Chapter 3

Bacterial Identification and Antibiotic Susceptibility of Clinical Isolates of Vibrio and Shigella Species

3 Bacterial identification and antibiotic susceptibility of clinical isolates of *Vibrio* and *Shigella* species

3.1. Introduction

Vibrio and *Shigella* spp. are the major etiological agents of gastroenteritis in humans and have been a menace to public health in the developing countries like India [Niyogi, 2005; Nair et al. 2007; Kanungo et al. 2010; Mandal et al. 2011; Sarkar et al. 2012; Pazhani et al. 2014; Ramamurthy et al. 2014]. *Vibrio* spp. have been associated with several outbreaks of diarrhea and sporadic infections throughout the world [Huq et al. 1980; Faruque et al. 1998; Thompson et al. 2004]. The genus *Vibrio* includes several species such as *V. cholerae*, *V. parahaemolyticus*, *V. fluvialis*, *V. vulnificus* and *V. mimicus* that have been reported to cause diarrheal illnesses in humans. Mixed infections due to two or more *Vibrio* species have also been reported [Hoge et al. 1989; Lesmana et al. 2002]. Rapid identification of the causative organisms up to the species level is very useful for research and epidemiological studies as it helps in determining the exact source of any outbreak and in devising the strategy to reduce the severity of the disease. However, the conventional identification techniques involving a series of biochemical tests and agglutination with specific antisera are time-consuming and ambiguous [Sakazaki, 1992]. Therefore, several monoplex and multiplex PCR methods have been developed for species-specific identification of *Vibrios* by targeting various housekeeping, virulence and regulatory genes [Fields et al. 1992; Olsvik et al. 1993; Chakraborty et al. 2006; Bauer and Rorvik, 2007; Tarr et al. 2007; Khuntia et al. 2008; Kim and Bang, 2008; Dalmasso et al. 2009; Yu et al. 2010; Izumiya et al. 2011]. Cholera is endemic in India, and the presence of *V. cholerae*, *V. parahaemolyticus* and *V. fluvialis* has been frequently reported from India as enteric/food-poisoning organisms [Thekdi et al. 1982; Okuda et al. 1997; Srinivasan et al. 2006; Nair et al. 2007; Rajpara et al. 2009; Bhattacharjee et al. 2010; Ceccarelli et al. 2011; Ghosh and Ramamurthy, 2011; Mandal et al. 2011; Chowdhury et al. 2012; Kanungo et al. 2012; Sarkar et al.

2012; Singh et al. 2012; Ramamurthy et al. 2014]. Similarly, *Shigella*, another important diarrhea-causing genus is again a common cause of morbidity and mortality in India [Sur et al. 2004; Niyogi, 2005; Taneja, 2007; Nandy et al. 2010]. The increased incidences of outbreaks of gastroenteritis caused by multidrug resistant *Vibrio* and *Shigella* species in India, in the last decade, increased the treatment complications [Sack et al. 2001; Niyogi, 2005; Ghosh and Ramamurthy, 2011].

Drug susceptibility of a microorganism may vary due to the clinical efficacy of drugs, selection of mutants and acquisition of resistance traits from other microbes. Determination of the drug sensitivity pattern of clinically important bacteria such as *Vibrio* and *Shigella* species is essential to understand the evolving drug resistance in microbes and also for the selection of most appropriate antimicrobial agents for treating the infections caused by them. Hence, determining the antibiotic susceptibility phenotype of infectious organisms is necessary to control the irrational use of antibiotics and the spread of these organisms. Quinolone class of antibiotics are the drugs of choice to treat illnesses caused by these diarrhea-causing bacteria, but the emergence of quinolone resistance in these bugs led to a serious public health threat [Sack et al. 2001]. As these drugs were in use in India and other countries for a long time for treating different infectious diseases, the emergence of fluoroquinolone resistance has been reported from early 2000 [Ghosh and Ramamurthy, 2011]. Keeping in view, the widespread use of the quinolone class of drugs in clinical settings and the increasing resistance of *Vibrio* and *Shigella* species to these drugs, the study described in this chapter was aimed at identification of clinical isolates used during the study and characterization of their antibiotic susceptibility patterns to understand the trend of quinolone resistance in these major diarrhea-causing pathogens.

3.2. Results

3.2.1. Bacterial isolates and their identification

As mentioned in section 2.1, 18 isolates of *V. fluvialis*, 119 isolates of *V. cholerae*, 58 isolates of *V. parahaemolyticus* and 18 isolates of *Shigella* species (9 *S. flexneri*, 3 *S. dysenteriae*, 4 *S. boydii* and 2 *S. sonnei*), used in this study, were procured from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. The isolates were obtained in the form of stabs of pure culture. The isolates of *Shigella* species were checked for their authenticity as described previously [Niyogi, 2005], by growing them in selective media such as Xylose-Lysine Deoxycholate (XLD) agar, MacConkey agar and Hektoen Enteric Agar (HEA) to observe the appearance of red, colorless and green colonies respectively. Subsequent to that, the authenticity of the isolates was confirmed by subjecting them to the triple sugar iron test (TSI) and observing the alkaline slant and acidic butt.

As the isolates belonging to *Vibrio* species were more in number, the conventional identification techniques involving a series of biochemical tests could be time-consuming and laborious. Hence, a triplex PCR assay was developed for rapid and species-specific identification of these three major *Vibrios*, *V. cholerae*, *V. parahaemolyticus* and *V. fluvialis* as described in section 2.2. The target genes selected for the identification were the central regulatory factor gene (*toxR*) of *V. fluvialis*, the hemolysin gene (*hlyA*) of *V. cholerae* and the heme-utilizing gene (*hutA*) of *V. parahaemolyticus*. For identification of *V. fluvialis*, the published primers used in the study were based on membrane tether region of the *toxR* gene [Chakraborty et al. 2006]. The primers for the genes *hlyA* of *V. cholerae* and *hutA* of *V. parahaemolyticus* were designed by exploiting their unique sequences after alignment of these genes from the three *Vibrio* species (Table 2.1).

