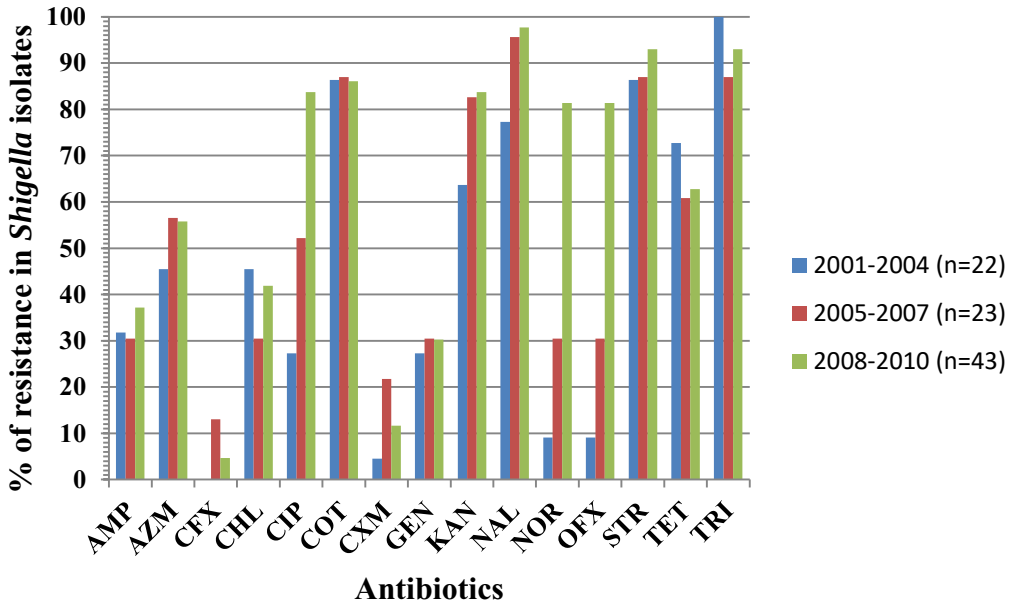


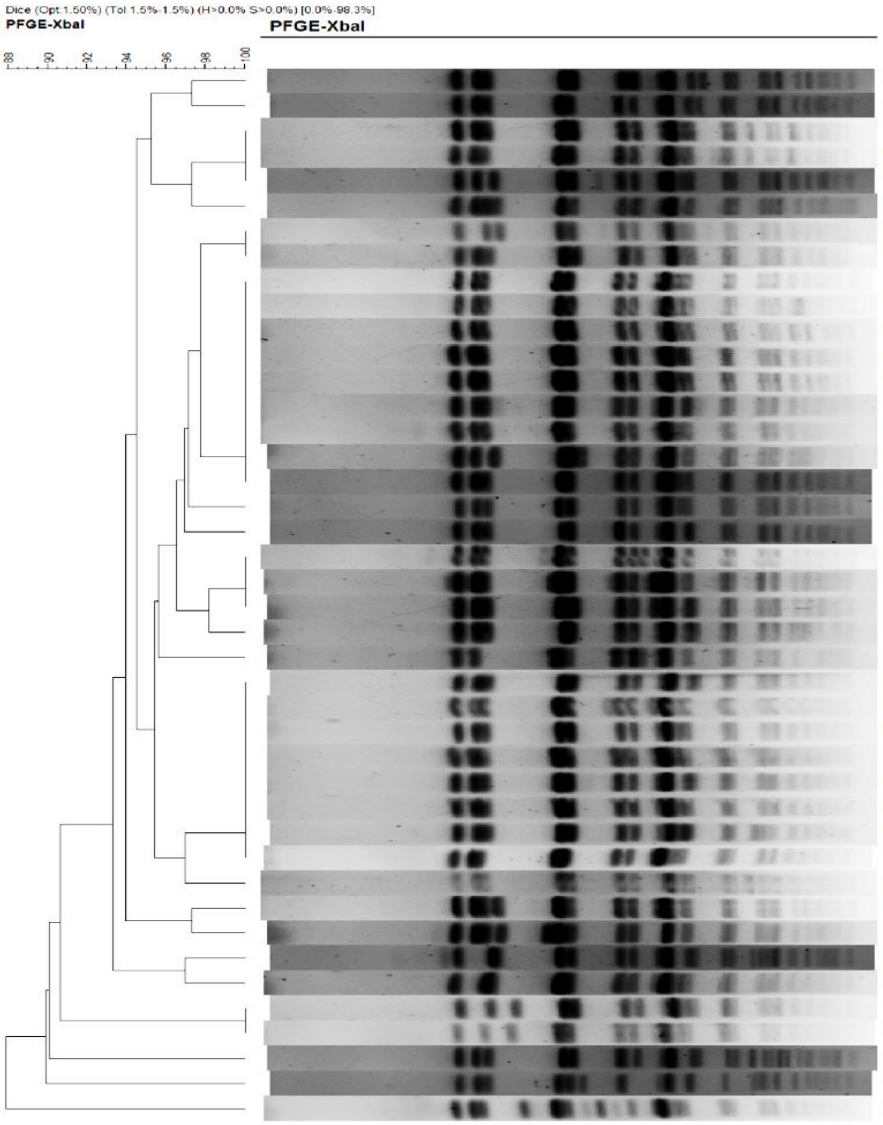
Figure 4.2. Year-wise percentage of drug resistance in *Shigella* isolates. AMP, ampicillin (10 µg); AZM, azithromycin (15 µg); CFX, ceftriaxone (30 µg); CHL, chloramphenicol (30 µg); CIP, ciprofloxacin (5 µg); GEN, gentamicin (10 µg); CXM, cefuroxime (30 µg); NAL, nalidixic acid (30

μg); NOR, norfloxacin (10 μg); KAN, kanamycin (30 μg); STR, streptomycin (10 μg); OFX, ofloxacin (5 μg); TET, tetracycline (30 μg); TRI, trimethoprim (5 μg); COT, co-trimoxazole (1.25 μg trimethoprim/23.75 μg sulfamethoxazole).

Year-wise analysis of the above antibiotic susceptibility profiles revealed that during ten years, resistance to fluoroquinolone drugs such as ciprofloxacin, norfloxacin and ofloxacin was remarkably increased as compared to nalidixic acid (Figure 4.2). 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) in the years 2005 to 2007. This resistance decreased in later years.

