environments of Bay of Bengal lacked the pandemic serotype O3:K6 despite a significant proportion of the environmental strains possessed important epidemic marker genes such as *toxR*, *tdh*, *trh*, *tlh* but not the group specific (GS), and ORF 8. The DNA fingerprinting analysis employing randomly amplified polymorphic DNA (RAPD), enterobacterial repeatitive intragenic consensus (ERIC)-PCR, and pulsed-field gel electrophoresis (PFGE) showed the strains to be unrelated clonally and were diverse genetically. We also stated the need for further PFGE analysis of strains comprising more environmental and clinical strains of diverse geographical origins. After the progress report that we submitted in February 2007, we analyzed many *V. parahaemolyticus* strains of diverse geographical origins that included pathogenic (both pandemic and non-pandemic serotypes) to see their clonal origins. The dendrogram constructed using the *sfi*I-digested PFGE images of *V. parahaemolyticus* strains revealed them to be diverse clonally. The results also revealed that, although the regional strains fall mostly under the same clusters, there were overlapping between clusters, implying that they are somehow linked. Further study is under way.

**Purpose:**

*V. parahaemolyticus* naturally inhabits marine water and is present in higher concentrations during summer; it is a halophilic organism which causes gastrointestinal illness in humans and are the cause of approximately half of the food borne outbreaks in some Asian countries. V. *parahaemolyticus* gastroenteritis is a multi-serogroup affliction, and as many as 75 different combinations of O and K serotypes of *V. parahaemolyticus* are so far recognized and known to be associated with gastroenteritis. In Bangladesh, *V. parahaemolyticus* associated diarrhea and gastroenteritis has recently been reported from various coastal villages but no study has so far been conducted on the molecular characterization of *V. parahaemolyticus* in this regions. On the other hand, pandemic serogroup strains of *V. parahaemolyticus*, namely O3:K6, have been reported to be disseminating rapidly in recent years, although the molecular characteristics namely the DNA fingerprinting analysis of the *V. parahaemolyticus* strains occurring in the coastal aquatic environments of Bangladesh were limited because of the lack of standardized pulsed-field gel electrophoresis (PFGE) protocol in use. Since we have successfully

optimized the PFGE protocol for *V. parahaemolyticus* strains using both *NotI* and *SfiI* restriction enzymes in our laboratory conditions, we investigated the molecular fingerprinting of *V. parahaemolyticus* strains (*SfiI* restriction enzyme) of diverse geographical origins.

**Methodology:**

We used the standardized protocol as indicated in our prepared manuscript below:

*Vibrio parahaemolyticus* PulseNet PFGE protocol Working Group. International Evaluation and Validation of a PulseNet Standardized Pulsed-Field Gel Electrophoresis Protocol for Subtyping *Vibrio parahaemolyticus*: A Multi-Center Collaborative Study. (*To be Submitted by KM Kam of Public Health Laboratory, Hong Kong*).

**Casting Agarose Gel and Loading Restriction Plug Slices on the Comb:**

One gm of Seakem Gold (SKG) Agarose (Bio-Rad) was added to 100 ml of 0.5X TBE buffer in a 500 ml Erlenmeyer flask. The slurry was heated in the microwave oven until the agarose was dissolved completely. The temperature of the slurry was equilibrated to 54-58 $^{0}$ C in a water bath. The casting apparatus of the PFGE was assembled according to the instruction manual (Bio-Rad). The comb was put on bench top and the plugs were loaded on the bottom of the comb teeth; The Salmonella ser. Braenderup standard plug slices were put on teeth, 1, 5 and 10 and the samples were loaded on the remaining teeth. Using a Pasteur pipette, the edges of the casting platform were sealed with a small quantity of the agarose solution and allowed to set. Then the remainder of the warm agarose solution was poured into the casting stand for a thickness of approximately 5-6 mm. The gel was allowed to solidify for 30 minutes at room temperature.