The multiplex PCR assay carried out with the reference strains (n =14) yielded fragments of the expected size from *toxR* (217 bp), *hlyA* (427 bp) and *hutA* (330 bp) as shown in Figure 3.1, lanes 1, 4 and 8 respectively. Subsequently, the

multiplex PCR assay was evaluated with a total of 221 *Vibrio* and non-*Vibrio* test strains (Table 3.1) that were identified earlier with standard biochemical techniques [Colwell et al. 1981; Huq et al. 1984]. These included 119 clinical *V. cholerae* isolates, 8 environmental *V. cholerae* non-O1/non-O139 isolates, 18 clinical *V. fluvialis* isolates, 58 clinical *V. parahaemolyticus* isolates and 18 clinical *Shigella* isolates. The reference strains *Aeromonas caviae* (MTCC 6832), *A. culicicula* (MTCC 3249), *A. hydrophila sub sp. hydrophila* (MTCC 1739 and MTCC 646), *A. liquefaciens* (MTCC 2654), *A. salmonicida* (MTCC 1522) and *V. vulnificus* (MTCC 1145 and MTCC 1146) were included for the evaluation of PCR as *Aeromonas* spp. and *V. vulnificus* share close similarity with *V. fluvialis.* All the *V. cholerae* test isolates showed a prominent band at the expected position of 427 bp and *V. fluvialis* and *V. parahaemolyticus* showed bands of 217 bp and 330 bp respectively as expected. PCR amplicons for some of the test isolates have been shown in Figure 3.1. All the other isolates of *Shigella, Aeromonas, V. vulnificus* and *V. alginolyticus* (MTCC 4439) showed no amplification which proved the specificity of this multiplex PCR. The multiplex was used to detect the presence of *V. cholerae* in two cases of mixed infections. One was a mixed infection of *V. cholerae* and *V. fluvialis* in a clinical sample where the PCR showed two bands corresponding to both the species (Fig. 3.1, lane 5). In the second case, a mixed infection of *V. cholerae* IDH02365 with *Providencia vermicola*. specifically detected only *V. cholerae* and not the *P. vermicola* strain (Fig. 3.2, Lane 6), which further confirmed that this multiplex did not show cross-reactivity with either *Shigella* spp., *Aeromonas* spp., *P. vermicola*, *V. vulnificus* or *V. alginolyticus*. Similarly, a *Vibrio* isolate BD81 which was mistakenly designated as *V. fluvialis* was found to be actually a *V. cholerae* isolate using this method (Fig. 3.2, Lane 4). The multiplex PCR results were 100% consistent with the conventional identification techniques (Table 3.1).

Table 3.1. Analysis of specificity of triplex PCR using a variety of isolates/strains from the family of *Enterobacteriaceae* and *Vibrionaceae*

S.No	Species	Source	No.	Consistency (%)[a]
	Test isolates (n=221)			
1	*V. cholerae* O1 ElTor	Clinical	119	100
2	*V. cholerae* nonO1/nonO139	Environmental	08	100
3	*V. parahemolyticus*	Clinical	58	100
4	*V. fluvialis*	Clinical	18	100
5	*Shigella* spp	Clinical	18	100
	Reference strains (n=14)			
6	*V.vulnificus*	MTCC	02	-
7	*V. alginolyticus*	MTCC	01	-
8	*V. cholerae* O1 ElTor N16961	NICED	01	-
9	*V. cholerae* classical 569B	NICED	01	-
10	*V. cholerae* O139 MO10	NICED	01	-
11	*V. fluvialis* BD123	NICED	01	-
12	*V. parahaemolyticus*	NICED	01	-
13	*Aeromonas* spp.	MTCC	06	-

MTCC= Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology, Chandigarh, India
NICED= National Institute of Cholera and Enteric Diseases (Kolkata, India)
[a] consistency of results from triplex PCR assays with conventional identification techniques.

The whole assay starting from a colony on the agar plate to the detection of PCR amplicons on agarose gel could be completed within 6 h, whereas, from the stool samples, the whole assay could be completed in less than 24 h. Using this multiplex PCR, amplicons could be detected in cell lysates diluted up to 10,000X (10 CFU), which further underlined the applicability of this multiplex as a highly sensitive assay with high detection power in addition to being specific, consistent and rapid.