4.2.2. Clonality in *Shigella* isolates

The PFGE experiments and gel analysis were carried out to study the clonality among these clinical isolates. The genotypes obtained by PFGE were analysed separately for each *Shigella* species; *S. sonnei* (Figure 4.3), *S. flexneri* (Figure 4.4), *S. boydii* (Figure 4.5) and *S. dysenteriae* (Figure 4.6). PFGE analysis of *Xba*I digested chromosomal DNA of the 42 *S. sonnei* strains yielded 21 reproducible PFGE patterns. As shown in Figure 4.3, two major PFGE patterns were shared by 9 isolates (21%) from the years 2002, 2004, 2006 and 2009, and by 8 isolates (19%) from the years 2007 and 2008. The similarity index for all *S. sonnei* isolates were 92-100%, suggesting that most of the *S. sonnei* strains were clonally related to each other. PFGE analysis of *S. flexneri* after digestion with *Not*I revealed serotype-specific clusters, with approximately 80–100% similarity within each serotype. *S. flexneri* exhibited three distinct clades (Figure 4.4). 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 approximately 80% similarity and *S. dysenteriae* isolates were also observed to be 80% clonally related to each serotype. As could be observed from Figures 4.3 and 4.4, 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.



	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	-	+

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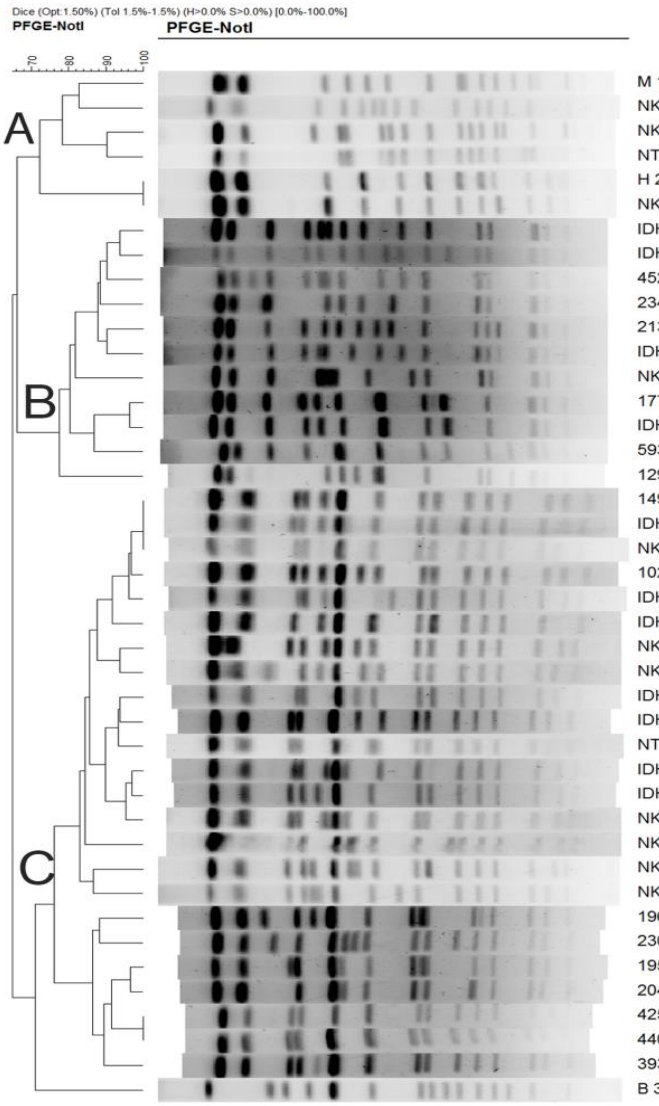
Figure 4.3. Dendrogram of *Xba*I- digested pulsed-field gel electrophoresis profiles of clinical isolates of *Shigella sonnei*. Scale bar indicates degree of similarity



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

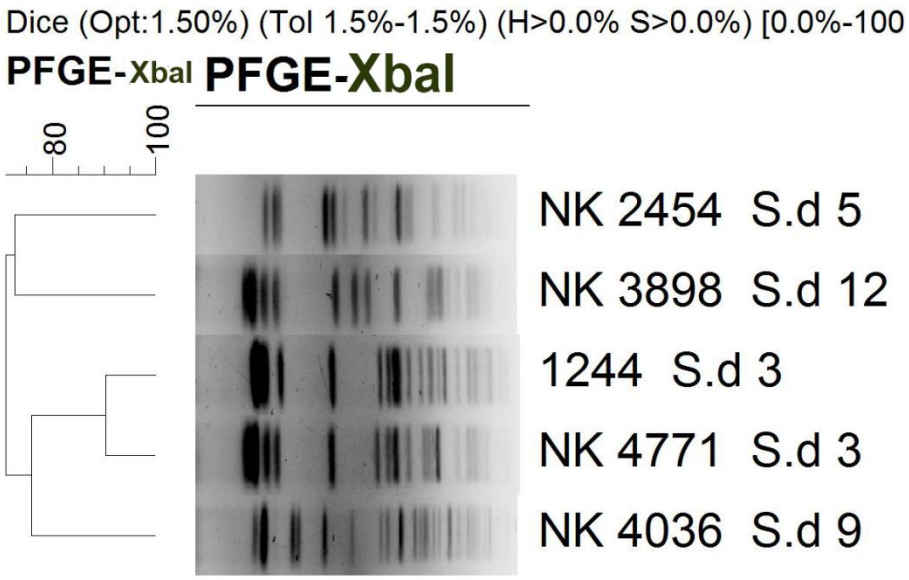


Figure 4.5. Dendrogram of *Xba*I- digested pulsed-field gel electrophoresis profiles of clinical isolates of *Shigella boydii*. Scale bar indicates the degree of similarity.

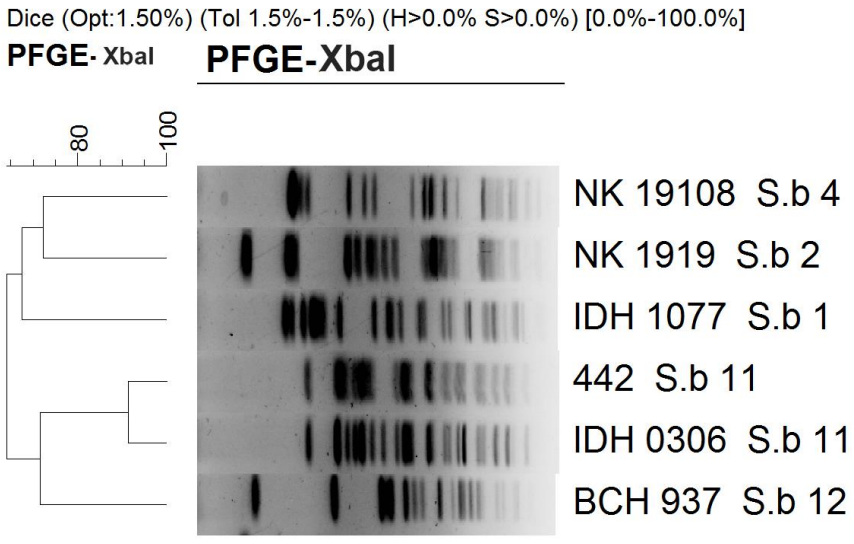


Figure 4.6. Dendrogram of *Xba*I- digested pulsed-field gel electrophoresis profiles of clinical isolates of *Shigella dysenteriae*. Scale bar indicates degree of similarity

4.2.3. Presence of class 1 and class 2 integrons

The presence of integrons and SXT elements as carriers of drug resistance genes was assessed in the clinical isolates of *Shigella* spp. by PCR (Section 2.7; Table 2.6; Figure 4.7 and 4.8). Analysis of integrons and SXT elements revealed that the presence of class 1 and class 2 integrons while class 3 integron and SXT element were absent.