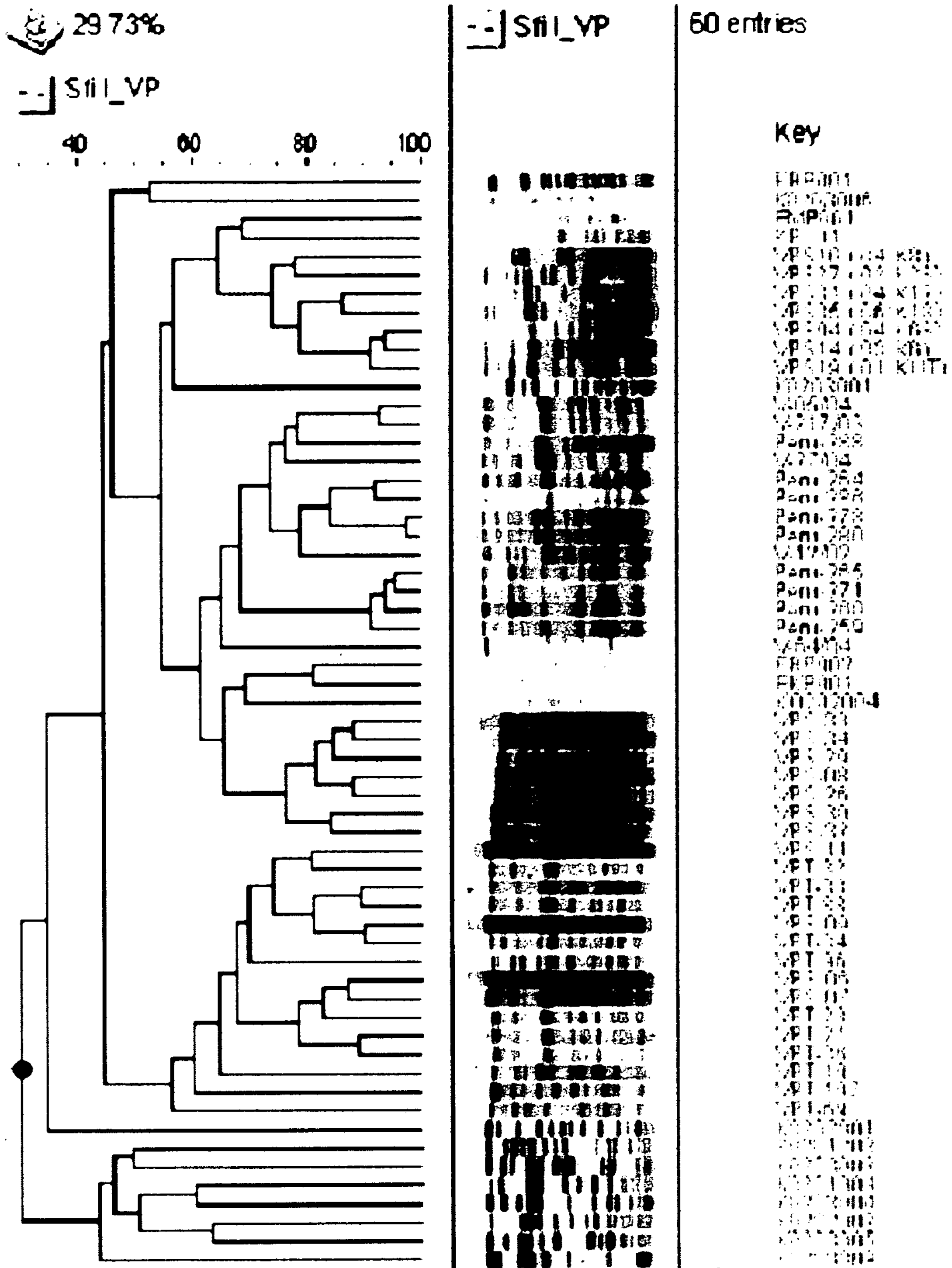


**Fig. 1.** PFGE images of *SfiI*-digested genomic DNA of *V. parahaemolyticus* strains that were analyzed by Bionumeric software to construct dendrogram.