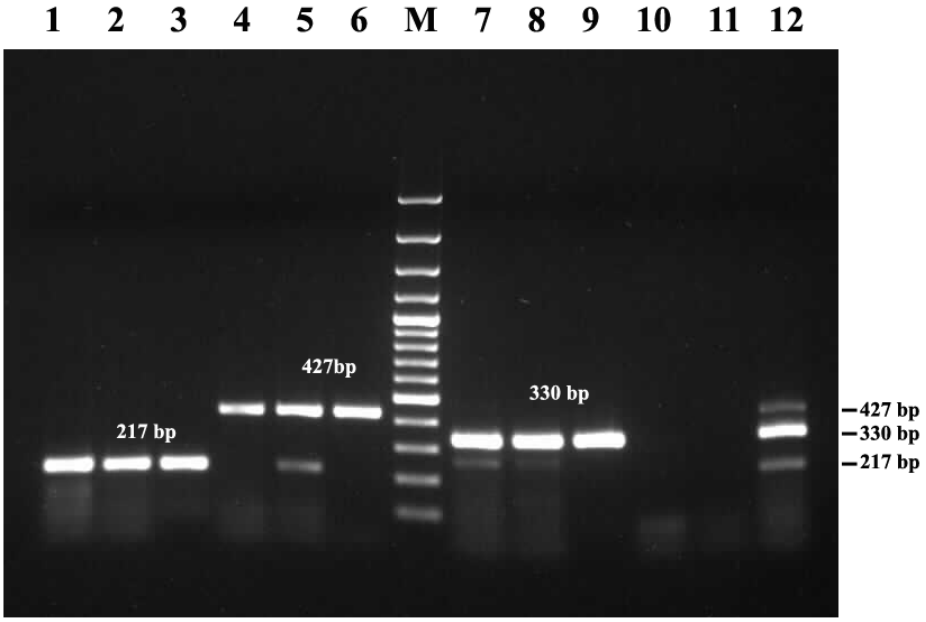

Figure 3.1. Agarose gel electrophoresis (1.2%) of triplex PCR discriminates *V. fluvialis*, *V. cholerae* and *V. parahaemolyticus* from each other by producing specific amplicons of 217 bp, 427 bp and 330 bp respectively. Lane 1, triplex PCR product of *V. fluvialis* BD123; lane 2, triplex PCR product of *V. fluvialis* L12482; lane 3, monoplex PCR for *toxR* gene in *V. fluvialis* BD123; lane 4, triplex PCR product of *V. cholerae* N16961; lane 5, triplex PCR product of *V. cholerae* IDH01426; lane 6, monoplex PCR for *hlyA* gene in *V. cholerae* 569B; lane M, 100 bp DNA ladder (Fermentas); lane 7, triplex PCR product of *V. parahaemolyticus* IDH02127; lane 8, triplex PCR product of *V. parahaemolyticus* IDH02033; lane 9, monoplex PCR for *hutA* gene in *V. parahaemolyticus* IDH02033; lane 10, triplex PCR product of *S. flexneri* type 2a NT4966; lane 11, triplex PCR product of *A. culicicula* MTCC 3249; lane 12, triplex PCR product of the cell lysates from the mixed cultures of *V. fluvialis*, *V. cholerae* and *V. parahaemolyticus*. In lane 5, mixed samples of *V. cholerae* and *V. fluvialis* could be detected with the multiplex PCR.

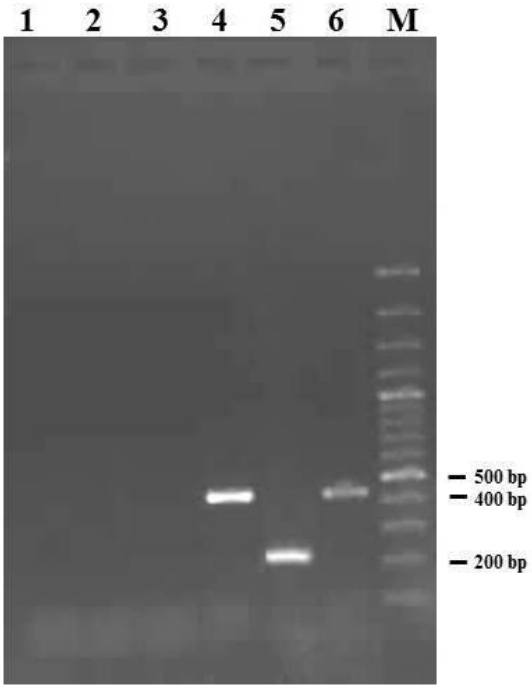

Figure 3.2. Agarose gel electrophoresis (1.0%) of triplex PCR of *Aeromonas* spp., *V. cholerae* BD81 and *V. cholerae* IDH2365. Lane 1, triplex PCR product of *A.hydrophilla* MTCC1739; lane 2, triplex PCR product of *A.salmonicida* MTCC1522; lane 3, triplex PCR product of *A. liquefaciens* MTCC2654; lane 4, triplex PCR product of *V. cholerae* BD81 which was mistakenly designated as *V. fluvialis*; lane 5, triplex PCR product of *V. fluvialis* CRC233; lane 6, triplex PCR product of *V. cholerae* IDH02365 which had mixed infection of *V. cholerae* with *Providencia vermicola*.; lane M, 100 bp DNA ladder (Fermentas).

3.2.2. Antimicrobial susceptibility of bacterial isolates

The antibiogram profiles were determined to assess the antibiotic susceptibility of the clinical isolates of *Vibrio* and *Shigella* species as mentioned in section 2.3. The antibiogram results revealed that the clinical isolates of *Vibrio* spp. and *Shigella* spp. were resistant to multiple drugs and quinolone resistance was prevalent among these isolates (shown in red color) (Fig. 3.3-3.6). In this representation of antibiogram profiles of *V. cholerae*, *V. fluvialis* and *Shigella* isolates, the complete resistance and intermediate resistance to the antibiotics were both considered as resistant. As *V. parahaemolyticus* isolates showed intermediate resistance to most of the antibiotics, the complete and intermediate resistance was shown separately.