As interpreted from the Table 4.2, typical class 1 integron was present only in one *S. sonnei* isolate IDH0734 that harboured ~750 bp variable region (Figure 4.7c). Atypical class 1 integron were found in *S. flexneri*, *S. dysenteriae* and *S. boydii* isolates (Table 4.2) and PCR products of their variable regions showed a band 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* (Figure 4.7d).

The analysis of integrase from class 2 integron revealed that 83.2% isolates (79/95) were found to be positive (Table 4.2; Figure 4.8a) and their variable region showed amplicons with varying sizes of 1.4 kb or 2.2 kb except two *S. sonnei* isolates (Figure 4.8b). One of these two harboured 2.4 kb variable region while the other one didn't show any amplification. The detailed sequence analysis of integrons was described in next section.

As depicted in the Table 4.2, there was simultaneous presence of both atypical class 1 and class 2 integrons in 30 isolates out of 95 isolates of *S. flexneri* (n=28) and *S. dysenteriae* (n=2) (Table 4.2), and similarly *S. sonnei* IDH0734 carried both typical class 1 and class 2 integron (Table 4.2). Importantly, barring five, 90 out of 95 isolates harbored integron(s) with drug resistance genes. This underlined the importance of integrons in governing the drug resistance phenotypes of this population of *Shigella* isolates from Kolkata region.

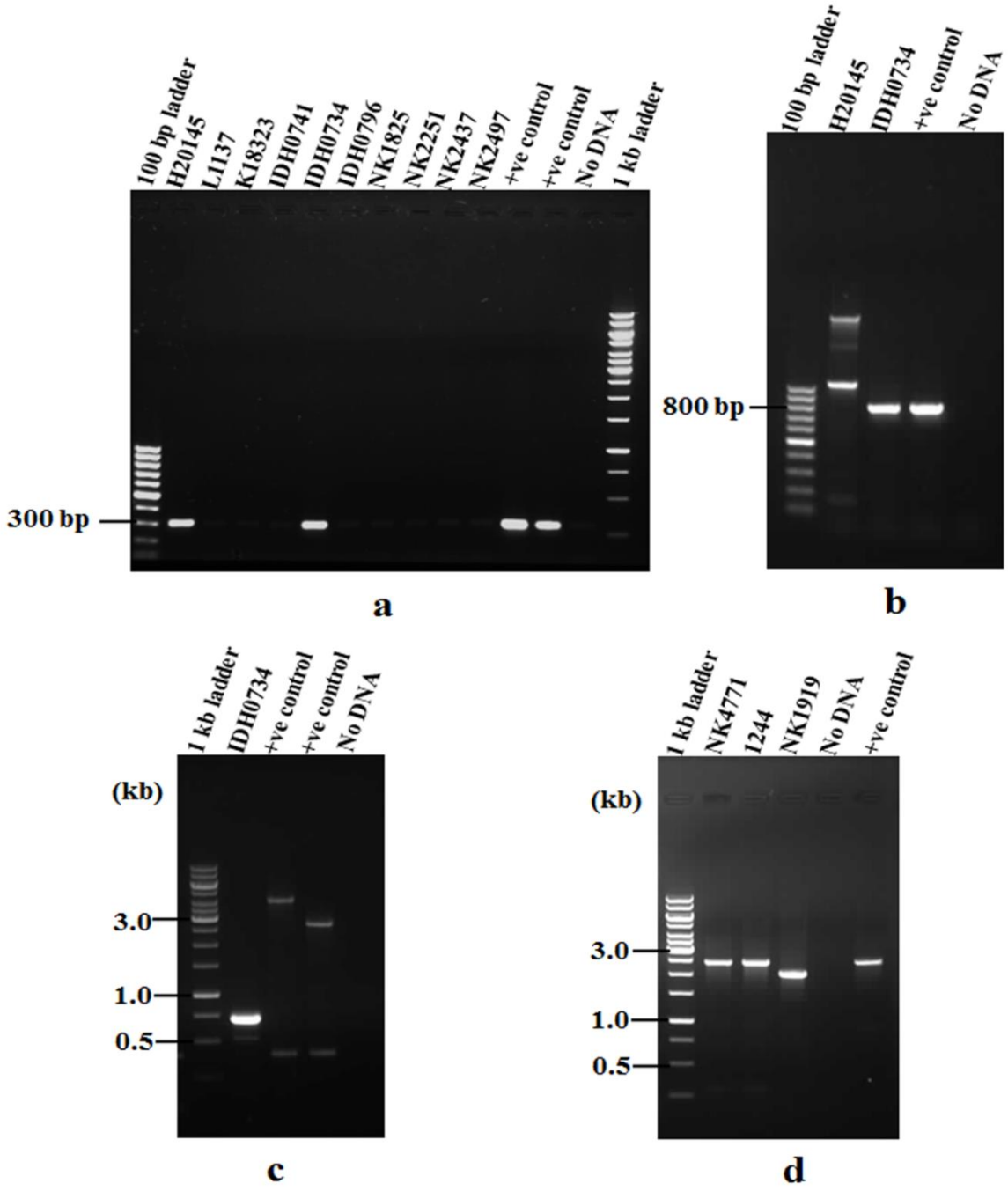


Figure 4.7. PCR analysis for class 1 integron. Agarose gel analysis of PCR amplicons obtained from: 5' conserved segment (5'CS) of class 1 integron with L2, L3 primers (a); 3' conserved segment (3'CS) of class 1 integron with qacEΔ1, sul1-B primers (b); variable region of typical class 1 integron with in F, in B primers (c); variable region of atypical class 1 integron with Int1CA F, IS1CA R primers (d). The sample identity has been indicated on top of each lane. 1 kb DNA ladder (Thermo Scientific) and 100 bp DNA ladder (Thermo Scientific) were used as markers