The *SfiI*–digested PFGE profiles of the *V. parahaemolyticus* strains showed a great deal of diversity in patterns. The number of bands up on *NotI* digestion ranged from 15 – 16, and PFGE results revealed 12 different subtypes among the 14 representative *V. parahaemolyticus* strains studied.

**Discussion:**

*V. parahaemolyticus* associated diarrhea and gastroenteritis has recently been reported from coastal villages of Bangladesh, however no study has so far been carried out on the molecular characterization of *V. parahaemolyticus* in this regions. The pandemic serogroup strains of *V. parahaemolyticus* have been reported to be disseminating rapidly in recent years, although the molecular characteristics such strains occurring in the coastal aquatic environments of Bangladesh are unknown. We planned a detailed molecular study on the *V. parahaemolyticus* strains occurring in the coastal areas of the Bay of Bengal, but for the fingerprinting analysis, there was no existing standard pulsed-field gel electrophoresis (PFGE) protocol for *V. papahaemolyticus*. So, before the *V. parahaemolyticus* test strains were analyzed by PFGE, we adopted and optimized the standardized PulseNet approved protocol in our laboratory by using genomic DNA isolated from seven *V. parahaemolyticus* strains and the standard PFGE protocol provided by the PulseNet Asia-Pacific coordinating Public Health Laboratory Centre (PHLC) at Hong Kong. We carried out extensive PFGE analysis of genomic DNA digested with *NotI* and *SfiI* restriction enzymes and finally succeeded in adopting the PFGE protocol in our laboratory conditions. Our results also revealed *NotI* to be the restriction enzyme for *V. parahaemolyticus* DNA that give better and clearer banding patterns. The PFGE images of both *NotI* and *SfiI*-digested *V. parahaemolyticus* DNA that we produced in our laboratory was submitted to the PulseNet Asia-Pacific coordinating laboratory at Hong Kong, and the images were accepted as optimally produced ones. We then analyzed the test strains of *V. parahaemolyticus* we had in our possession using the optimized PFGE protocol. The PFGE of *NotI*-digested genomic DNA revealed *V. parahaemolyticus* strains to be diverse clonally, although further and continued PFGE analysis is crucial for tracking the spread of pandemic pathogen in this region.

We also analyzed many *V. parahaemolyticus* strains of diverse geographical origins that included pathogenic (both pandemic and non-pandemic serotypes) to see their clonal origins. The dendrogram constructed using the *Not*I-digested PFGE images of *V. parahaemolyticus* strains also revealed them to be diverse clonally. The results also revealed that, although the regional strains fall mostly under the same clusters, there were overlapping between clusters, implying that the strains which were physically apart may also be linked clonally. Further study is under way.

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**List of Publications:**
*Vibrio parahaemolyticus* PulseNet PFGE protocol Working Group. International Evaluation and Validation of a PulseNet Standardized Pulsed-Field Gel Electrophoresis Protocol for Subtyping *Vibrio parahaemolyticus*: A Multi-Center Collaborative Study. (*To be Submitted by KM Kam of Public Health Laboratory, Hong Kong*).

**More manuscripts are under preparation.**

# 1. Title: Phenotypic and Genotypic Characterization of Common Enteric Pathogens Isolated from Diarrheal Patients: a National Study