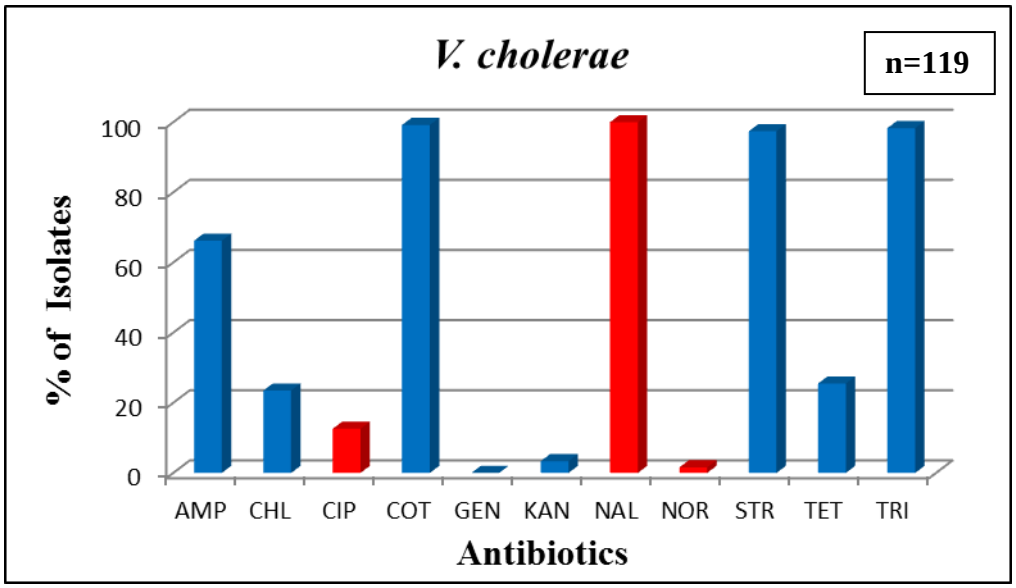

Figure 3.3. Antimicrobial resistance pattern in *Vibrio cholerae* isolates. AMP- Ampicillin, CHL- Chloramphenicol, CIP- Ciprofloxacin, COT- Co-Trimoxazole, GEN- Gentamicin, KAN- Kanamycin, NAL- Nalidixic acid, NOR-Norfloxacin, STR- Streptomycin, TET- Tetracycline, TRI- Trimethoprim. Both the complete and intermediately resistant isolates to antibiotics were considered as resistant. The red bars indicate the isolates resistant to quinolone antibiotics (NAL, NOR and CIP) whereas blue bars represent the isolates resistant to other classes of antibiotics.

The *V. cholerae* isolates majorly showed resistance to antibiotics such as nalidixic acid (100%), co-trimoxazole (99.2%), trimethoprim (98.3%) and streptomycin (97.4%) followed by ampicillin (66.2%), tetracycline (25.5%), chloramphenicol (23.5%), ciprofloxacin (12.6%), kanamycin (3.36%) and norfloxacin (1.6%) (Fig. 3.3). *V. fluvialis* isolates showed wider spectrum of MDR as compared to *V. cholerae* clinical isolates used in this study (Fig. 3.4). *V. fluvialis* isolates were resistant to ampicillin (88.8%), kanamycin (72.2%), ciprofloxacin (55.5%), nalidixic acid (50%), streptomycin (50%), trimethoprim (44.4%), co-trimoxazole (44.4%), chloramphenicol (33.3%), norfloxacin (33.3%), tetracycline (16.6%) and gentamicin (16.6%) (Fig. 3.4). In *V. parahaemolyticus* isolates, the complete resistance was found for ampicillin (96.5%), trimethoprim (77.5%), kanamycin (5.1%) and streptomycin (1.7%) and intermediate resistance for the antibiotics kanamycin (94.8%), streptomycin (82.7%), ciprofloxacin (36.2%), gentamicin (22.4%), trimethoprim (22.4%), ampicillin (3.4%), nalidixic acid (3.4%) and

norfloxacin (3.4%) (Fig. 3.5). *Shigella* isolates also showed a wide spectrum of MDR phenotype.

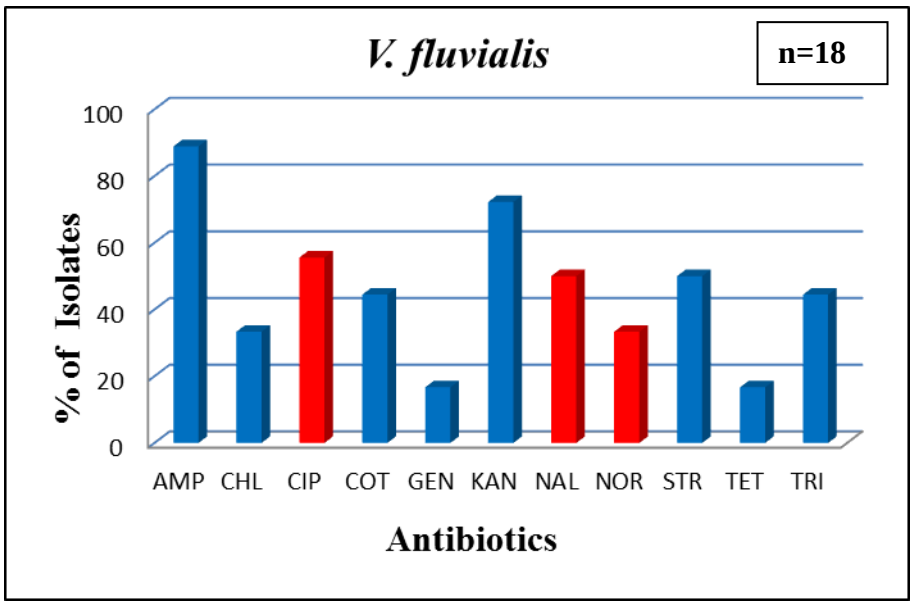

Figure 3.4. Antimicrobial resistance pattern in *Vibrio fluvialis* isolates. AMP- Ampicillin, CHL- Chloramphenicol, CIP- Ciprofloxacin, COT- Co-Trimoxazole, GEN- Gentamicin, KAN- Kanamycin, NAL- Nalidixic acid, NOR-Norfloxacin, STR- Streptomycin, TET- Tetracycline, TRI- Trimethoprim. Both the complete and intermediately resistant isolates to antibiotics were considered as resistant. The red bars indicate the isolates resistant to quinolone antibiotics (NAL, NOR and CIP) whereas blue bars represent the isolates resistant to other classes of antibiotics.