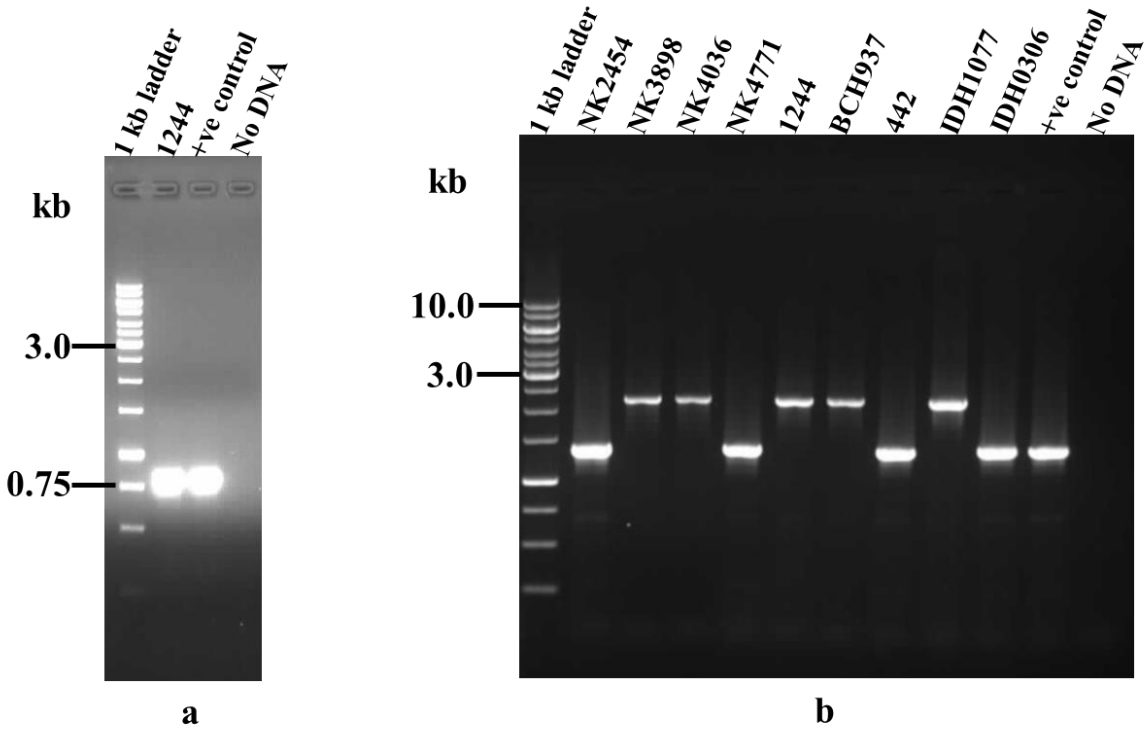


Figure 4.8. PCR analysis for class 2 integron. Agarose gel analysis of PCR amplicons obtained from: 5' conserved segment (5'CS) of class 2 integron with Int 2 F, Int 2 R primers (a); variable region of class 2 integron with Int 2 VA F, Int 2 VA R primers (b). The sample identity has been indicated on top of each lane. 1 kb DNA ladder (Thermo Scientific) was used as a marker.

Table 4.2. Presence of integrons in *Shigella* isolates.

Isolates	Total no. of isolates	Presence of Typical Class 1 Integron (Primers L2/L3, In F/In B, qac EΔ1/Sul1B)	Variable region (Band Size in kb)	Gene cassettes found on Class 1 Integron	Presence of Atypical Class 1 Integron (Primers L2/L3, IntCA F/IS1CA R)	Variable region (Band Size in kb)	Gene cassettes found on Atypical Class 1 Integron	Presence of Class 2 Integron (Primers Int 2 F/Int 2 R, Int 2 VA F/Int 2VA R)	Variable region (Band Size in kb)	Gene cassettes found on Class 2 Integron	Presence of both the integrons Class 1 (typical/atypical) and Class 2	Isolates without any integrons
S. 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
S. sonnei	42	1	0.75	*dfrV*	0	0	0	41	1.4, 2.4	*dfrA1-sat, InsE-InsO-dfrA1-sat*	1	1
S. boydii	6	0	0	0	1	2.0	*dfrA1-aadA*	4	2.2, 1.4	*dfrA1-sat-aadA, dfrA1-sat*	0	1
S. 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**

4.2.4. Sequence analysis of integrons

DNA sequences of amplicons corresponding to conserved segments of integrons, variable regions of class 1 (750 bp) or atypical class 1 integron (2.4 kb and 2.0 kb) and variable regions of class 2 integrons (1.4 kb, 2.2 kb and 2.4 kb) were analysed and submitted to GenBank as described in section 2.10.

Sequences corresponding to 5'CS of class 1 and atypical class 1 integrons, and 3'CS of class 1 integron were analysed and submitted to GenBank (**KX768278-KX768283; KX777252**). Sequence analysis of the variable region of typical class 1 integron from *S. sonnei* IDH0734 revealed that it encoded *dfrV* responsible for trimethoprim resistance (Table 4.3). The 2.0 kb variable region of atypical class 1 integron in *S. flexneri* 593 and *S. boydii* NK1919 showed the cassettes *dfrA1-aadA* (Table 4.2 and Table 4.3). The 2.4 kb variable region of atypical class 1 integron from *S. flexneri* 102 and *S. dysenteriae* 1244 carried bla_{oxa}-*aadA* gene cassettes (Table 4.2 and Table 4.3). 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 analysed and submitted to GenBank (**KX536824-KX536827**; **KX463270-KX463271**). Results revealed that these were the genes encoding non-functional class 2 integrase as they carried an internal stop codon TAA. This was in accordance with the 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 [Hansson et al., 2002]. Sequences of variable regions of class 2 integrons were analysed from the representative isolates of each species. The 1.4 kb band from *S. flexneri* H20145, *S. boydii* 442 and *S. sonnei* L1137 harbored *dfrA1-sat* cassettes while 2.2 kb band from *S. flexneri* 102 and *S. dysenteriae* 1244 carried *dfrA1-sat-aadA* gene cassettes (Table 4.3). *S. sonnei* NK4846 harbored a new cassette array *InsE-InsO-dfrA1-sat* of 2.4 kb band size on class 2 integron (Table 4.3). 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.