**2. Name of the Researcher:** T. Ramamurthy

**3. Affiliation:** National Institute of Cholera and Enteric Diseases, Kolkata, India

**4. Summary:**

Active surveillance on acute diarrheal cases admitted in the Infectious Diseases Hospital has suggested that the incidence pattern of *V. cholerae* non-O1, non-O139 serogroups is increasing recently in Kolkata. In this study, we have employed a PCR assay for the specious-specific identification of *V. cholerae* targeting the *ompW* gene. Serotyping results showed that the non-cholera vibrios belong to multi serogroups and does not have any marker virulence associated gene found among toxigenic counterparts. During 2007, we have encountered two cholera outbreaks, one in Orissa and the other in Assam. *V. cholerae* O1 Ogawa was identified as the causative agent of these outbreaks. DNA sequence analysis revealed that all the tested O1 strains have classical type of *ctxB* gene and instantly, this was the first detection of classical Ctx allele in India. Our results were confirmed with a newly designed mismatch amplification mutation assay (MAMA) PCR. Incidence of O139 sergroup was very less in India since 2004. However, focal incidence of O139 mediated cholera was recorded in Delhi during 2004-06. Interestingly, the O139 strains also had mutations in the *ctxB* gene with allelic changes that represents classical Ctx or a new type CT allele (CT type 5).

*Shigella* species represent one of the growing numbers of antimicrobial resistant bacteria in developing countries. Fluoroquinolone resistant strains of *S. dysenteriae* type1 and *S. flexneri* type 2a emerged in India during 2002 and 2003, respectively. Sixty strains of *Shigella* from different parts of India were analysed for antimicrobial susceptibility, the presence of the *qnr* plasmid, mutations in the quinolone resistance determining regions (QRDRs), fluoroquinolone accumulation, and other genes encoding resistance to various antimicrobials. Fluoroquinolone resistant strains had mutations in *gyrA* and *parC* genes and had an active efflux system. They were also resistance to several other antimicrobials but were susceptible to azithromycin and ceftriaxone. Majority of the strains harboured genes encoding resistance for ampicillin (97%), tetracycline (95%), streptomycin (95%) and chloramphenicol (94%). Pulsed-field gel electrophoresis (PFGE) analysis revealed clonality among strains of *S. dysenteriae* types 1 and 5, *S. flexneri* type 2a, and *S. boydii* type 12.

## 5. Purpose:

Diarrheal diseases are caused by an array of enteric pathogens. Its virulence and their distribution in many geographic areas are governed by many ecological and genetic factors. Different virulence and their regulatory genes are involved in the pathogenesis of the enteric organisms associated with infectious diarrhea. As seen in many developing countries, morbidity due to diarrhea is common in many states of India. This study was initiated to understand the incidence of different enteric bacteria among diarrheal patients in India and to study the phenotypic and genotypic features.

## 6. Methods:

### Hospital surveillance:

The National Institute of Cholera and Enteric Diseases (NICED) monitors incidence of *Vibrio cholerae* and other enteric pathogens through a surveillance program at the Infectious Diseases Hospital (IDH), Kolkata. Stool specimens were obtained from patients admitted to the IDH, Kolkata, in a McCartney bottles using sterile catheters. Rectal swabs were taken using sterile cotton-tipped swabs from patients from whom stool samples could not be obtained. Stool specimens and rectal swabs in Cary-Blair medium were transported within 1 h of collection and were examined within 1 h of arrival at the laboratory. Stool specimens collected from the diarrhoeal patients were processed for common enteric pathogens following standard methods. The vibrios were grown on thiosulfate citrate bile salts sucrose agar (Eiken Chemical Co. Ltd, Tokyo, Japan) at 37°C for 16-18 h and the *Vibrio cholerae* O1 and O139 serogroups were confirmed using polyvalent and monospecific antisera prepared at the NICED. In addition to the surveillance, we have also investigated different cholera outbreaks in different States of India.