Shigella isolates were resistant to kanamycin (82.3%), streptomycin (82.3%), trimethoprim (82.3%), nalidixic acid (76.6%), tetracycline (70.5%), ciprofloxacin (47%), cotrimoxazole (47%), ampicillin (41.1%), chloramphenicol (41.1%), norfloxacin (35.2%) and gentamicin (11.7%) (Fig. 3.6).

3.2.3. Quinolone resistance phenotypes in the isolates of *Vibrio* and *Shigell*a spp.

As the focus of this study was quinolone resistance prevalent among these clinical isolates, some of the representative quinolone resistant strains were chosen for further studies. Hence, nine *V. cholerae*, eleven *V. fluvialis*, seven *V. parahaemolyticus* and ten *Shigella* isolates (seven *S. flexneri*, two *S. dysenteriae* and one *S. sonnei*) were selected for further analysis. Additionally, a quinolone

sensitive strain from each of the above organism type was included in the study, to serve as a control and is shown in bold face in Table 3.2.

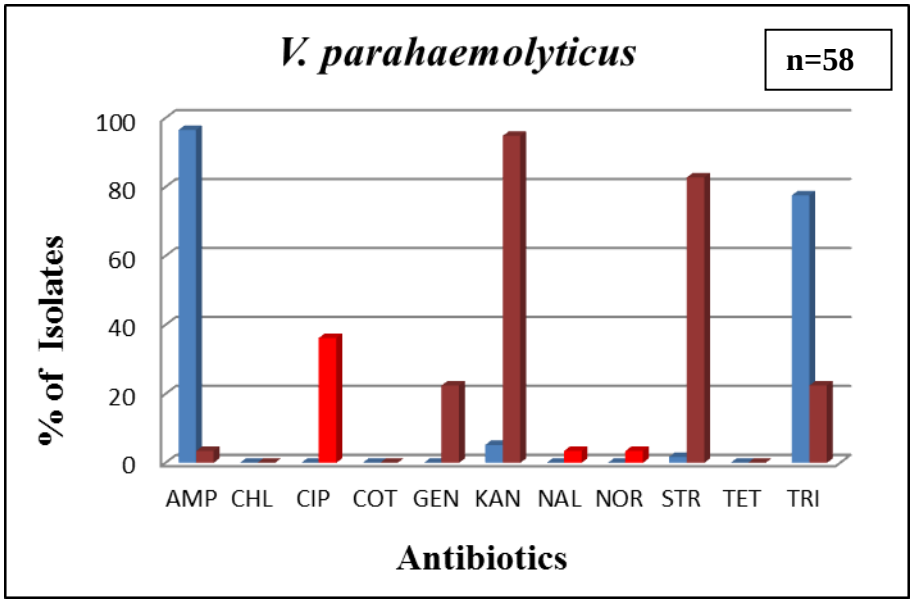

Figure 3.5. Antimicrobial resistance pattern in *Vibrio parahaemolyticus* isolates. AMP- Ampicillin, CHL- Chloramphenicol, CIP- Ciprofloxacin, COT- Co-Trimoxazole, GEN- Gentamicin, KAN- Kanamycin, NAL- Nalidixic acid, NOR-Norfloxacin, STR- Streptomycin, TET- Tetracycline, TRI- Trimethoprim. The complete and intermediately resistant isolates to antibiotics were represented distinctly using blue and brown bars repectively. The red bars indicate the isolates showing intermediate resistance to quinolone antibiotics (NAL, NOR and CIP).

The selected quinolone resistant isolates were further subjected to antibiogram analysis for an extended panel of quinolones including some higher generation quinolones. Therefore, the enire panel of quinolones consisted of nalidixic acid representing the first generation, norfloxacin, ciprofloxacin, ofloxacin, lomefloxacin and levofloxacin representing the second generation and sparfloxacin and gatifloxacin for the third generation of quinolones (Table 3.2). It was observed that *V. fluvialis* and *Shigella* isolates showed resistance to most of the higher generation quinolones (Fig. 3.7). Lomefloxacin resistance was common among *V. cholerae* isolates whereas *V. parahaemolyticus* did not show any significant quinolone resistance phenotype (Fig. 3.7). These results clearly indicated the widespread quinolone resistance in the pathogens belonging to

Vibrio and *Shigella* spp. The complete quinolone resistance profile for the selected quinolone resistant isolates is shown in Table 3.2.