Table 4.3. Integron analysis in *Shigella* isolates

Strain	Strain ID NO.	Antibiogram		Types of Integrons	Band size	Cassettes	GenBank accession no.
		Resistant	Intermediate				
S. flexneri 2a	102	AMP, CHL, CIP, NAL, NOR, STR, OFX, TET, TRI, COT	CXM, KAN	Atypical class 1, class 2	2.4 kb 2.2 kb	bla_{oxa}-*aadA*, *dfrA1-sat-aadA*	KX817769 KX817767
S. flexneri 3a	593	CHL, CIP, NAL, NOR, OFX, TET, TRI, COT	KAN, STR	Atypical class 1	2.0 kb	*dfrA1-aadA*	KX817770
S.flexneri 1b	H20145	AMP, CHL, STR, TET, TRI, COT	NAL, KAN	Atypical class 1, class 2	2.4 kb 1.4 kb	bla_{oxa}-*aadA, dfrA1-sat*	KX817766
S.sonnei	NK4846	AZM, NAL	CFX, STR, GEN, KAN, TET	Class 2	2.4 kb	*InsE-InsO-dfrA1-sat*	KX781232
S. sonnei	IDH0734	NAL, NOR, KAN, STR, TRI, COT	AZM, CIP, OFX	Class 1, Class 2	0.75 kb 1.4 kb	*dfrV dfrA1-sat*	KX777251
S. sonnei	L1137	NAL, STR, TRI, COT	AMP, AZM, CHL, CXM, KAN	Class 2	1.4 kb	*dfrA1-sat*	KX781233
S.boydii 11	442	NAL, STR, TRI, COT	AZM, GEN, KAN	Class 2	1.4 kb	*dfrA1-sat*	KX817768
S.boydii 2	NK1919	CHL,TET,TRI	AZM,STR	Atypical class 1	2.0 kb	*dfrA1-aadA*	KX817771
S. dysenteriae 3	1244	AMP,CHL, NAL, KAN, TET, TRI, COT	AZM,GEN, STR	Atypical class 1, class 2	2.4 kb 2.2 kb	bla_{oxa}-*aadA*, *dfrA1-sat-aadA*	KX951422 KX792556

4.2.5. Quinolone resistance

Out of 95 isolates, 85 isolates were resistant to quinolones. These 85 quinolone resistant isolates were analysed for plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrD*, *qnrB*, *qnrS*, *oqxA*, *aac(6')-Ib-cr* and *qnrC*) by multiplex PCR (section 2.7). Positive results were re-confirmed in a monoplex PCR. Results of multiplex PCR/monoplex PCR revealed that the PMQR genes were absent in these isolates except *S. flexneri* isolate M11560 that harbored a *qnrS* gene (Figure 4.9).

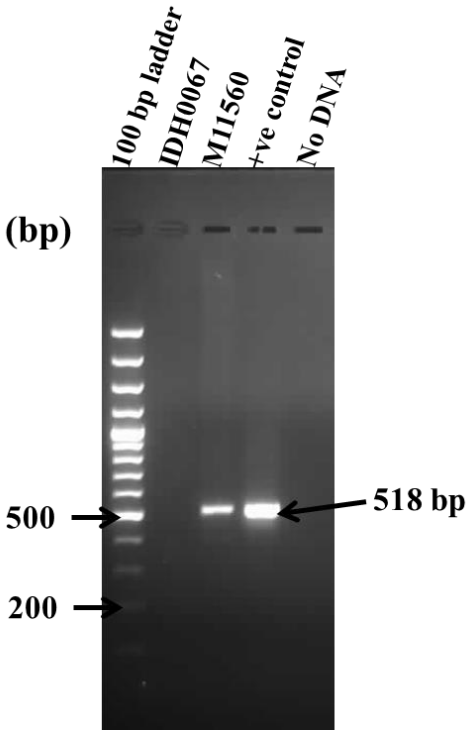


Figure 4.9. Agarose gel analysis (2%) of PCR products of the *qnrS* gene. Lane IDH0067 and Lane M11560: PCR amplicons of *qnrS* gene from these *Shigella* isolates; Lane 4, positive control; Lane 5, negative control. The sample identity has been indicated on the top of each lane. The position of amplicon size has been indicated to the right.

Mutations in a region called the quinolone resistance determining regions (QRDRs) of subunits of DNA gyrase or topoisomerase IV, results in reduced drug affinity to these enzymes [Kakinuma et al. 2012]. The substitution of hydrophilic amino acid (serine) by hydrophobic residues (leucine or isoleucine), or substitution of acidic residue (aspartic acid) by basic amino acid (asparagine) or substitution of negatively charged amino acid (glutamic acid) by positively charged residue (lysine)

are responsible for the conformational change of quinolone binding pocket (the region where quinolones interact with both QRDR of enzyme and cleaved DNA) [Heddle and Maxwell, 2002]. This conformational change reduces the susceptibility of the target enzymes to the quinolones.