### Bacterial strains

In addition to the isolation of *V. cholerae*, *Shigella* strains received from different parts of India were also included in this study. We examined 60 strains of *Shigella* spp. (20 *S. dysenteriae*, 16 *S. flexneri*, 7 *S. boydii* and 17 *S. sonnei*) isolated from dysentery outbreaks from different parts of India and sporadic hospitalized cases of shigellosis in Kolkata and Goa between 2001 and 2004. Strains were confirmed as *Shigella* spp. by standard biochemical tests (WHO, 1987) and serotyped using commercially available antisera (Denka Seiken). *Shigella* strains were serotyped using commercial antisera kit (Denka Seiken, Tokyo, Japan). The Clinical and

Laboratory Standards Institute's antimicrobial susceptibility test (NCCLS) was adapted for testing the bacteria isolates with commercially available discs (Becton Dickinson Co, Sparks, MD).

**PCR assay for the detection of *ctxB* alleles among *V. cholera* O1 and O139 strains**

Representative strains of *V. cholerae* O1 and O139 were screed for *ctxB* alleles using a mismatch amplification mutation assay (MAMA) PCR (Morita *et al.* Unpublished data). The full gene of *ctxB* was also amplified and the mutation in the nucleotide as well as amino acid sequences was checked after DNA sequencing of the each amplicon.

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility tests were performed by a disk diffusion method in accordance with National Committee of Clinical Laboratory Standards (NCCLS, 2004) for ampicillin (A, 10 μg), co-trimoxazole (Co, 25 μg), tetracycline (T, 30 μg), chloramphenicol (C, 30 μg), streptomycin (S, 10 μg), nalidixic acid (Na, 30μg), ciprofloxacin (Cf, 5μg), norfloxacin (Nf, 10μg), ofloxacin (Of, 5μg), ceftriaxone (Cr, 30 μg) and azithromycin (Az, 15 μg) (Becton Dickinson). *Escherichia coli* ATCC 25922 was used for quality control in each batch of tests. Minimal inhibitory concentrations (MICs) of nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin and azithromycin were determined for selected strains by the E-test (AB Biodisk).

**Screening of antimicrobial resistance genes**

PCR was performed to detect genes encoding resistance for ampicillin ($bla_{TEM}$), gentamicin (*aadB*), streptomycin (*aadA1, strA*), kanamycin (*aphA1-Ia*), chloramphenicol (*catA1*), tetracycline (*tetA, tetB, tetC, tetD, tetE* and *tetY*), betalactams ($bla_{OXA-1}$, $bla_{OXA-7}$, $bla_{SHV}$, $bla_{PSE-4}$, and $bla_{CTXM-3}$) and plasmid mediated quinolone resistance (*qnr*) as published for other organisms (Maidhof *et al.*, 2002). The newly designed primers (FMEF 5'-GCA ACG CAA AAA CAA AGT TAG G-3'; FMER 5'- GTG TTT GAA CCA TGT ACA - 3') were used to detect *aac (6')-1b* variants. All assays were carried out as single PCR assays except for *qnr* gene which was performed in a multiplex format, targeting all the three variants of *qnrA, qnrB* and *qnrS.* Template DNA was prepared by boiling the cultures grown in Luria-Bertani (LB) broth medium

(Difco) for 10 min, rapidly cooled on ice followed by brief centrifugation at 5000 rpm; the supernatant was retained for PCR assays.

**Nucleotide sequencing of the PCR products**

PCR products were purified with QIAquick PCR purification column (Qiagen), and sequencing reactions were carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Nucleotide sequence was performed in both directions with the same PCR primers used for the amplification of the target genes in an automatic sequencer (ABI Prism 3200, Applied Biosystems). Contig sequences were edited with DNA STAR (Lasergene Inc) and compared in BLAST of the NCBI database.

**Fluoroquinolone accumulation assay**

Fluoroquinolone sensitive and resistant strains of *Shigella* were grown to mid-log phase in LB ($OD_{600}$ = 0.4), harvested, and suspended in 0.2M morpholine propane sulfonic acid (MOPS) -Tris buffer (pH 7.0) to an optical density at 600 nm of 20 per ml. Cells were energized with 0.2% glucose for 20 min and fluoroquinolones were added at a concentration of 10 μg/ml. Aliquots of this mixture were taken and suspended in 1 ml of 100 mM glycine-HCl (pH 3.0), shaken for 1 h at room temperature, and the amount of released fluoroquinolone was determined spectrofluromettrically with excitation at 277 nm and emission at 448 nm. Experiments were performed in triplicate after the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to the assay mixture, as an inhibitor of the proton-motive force at a final concentration of 100 μM.