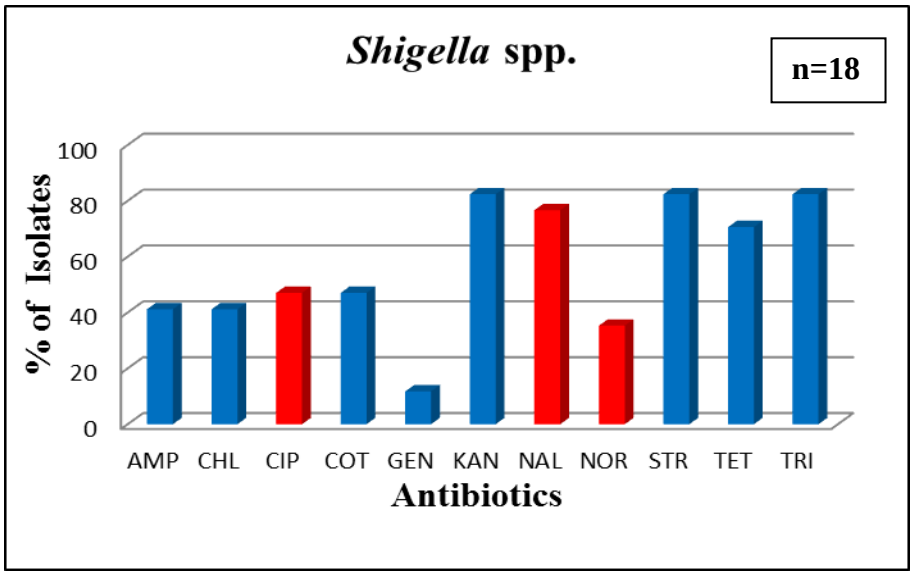

Figure 3.6. Antimicrobial resistance pattern in *Shigella* isolates. AMP- Ampicillin, CHL- Chloramphenicol, CIP- Ciprofloxacin, COT- Co-Trimoxazole, GEN- Gentamicin, KAN- Kanamycin, NAL- Nalidixic acid, NOR-Norfloxacin, STR- Streptomycin, TET- Tetracycline, TRI- Trimethoprim. Both the complete and intermediately resistant isolates to antibiotics were considered as resistant. The red bars indicate the isolates resistant to quinolone antibiotics (NAL, NOR and CIP) whereas blue bars represent the isolates resistant to other classes of antibiotics.

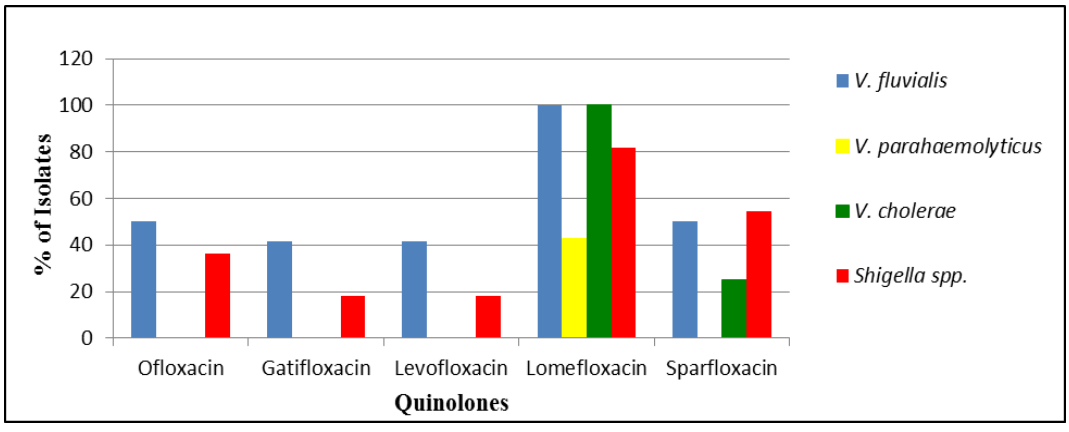

Figure 3.7. Resistance pattern in *Vibrio* and *Shigella* spp. to the extended panel of quinolone antibiotics. Both the complete and intermediately resistant isolates to antibiotics were considered as resistant. The numbers of isolates included in this study were: *V. cholerae* (n=9), *V. fluvialis* (n=11), *V. parahaemolyticus* (n=7) and *Shigella* spp. (n=10).

Table 3.2. Quinolone resistance profile for selected quinolone resistant isolates of *Vibrio* and *Shigella* spp.

Bacteria	Strain	Resistance	Intermediate
V. cholerae	IDH02365	NAL, LOM	CIP
	IDH02233	NAL, LOM	-
	IDH02118	NAL, LOM	NOR
	IDH02101	NAL, LOM	CIP, SPA
	IDH02087	NAL, LOM	NOR, SPA
	IDH01957	NAL, LOM	-
	IDH01738	NAL, LOM	CIP, SPA
	IDH01681	NAL, LOM	SPA
	BD81	NAL, LOM	-
	N16961	-	-
V. fluvialis	**L13828**	-	-
	L13230	LOM	CIP
	L98411	-	CIP, LOM
	L10734	NAL, LOM	CIP, OFX, GAT, LEV, SPA
	L9077	LOM	NAL
	L12387	NAL, LOM	NOR, CIP, SPA
	L9978	LOM	CIP
	L15318	NAL, LOM	NOR, CIP, OFX, GAT, LEV
	BD146	NAL, NOR, CIP, LOM, SPA	OFX, GAT, LEV
	BD123	NAL, LOM	CIP, NOR, SPA
	PL78/6	NAL,CIP, OFX, LOM, SPA	NOR, GAT, LEV
	PL171b	NAL, NOR, LOM	CIP, OFX, GAT, SPA
V. parahaemolyticus	IDH01402	-	NAL, NOR, CIP, LOM
	IDH01415	-	-
	IDH01473	-	CIP, LOM
	IDH01998	-	CIP
	IDH02068	-	CIP
	IDH02189	-	NOR, CIP
	IDH02191	-	NAL, CIP
	IDH02208	-	CIP, LOM
S. flexneri	NT4966	NAL, NOR, CIP, LOM, SPA	OFX
	M11560		NAL, CIP, LOM
	NT5120	-	NAL
	B36	NAL, NOR, CIP, OFX, LOM, SPA	GAT, LEV
	NK4/08	-	-
	NK05/08	NAL, NOR, CIP, LOM, SPA	OFX
	IDH00177	NAL, NOR, CIP, LOM, SPA	OFX
	NK23/08	-	NAL
S. dysenteriae	NK4771	NAL, LOM	CIP
	NK3898	NAL	LOM
S. sonnei	NK2070	NAL	LOM