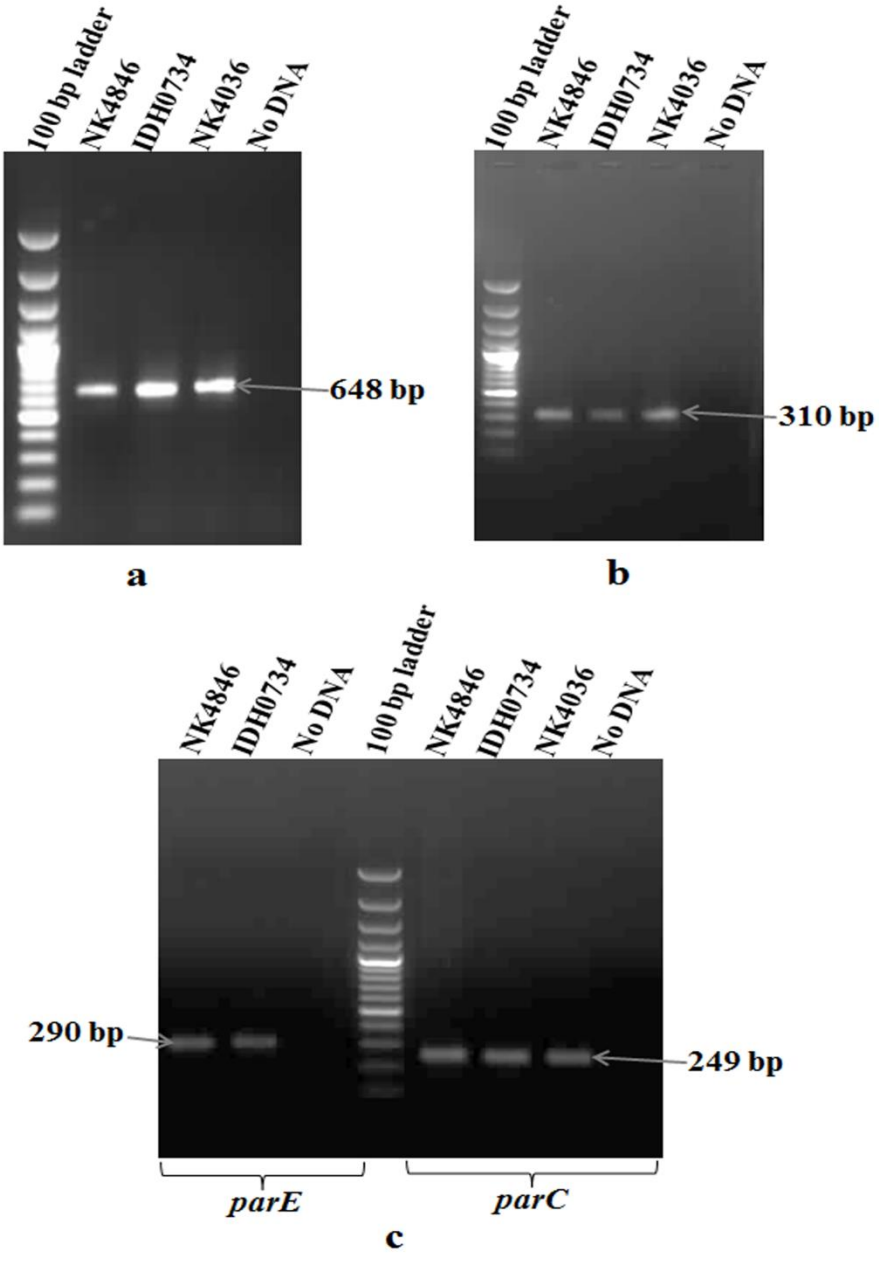


Figure 4.10. Agarose gel analysis of quinolone resistance determining regions of topoisomerases: *gyrA* (a); *gyrB* (b); *parE* and *parC* (c). The DNA samples used as templates are indicated on top of each lane. 100 bp DNA ladder (Thermo Scientific) was used as a marker.

For deciphering the quinolone resistance due to mutations, sequences from the QRDRs of topoisomerases i.e. GyrA, GyrB, ParC and ParE were analyzed from the quinolone resistant/sensitive isolates of each *Shigella* species and submitted to GenBank (Figure 4.10; Table 4.4). These isolates were also analysed for MICs of four quinolones [nalidixic acid (NAL), ciprofloxacin (CIP), ofloxacin (OFX) and norfloxacin (NOR)] in order to correlate the effect of mutations on their quinolone resistance phenotypes. As depicted in the Table 4.4, the increase in a number of mutations directly correlated with an increase in resistance to the spectrum of quinolones. A single mutation S_{83}→L or D_{87}→Y in QRDR region of GyrA were observed in four isolates (1244, 442, NK4219 and NK4846) and these were correleted with their high MIC values of NAL. Multiple mutations in QRDR regions of GyrA (S_{83}→L and D_{87}→N) and ParC (S_{80}→I and E_{84}→G) in *S. flexneri* 2a strain 102 was responsible for its high MIC values of >256 µg/mL for CIP, NAL, NOR and >32 µg/mL for OFX. Similarly, two mutations in GyrA (S_{83}→L and D_{87}→G) and one mutation S_{80}→I in ParC regions of *S. sonnei* isolates IDH1694 and IDH0734 was associated with their moderate resistance to quinolones.

Novel mutations H_{211}→Y or V_{196}→A outside the QRDR region of GyrA were reported earlier [Taneza et al., 2015; Azmi et al., 2014]. Either of these two was also detected in *S. flexneri* isolates 102 and NK2640, and *S. sonnei* isolates NK4219 and NK1694 but it could not be correlated with the decreased susceptibility of quinolones.

Therefore, mutations in GyrA and ParC played a major role in quinolone resistance in this isolates.

Table 4.4. Topoisomerase mutations in quinolone resistance determining regions of *Shigella* isolates.

Strain	Strain ID no.	Quinolone resistance	MIC				Mutations in QRDR regions of Topoisomerase		GenBank Accession No.
			CIP	NAL	NOR	OFX	GyrA	ParC	
S. flexneri 2a	102	CIP, NAL, NOR, OFX	>256	>256	>256	>32	S_{83}→ L D_{87}→ N H_{211}→Y	S_{80} → I E_{84} → G	KX817788; KX817790; KX817792; KX817794
S. flexneri 2a	NK2640	Sensitive to all quinolones	0.032	1	0.125	0.047	V_{196}→A	No Mutation	KX817789; KX817791; KX817793; KX817795
S. sonnei	NK4219	NAL	0.094	>256	0.38	0.19	D_{87}→ Y V_{196}→A	No Mutation	KX583660; KX583664; KX583668; KX583672
S. sonnei	IDH1694	CIP, NAL, NOR, OFX	32	>256	48	8	S_{83}→ L D_{87}→G V_{196}→A	S_{80} → I	KX583661; KX583665; KX583669; KX583673
S. sonnei	NK4846	NAL	0.125	>256	0.25	0.25	S_{83}→ L	No Mutation	KX583663; KX583667; KX583671; KX583675
S. sonnei	IDH0734	CIP, NAL, NOR, OFX	24	>256	24	12	S_{83}→ L D_{87}→G	S_{80} → I	KX583662; KX583666; KX583670; KX583674
S. boydii 11	442	NAL	0.19	48	0.38	0.19	S_{83}→ L	No Mutation	KX817781; KX817783; KX817785; KX817787
S. boydii 2	NK1919	Sensitive to all quinolones	0.047	1.5	0.125	0.047	No Mutation	No Mutation	KX817780; KX817782; KX817784; KX817786
S.dysenteriae 3	1244	NAL	0.19	>256	0.38	0.50	S_{83}→ L	No Mutation	KX817773; KX817775; KX817777; KX817779
S.dysenteriae 9	NK4036	Sensitive to all quinolones	0.047	1	0.064	0.064	No Mutation	No Mutation	KX817772; KX817774; KX817776; KX817778

Note: no mutations were observed in GyrB and ParE from all these isolates

CIP, ciprofloxacin; NAL, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin

4.2.6. Efflux pump activity

The role of efflux pumps in the antibiotic resistance phenotype of the isolates used in above studies was assessed using synergy test. MIC for a different group of antibiotics was evaluated with or without efflux pump inhibitor CCCP and change in MIC was observed (Table 4.5). As interpreted from Table 4.5, 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. It was also observed that the *S. flexneri* 2a isolate 102 had very high MICs in all the drugs tested.