**Pulsed-field gel electrophoresis (PFGE)**

DNA fingerprinting was carried out by PFGE with *Xba*I restriction enzyme (Takara) according to a standard procedure (CDC, 2000). PFGE run conditions were generated by the autoalgorithm mode of CHEF Mapper system with a size range of 30 to 600 kb (Bio-Rad). After gel electrophoresis, gels were stained with ethidium bromide for 30 min and destained for 30 min with distilled water. The gel images were digitalized for computer-aided analysis (Gel Doc 2000; Bio-Rad). A DNA size standard (lambda ladder; New England Biolabs, Beverly, Mass) or *Salmonella* serovar Braenderup H9812 strain was used as the molecular mass standard.

**Cluster analysis**

PFGE gel images were retrieved and aligned to generate composite images containing the banding profiles of all the strains. The images were analysed with Diversity Database fingerprinting software (version 2.2.0; Bio-Rad) to determine the relatedness of the strains. Bands ranging from 48.5 to 600 kb were considered for the construction of dendrograms. Degrees of homology were determined by comparison of the Dice coefficient, and clustering correlation coefficients were calculated by an unweighed pair-group method with arithmetic means (UPGMA). A dendrogram showing the hierarchical representation of the level of linkage between the strains was drawn to predict the degree of clonal relatedness.

**7. Results and Discussion:**

**7.1 Phenotyping and genotypic characteristic of *V. cholerae***

A total of 17,249 patients hospitalized with acute secretary diarrhoea from 1997 to 2006 were examined for the presence of various enteric pathogens. The incidence of various serogroups of *V. cholerae* is shown in Table 1. Isolation rate of non-cholera vibrios ranged between 1.3 and 3.4% with peaks during 1998, 1999 and 2003. Serotyping results showed that there is no dominance of any particular serogroup. However, large number of strains remained untypable with exiting 206 antisera (Table 2). Representative O1 strains isolated between 2007 and 2008 were screened for the ctxB gene alleles using MAMA-PCR. In this PCR assay, all the test strains gave *ctxB* amplicon confirming the presence of classical type of CT (Table 3).

The isolation status of *V. cholerae* serogroup O139 is very low in most parts of India. However, in Delhi and its surroundings, sporadic cholera due to this serogroup still exists. We have included eight *V. cholerae* O139 isolated Delhi for molecular characterization in this study. The CT types are used as a marker to study the epidemiological difference in the O139 serogroup isolated during 2001 and 2004 to 2005. Of the 8 representative O139 strains 6 strains isolated during 2001, 2004, and 2006 had CT type 5 (Table 4). Interestingly, two strains isolated between 2004 and 2005 had classical type CT (type 1) (Table 4). Nucleotide sequence analysis of the *ctxB* gene of 8 representative *V. cholerae* O139 strains isolated between 2001 and 2006 showed interesting results. The amino acid sequence of 5 strains isolated during 2001, 2004 and 2006 has alanine at position 28, but both the El Tor and classical reference strains have aspartate at the same position but has histidine, phenylalanine and threonine at positions 39, 46 and 68,

respectively same as classical strain 569B of CT genotype 1. The O139 strains isolated during 2004 and 2005 had identical sequence of classical *ctxB* (genotype 1). With the available information it seems that most of the O139 strains isolated in Delhi had new *ctxB* genotype 4.