NAL – Nalidixic acid; NOR – Norfloxacin; CIP – Ciprofloxacin; OFX- Ofloxacin, GAT- Gatifloxacin; LEV-Levofloxacin; LOM- Lomefloxacin; SPA- Sparfloxacin

Following the antibiogram results, to understand the extent of resistance quantitatively, MIC assay was carried out for the selected quinolone resistant isolates against some of the representative quinolones such as nalidixic acid, norfloxacin, ciprofloxacin and ofloxacin using the Ezy MIC strip method described in the section 2.4.1. The isolates showed varying levels of resistance to the tested quinolones (Tables 3.3 to 3.6). *V. cholerae* isolates showed high MIC values (>256 µg/mL) for nalidixic acid (Table 3.3).

Table 3.3. MIC of quinolones for *Vibrio cholerae* isolates

No.	***V. cholerae* isolate**	**Antibiogram profile**	**MIC (µg/mL)**		
			NAL	**NOR**	**CIP**
1.	N16961*	**S**-NAL, NOR, CIP	0.75	0.094	0.032
2.	IDH02365	**R**-NAL, **I**-CIP	≥256	1.5	3-4
3.	IDH02233	**R**-NAL	≥256	-	-
4.	IDH02118	**R**-NAL, **I**-NOR	≥256	0.75	-
5.	IDH02101	**R**-NAL, **I**-CIP	>256	-	3-4
6.	IDH02087	**R**-NAL, **I**-NOR	>256	2	-
7.	IDH01957	**R**-NAL	≥256	-	-
8.	IDH01738	**R**-NAL, **I**-CIP	>256	-	3-4
9.	IDH01681	**R**-NAL **S**- NOR, CIP	>256	0.75-1.5	3
10.	BD81	**R**-NAL	>256	-	-

NAL – Nalidixic acid; NOR – Norfloxacin; CIP – Ciprofloxacin.
R- Resistant; **I**-Intermediate resistant; **S**- sensitive.
*Sensitive strain

Among the *V. fluvialis* isolates, BD146 showed highest resistance to all the four antibiotics, followed by PL78/6, PL171b and L15318 (Table 3.4). *V. fluvialis* BD123, L12387 and L10734 isolates showed elevated MIC for more than two quinolones when compared with sensitive isolate L13828 (Table 3.4). This indicated the possible involvement of more than one type of quinolone resistance mechanism in these isolates. The minimal resistance to ciprofloxacin showed by *V. fluvialis* L9978 (Table 3.4) showed the possibility of the presence of any PMQR determinants or involvement of efflux pumps in these isolates.

Table 3.4. MIC of quinolones for *Vibrio fluvialis* isolates

No.	***V. fluvialis* strain**	**Antibiogram profile**	**MIC (µg/mL)**			
			NAL	**NOR**	**CIP**	**OFX**
1.	L13828*	**S**- NAL, NOR, CIP, OFX	1.5	0.25-0.38	0.125	0.75
2.	L13230	**I**-CIP	-	-	0.125	-
3.	L98411	**I**-CIP	-	-	0.19	-
4.	L10734	**R**-NAL **I**-CIP, OFX	>256	-	16	24
5.	L9077	**I**-NAL	1.5	-	-	-
6.	L12387	**R**-NAL **I**-NOR, CIP	>256	8	8-12	-
7.	L9978	**I**-CIP	-	-	0.25	-
8.	L15318	**R**- NAL **I**-NOR, CIP, OFX	>256	16	16-24	16
9.	BD146	**R**-NAL, NOR, CIP **I**-OFX	>256	32	48	32
10.	BD123	**R**- NAL **I**-NOR, CIP	>256	8-12	12	-
11.	PL78/6	**R**-NAL, CIP **I**-NOR, OFX	>256	12	24	32
12.	PL171b	**R**-NAL, NOR **I**-CIP, OFX	>256	12	16-24	32

NAL – Nalidixic acid; NOR – Norfloxacin; CIP – Ciprofloxacin; OFX – Ofloxacin. **R**- Resistant; **I**-Intermediate resistant; **S**- sensitive. *Sensitive strain

A very low-level resistance to quinolones was found in *V. parahaemolyticus* isolates (Table 3.5). Among *Shigella* isolates, *S. flexneri* B36 showed the highest degree of resistance to all the four tested quinolones, followed by *S. flexneri* NK05/08, NT4966 and IDH00177, again indicating the possible involvement of more than one genetic determinant (Table 3.6). The *S. flexneri* M11560 showed a low-level elevation in the MIC of nalidixic acid and ciprofloxacin in comparison to the sensitive isolate NK4/08 suggesting the involvement of PMQR determinant(s) or any quinolone specific efflux pump (Table 3.6). *S. dysenteriae* NK3898 showed an average level of resistance to nalidixic acid with MIC of 48 µg/mL as opposed to B36 showing high MIC value of >256 µg/mL (Table 3.6).