Table 4.5. Efflux pump activity in *Shigella* isolates by synergy test

Antibiotics	**Antibiotics + CCCP (4 mg/L)**	***S. flexneri* 102**	***S. flexneri* 129**	***S. sonnei* NK4846**	***S. sonnei* IDH0734**	***S. dysenteriae* 1244**	***S. boydii* 442**
Ampicillin	**-**	>256	1.5	ND	ND	>256	ND
	+	>256	1	ND	ND	>256	ND
Fold change		1	**1.5**	-	-	1	-
Chloramphenicol	**-**	>256	1.5	ND	ND	>256	ND
	+	>256	1	ND	ND	>256	ND
Fold change		1	**1.5**	-	-	1	-
Ciprofloxacin	**-**	>256	0.125	0.19	6	0.25	ND
	+	>256	0.19	0.19	6	0.125	ND
Fold change		1	**0.65**	1	1	**2**	-
Trimethoprim	**-**	>32	0.75	ND	>32	>32	>32
	+	>32	0.75	ND	>32	>32	>32
Fold change		1	1	-	1	1	1
Tetracycline	**-**	>256	1.5	3	ND	>256	ND
	+	>256	1	3	ND	>256	ND
Fold change		1	**1.5**	1	-	1	-
Streptomycin	**-**	>256	4	4	128	48	>256
	+	>256	4	4	128	48	>256
Fold change		1	1	1	1	1	1

Note: Bold values indicate the antibiotics for which the efflux pumps were minimally active.

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)

4.2.7. Transfer of resistance by conjugation

Shigella isolates harbored multiple plasmids ranging from 1.0 kb to 23 kb as observed on an agarose gel (Figure 4.11 for selected isolates). Based on antibiotic susceptibility, plasmid profile and presence of integrons, six isolates were selected from the present population to examine the transferability of their resistance traits through conjugation (Table 4.6). The choice of recipient *E. coli* was based on the resistance profile of donor isolates. For example, 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 in the range of 10^{-6} to 10^{-7} transconjugants per recipient cell.

These experiments showed the transferability of resistance traits such as ampicillin, azithromycin, chloramphenicol, ciprofloxacin, co-trimoxazole, kanamycin, nalidixic acid, ofloxacin, streptomycin, tetracycline and trimethoprim to recipient *E. coli* establishing the role of plasmids in horizontal gene transfer (Table 4.6). Most commonly, the resistance traits for co-trimoxazole and trimethoprim were transferred in all the cases confirming the carriage of resistance for these genes on plasmids. Transconjugants were further analysed for plasmid profile and for the presence of mobile genetic elements as described in section 2.5.2.1 and 2.7. The plasmid profile of transconjugants revealed that multiple plasmids were transferred during conjugation (Figure 4.12). Integron analysis revealed that atypical class 1, typical class 1 and class 2 integrons were transferable during conjugation from donor isolates to the *E. coli* recipients. Conjugation experiments between *S. sonnei* NK4846 and *E. coli* J53 revealed that this isolate did not carry any conjugable plasmid.

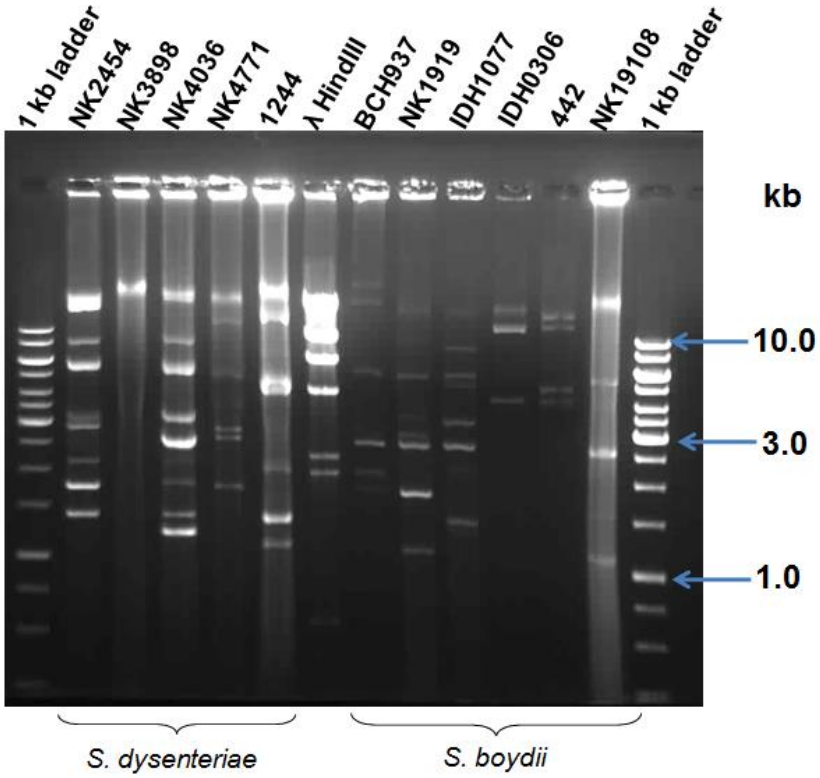


Figure 4.11. Plasmid DNA profile of *Shigella dysenteriae* and *Shigella boydii*

Table 4.6. Transferable resistance traits of transconjugants from *Shigella* isolates

Conjugation				Resistance traits transferred to the trans-conjugants	Transferable mobile genetic element
Donor	**Resistance phenotype of donor**	**Recipient**	**Resistance phenotype of recipient**		
S. boydii 442	AZM, COT, GEN, KAN, NAL, STR, TRI, Sodium azide	*E. coli* XL1Blue	NAL, TET	AZM, COT, KAN, STR, TRI	Plasmids and class 2 integron
S. dysenteriae 1244	AMP, AZM, CHL, CIP, COT, GEN, KAN, NAL, STR, TET, TRI	*E. coli* J53	Sodium azide	AMP, CHL, COT, NAL, KAN, TET, TRI	Plasmids, atypical class 1 and class 2 integron
S. flexneri NK2220	AMP, CHL, COT, KAN, NAL, STR, TET, TRI	*E. coli* J53	Sodium azide	AMP, CHL, COT, NAL, TET, TRI	Plasmids, atypical class 1 and class 2 integron
S. flexneri 102	AMP, CHL, CIP, COT, CXM, KAN, NAL, NOR, OFX, STR, TET,TRI	*E. coli* J53	Sodium azide	AMP, CHL, CIP, COT, KAN, NAL, NOR, OFX, STR, TET, TRI	Plasmids, atypical class 1 and class 2 integron
S. sonnei IDH0734	AZM, CIP, COT, KAN, NAL, NOR, OFX, STR, TRI	*E. coli* J53	Sodium azide	CIP, COT, KAN, NAL, OFX, STR, TRI	Plasmids, class 1 and class 2 integron
S. sonnei NK4846	AZM, CFX, CIP, GEN, KAN, NAL, STR,TET	*E. coli* J53	Sodium azide	Non-conjugable Strain	-

AZM, azithromycin; CIP, ciprofloxacin; COT, co-trimoxazole; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; STR, streptomycin; TET, tetracycline; TRI, trimethoprim