**Antimicrobial resistance in *Shigella* spp**

**Antimicrobial resistance:**

Table 5 shows the breakdown of the serotypes of the 60 *Shigella* strains, their antimicrobial resistance profiles and resistance gene complement. *S. dysenteriae* type 1 strains (n=17) were uniformly resistant to all the tested antimicrobials, except for azithromycin and ceftriaxone. *S. dysenteriae* type 1 strains HU8 and BCH518 isolated during 1988 and 1995 from a dysentery outbreak and sporadic infections respectively, had similar resistance profiles. The two *S. dysenteriae* type 5 strains were susceptible to ampicillin, fluoroquinolone, azithromycin and ceftriaxone and had a resistance profile of CoTCNaS. Except for two strains (NK2685 and NK2683), all the tested *S. flexneri* strains (n=16) were resistant to cotrimoxazole, tetracycline and streptomycin. Three strains of *S. boydii* serotype 12 and the majority (94%) of the *S. sonnei* strains had an identical resistance pattern (CoTNaS). The MIC for azithromycin resistant *S. flexneri* type 3b (NK2788) and *S. boydii* type 1 (G24371) was 192 and 128 μg/ml, respectively. None of the other *Shigella* strains proved to be resistant to azithromycin and ceftriaxone in contrast to a recent report from Bangladesh of resistance to these agents among *Shigella* spp. (Rahman *et al.*, 2004).

**Fluoroquinolone resistance and resistance mechanisms**

Ciprofloxacin, norfloxacin and ofloxacin are broad-spectrum fluoroquinolone agents that have excellent activity against most enteric pathogens. Clinical studies have underlined their safe use in adults and children (Bhattacharya *et al.*, 1997, Salam & Bennish 1998). In this study, 30% of the *Shigella* strains were resistant to fluoroquinolnes and a *S. boydii* serotype 1 strain (G24371) was resistant to each of the four compounds tested (Table 6). Due to the unrestricted use of fluoroquinolones in Kolkata for the treatment of diarrhoea and other infectious diseases, resistance to these drugs has been reported among enteric pathogens (Garg *et al.*, 2001; Sinha *et*

*al.*, 2004). In India, this trend has been increasing year on year since 2002 among *Shigella* spp. (Pazhani *et al.*, 2005). Fluoroquinolone resistance has also been identified in *Shigella* isolates in many Asian countries (MoezArdalan *et al.*, 2003; Talukder *et al.*, 2004; Von Seidlein *et al.*, 2006) and Canada (CCDR, 2005). At present, fluoroquinolone resistant *S. dysenteriae* type 1 and *S. flexneri* 2a strains remain susceptible to azithromycin and ceftriaxone, which have been reported to be effective against shigellosis in many countries (Khan *et al.*, 1997; Ashkenazi *et al.*, 2003) although cephalosporin resistance has been reported from Spain (Vila *et al.*, 1994) and Argentina (Radice *et al.*, 2001).

*S. dysenteriae* type 1 strains isolated from sporadic cases of dysentery from Calcutta (BCH518, NK2678 and H16576) Goa (12567) and outbreak cases from Calcutta, Aizal, and Chandigarh (D2, 21, AZ11, and 115, respectively) were tested for mutations in the *gyrA* and *parC* genes. For comparison, we included nalidixic acid resistant and fluoroquinolone susceptible *S. dysenteriae* type 1 strains isolated during 1988 (HU8) and 1995 (BCH518) All fluoroquinolone resistant strains had a uniform mutation in GyrA at position 83 (replacement of serine with leucine) and the majority of strains had a second mutation at position 87 with replacement of aspartic acid either with glycine or asparagine (Table 6). However, the *S. dysenteriae* type 1 strain BCH518, isolated during 1995 had a single mutation in the GyrA at position 83.but strain HU8 isolated in Tripura during 1988 had no mutation in *gyrA* and *parC* and showed reduced susceptibility to nalidixic acid although susceptible to fluoroquinolones (Table 6). However, *S. flexneri* (NK2788), *S. boydii* (G24371) and a *S. dysenteriae* type 1 strain from the Aizwal outbreak showed amino acid replacement at position 87 (D→N). To our knowledge, this is the first report of *S. boydii* having a mutation in the *gyrA*. All the fluoroquinolone resistant strains had a single mutation in ParC at position 80 (replacement of serine with isoleucine). In a nalidixic acid resistant *S. sonnei* (NK2017), a mutation was identified at position 83 (replacement of serine with leucine). Fluoroquinolone resistant strains of *S. boydii* (G24371), *S. flexneri* (NK2788) and a representative *S. dysenteriae* type 1 (12567) strongly exhibited fluoroquinolone efflux (Table 7). The steady sate accumulation of norfloxacin and ciprofloxacin was 2 to 4 fold lower in the resistant strains compared to that in the case of sensitive strains, C152 (Table 7). This suggests that the lower accumulation of fluoroquinolones can also account for the resistance of these strains. After the disruption of the efflux pump with the proton motive force uncoupler, m-chlorophenylhydrazone (CCCP), the accumulation was