Table 3.5. MIC of quinolones for *Vibrio parahaemolyticus* isolates

No.	***V. parahaemolyticus* strain**	**Antibiogram profile**	**MIC (µg/mL)**		
			NAL	**NOR**	**CIP**
1.	IDH01415*	**S**- NAL, NOR, CIP	1	0.19	0.19
2.	IDH01402	**I**-NAL, NOR, CIP	1-1.5	0.125	0.125
3.	IDH01473	**I**-CIP	-	-	0.25
4.	IDH01998	**I**-CIP	-	-	0.19-0.25
5.	IDH02068	**I**-CIP	-	-	0.19-0.25
6.	IDH02189	**I**-NOR, CIP	-	0.125	0.25
7.	IDH02191	**I**-NAL, CIP	1.5	-	0.19
8.	IDH02208	**I**-CIP	-	-	0.19

NAL – Nalidixic acid; NOR – Norfloxacin; CIP – Ciprofloxacin.
I-Intermediate resistant; **S**- sensitive.
*Sensitive strain

Table 3.6. MIC of quinolones for *Shigella* isolates

No.	Strain name	Antibiogram profile	MIC (µg/mL)			
			NAL	**NOR**	**CIP**	**OFX**
1.	*S. flexneri* 1a* NK 4/08	**S**-NAL, NOR, CIP, OFX	1.5	0.064	0.064	0.19
2.	*S. flexneri* 2a NT 4966	**R**-NAL, NOR, CIP **I**-OFX	>256	16-24	24	24
3.	*S. flexneri* 1b M 11560	**I**-NAL, CIP	6	-	0.50	-
4.	*S. flexneri* 1a NT 5120	**I**-NAL	1.5	-	-	-
5.	*S. flexneri* 6 B 36	**R**-NAL, NOR, CIP, OFX	>256	32	48	≥32
6.	*S. flexneri* 2a NK 05/08	**R**-NAL, NOR, CIP **I**-OFX	>256	24-32	32	32
7.	*S. flexneri* 3a IDH 00177	**R**-NAL, NOR, CIP **I**-OFX	>256	24	24-32	24
8.	*S. flexneri* 4a NK 23/08	**I**-NAL	1.5	-	-	-
9.	*S. dysenteriae* 12 NK 3898	**R**-NAL	48	-	-	-
10.	*S. dysenteriae* 3 NK 4771	**R**-NAL **I**-CIP	>256	-	0.38	-
11.	*S. sonnei* NK 2070	**R**-NAL	≥256	-	-	-

NAL – Nalidixic acid; NOR – Norfloxacin; CIP – Ciprofloxacin; OFX – Ofloxacin.
R- Resistant; **I**-Intermediate resistant; **S**- sensitive.
*Sensitive strain

3.3. Discussion

In this part of the study, the identity of all the clinical bacterial isolates was authenticated and subsequently, they were characterized for their antibiotic resistance phenotypes. Identification of all the *Vibrio* isolates was confirmed using triplex PCR assay designed in this study. Most of the research carried out to

develop PCR-based molecular identification techniques has either aimed at their utility in detection of pathogenic *Vibrios* in the food industry or in research and clinical diagnostics for the identification of *Vibrios* and detection of their serotypes, biotypes and regulatory genes. All these reports have used a single gene like *rpoA* (encoding RNA polymerase subunit A), *rpoB* (encoding RNA polymerase subunit B), *toxR*, *atpA* (encoding ATP synthase subunit alpha) or 16S-23S intergenic spacer region [Chakraborty et al. 2006; Tarr et al. 2007; Kim and Bang, 2008; Dalmasso et al. 2009; Izumiya et al. 2011] and some of them involved techniques like real-time PCR or sequencing of the amplified fragments [Olsvik et al., 1993; Tarr et al., 2007; Tracz et al., 2007; Dalmasso et al., 2009] that would increase the cost as well as time for the final detection. The triplex developed during this study described a reliable, sensitive, specific, rapid and cost-effective method based on three different genes to unambiguously detect *V. cholerae*, *V. fluvialis* and *V. parahaemolyticus* from a pool of pathogens. To the best of our knowledge, this was the first report describing the identification of the above combination of pathogens based on three different genes. Therefore, the identification of clinical samples belonging to *Vibrio* spp. and *Shigella* spp. was confirmed before proceeding for antibiogram analysis. This part of the study was published during 2013 [Vinothkumar et al. 2013]. After publication of this work, there were two reports pertaining to the rapid identification of *Vibrio* spp. [Hossain et al. 2014; Kim et al. 2015]. Hossain and co-workers developed an assay that involved PCR of *groEL* gene followed by digestion using *Nru*I or *Xba*I enzymes and based on their unique digestion pattern, 10 *Vibrio* spp. could be identified. Kim et al. developed a multiplex PCR method using six sets of primers that enabled the detection of *Vibrio* genus and five pathogenic *Vibrio* species viz. *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. mimicus*.

The clinical isolates tested in this study were found to be resistant to multiple drugs and showed extensive quinolone resistance phenotypes, indicative of the indiscriminate clinical use of these drugs. *V. fluvialis* and *Shigella* isolates showed resistance to most of the quinolones, including higher generation quinolones and their spectrum/level of resistance varied. Nalidixic acid resistance was common

among *V. cholerae* isolates. *V. parahaemolyticus* did not show any significant quinolone resistance phenotype. In a nutshell, quinolone resistance was widespread among all the isolates pursued in this study, though the degree of resistance varied. Different levels of resistance shown by the resistant isolates reflected the involvement of more than one kind of mechanism and their interplay.

Following the qualitative as well as quantitative estimation of quinolone resistance phenotypes in the selected quinolone resistant clinical isolates of *Vibrio* and *Shigella* spp. described in this chapter, for cogent reasons, the next step was to unravel the genetic determinants resident in these isolates and responsible for observed quinolone resistance. Correlation of the presence of these determinants with the observed quinolone resistance phenotypes would then explain the susceptibility profiles of these isolates. Such results have been described in the next chapter.