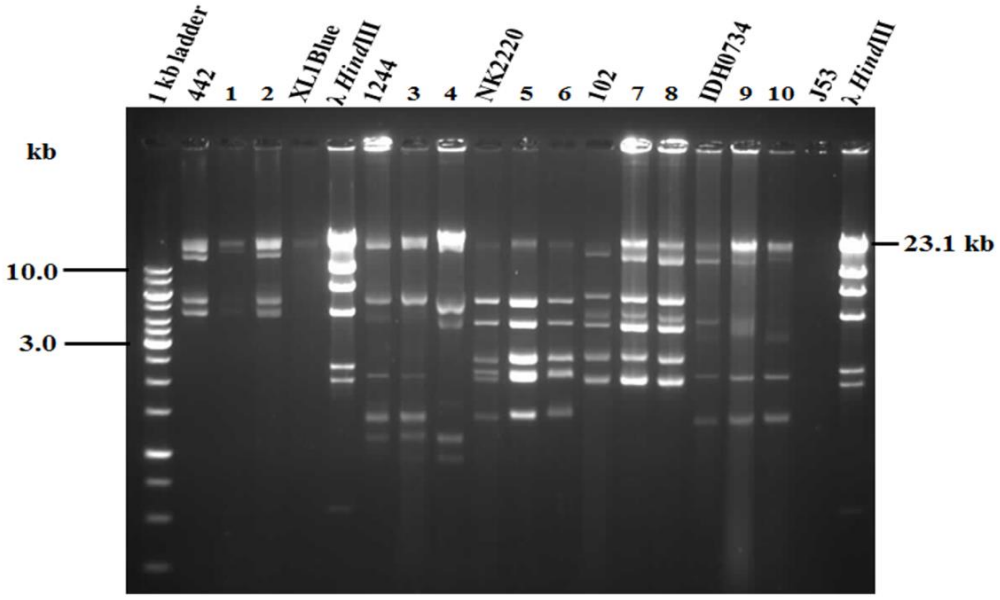


Figure 4.12. Agarose gel analysis (1%) of plasmid DNA from representative *Shigella* spp., their transconjugants, and recipient *E. coli* XL1Blue and J53. The sample identity has been indicated on the top of each lane. The position of amplicon size has been indicated on the left. Lanes 1 and 2: plasmid preparations of transconjugants from *S. boydii* 442; Lanes 3 and 4: plasmid preparations of transconjugants from *S. dysenteriae* 1244; Lanes 5 and 6: plasmid preparations of transconjugants from

S. flexneri NK2220; Lanes 7 and 8: plasmid preparations of transconjugants from *S. flexneri* 102; Lanes 9 and 10: plasmid preparations of transconjugants from *S. sonnei* IDH0734.

4.3. Discussion

Antibiotic resistance within the wide range of pathogenic bacteria is a growing public health concern globally. It hampers the effective treatment of the infectious diseases. Therefore, to mitigate the problem of MDR, it becomes pertinent to decipher the various factors/mechanisms involved in the antibiotic resistance of these infectious bacteria. This study was carried out to determine the patterns of antimicrobial resistance in 95 clinical isolates of *Shigella* (years 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 four or more antibiotics out of 15 antibiotics tested. This enormity of MDR was in accordance with the WHO reports published recently [WHO, 2017; WHO, 2014]. 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 the post-antibiotic era [WHO, 2014]. A WHO report published during February 2017 has included *Shigella* in the list of priority pathogens for which new antibiotics are urgently needed [WHO, 2017]. Our study presented here clearly shows the prevalence of multiply drug resistant *Shigella*, which as specified by WHO, could be a matter of serious concern. Results of the present study showed that resistance to quinolone drugs used for the treatment of diarrhoeal diseases was markedly increased through years. Emerging fluroquinolone resistance has also been earlier reported in *Shigella* spp. from India [Pazhani et al., 2008; Taneza, 2007]. 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 [Bhattacharya et al., 2012; WHO, 2005]. 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 ESBL producers from many countries [Gu et al., 2015]. 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 harboured by their variable regions conferred drug resistance traits on the parent isolates. Out of 95, 31 isolates harboured both class 1 and class 2 integrons. In addition to integrons, the presence of multiple plasmids in all the isolates was also the source of drug resistance as supported by conjugation experiments where a battery of resistance traits were transferred via plasmids carrying integrons on them. 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 integrons were found in *S. flexneri*, *S. dysenteriae* and *S. boydii*. Class 2 integrons were 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 [Gu et al., 2008; Hansson et al., 2002]. 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 [Gassama Sow et al., 2008]. In another report, a class 2 integron with an IS630 element was found in *S. flexneri* isolate, and a third report described a class 2 integron with an IS1 in an *E. coli* [Dubois et al., 2007; Biskri and Mazel,2003]. *S. sonnei* NK4675 harboring the class 2 integrase did not show amplification with primers specific to the variable region of class 2 integron suggesting the presence of null integron or mutations in the regions where primers annealed 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 the dissemination of drug resistance in these isolates.

Previously *Shigella* spp. were susceptible to co-trimoxazole but on the emergence of resistance to this antimicrobial, treatment recommendations were shifted to quinolone group of antibiotics and azithromycin [WHO, 2014]. Eventually, these bacteria also developed quinolone resistance [Taneza and Mewara, 2016; WHO, 2014; Gu et al., 2012; Dutta et al., 2005]. In this study, the majority of the isolates were resistant to nalidixic acid and resistance to other quinolone antibiotics such as ciprofloxacin, norfloxacin and ofloxacin were higher in *S. flexneri* as compared to *S. sonnei.* Resistance to quinolones is generally caused due to mutations in topoisomerase genes, efflux pump activity, *qnr* and *aac(6')-Ib-cr* genes [Bhardwaj et al., 2014]. In this study, an interesting array of mutations was observed in the QRDR regions of topoisomerases. Clearly, a mutation in GyrA S_{83} or D_{87} positions were chiefly responsible for resistance to nalidixic acid. Mutations in GyrA have been shown to be a major reason for resistance to quinolone in various organisms such as *Vibrios*, *Shigella* and *Salmonella* [Taneza and Mewara, 2016; Bhardwaj et al., 2014; Kutar et al., 2013; Singh et al., 2012; Lunguya et al., 2012]. 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 ciprofloxacin susceptibility [Lunguya et al., 2012]. Wherever mutations were detected in both the topoisomerases GyrA and ParC, a 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 towards quinolone resistance.

The use of an efflux pump inhibitor CCCP abolished the efflux activity but representative *Shigella* isolates did not show any decrease in MIC values on the addition of CCCP indicating that the resistance was due to factors other than efflux pumps. Such phenomenon (no change in MICs) was also observed in earlier studies especially for quinolone drugs [Taneja et al., 2015; Azmi et al., 2014]

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