almost at the same level in all the tested strains. This clearly suggests the role of efflux pumps as one of the responsible factors for the development of resistance.

*S. dysenteriae* type 1 strains from South Asia and Canada had uniform mutations in *gyr*A and *par*C (Talukder *et al.*, 2004; CCDR, 2005). In a *S. dysenteriae* strain, (HU8), which was susceptible to fluoroquinolone but resistant to nalidixic acid had identical mutation in the *gyrA*, similar to that of a 1995 strain isolated in Calcutta (Ahmed et al., 1999). Similarly, mutations in the *gyrA* of *S. flexneri* and *S. sonnei* were identical to those reported from other studies (Jeong *et al.*, 2003; Navia *et al.*, 2005). In shigellae, nalidixic acid resistance is not only due to mutations in the QRDR region, but also to an active efflux system (Ahamed *et al.*, 1999). Novel mechanisms for quinolone and fluoroquinolone resistance in *Enterobacteriaceae* are emerging all the time but high-level fluoroquinolone resistance among *S. dysenteriae* due to a proton motive force dependent efflux system was reported almost a decade ago in strains from Calcutta, which were devoid of any *gyrA* mutations (Ghosh *et al.*, 1998).

Plasmid-mediated quinolone resistance due to DNA gyrase protection by a protein from the pentapeptide repeat family called Qnr has recently been described in many clinical isolates of several species (Martinez-Martinez *et al.*, 2003, Jonas *et al.*, 2005). Indeed, clinical strains of *S. flexneri* type 2b from Japan were found to carry a transferable plasmid, which had 56% amino acid identity with Qnr (Hata *et al.*, 2005). Based on the aminoacid sequence, three subtypes of *qnr* genes such as *qnrA, qnrB* and *qnrS* and six variants each in *qnrA, qnrB* and two in *qnrS* have also been reported (Nordmann & Poirel, 2005). In this study, none of the strains harboured the *qnr* or its alleles. A novel ciprofloxacin-modifying enzyme (aminoglycoside acetyltransferase) encoding gene *aac (6')-1b-cr* was found in members of the *Enterobacteriaceae*, resistant to fluoroquinolones. We have identified the *aac(6')-1b-cr* gene in *S. boydii* type 1 (G24371) and *S. flexneri* 3b (NK 2788) strains and to our knowledge, this gene has not been reported previously in these *Shigella* species.

**Resistance to other antimicrobials and resistance genes**

All the *S. dysenteriae* type 1 strains harboured $bla_{oxa-1}$, *catA1*, *tet*(B), and *str*A genes, encoding resistance for ampicillin, chloramphenicol, tetracycline, streptomycin, respectively (Table 5). The, *tet*(B) gene was more common (90%) than *tet*(A) (10%) in *S. dysenteriae*. Irrespective of serotypes, *S. flexneri* strains harboured *tet*B as well as the $bla_{oxa-1}$ genes and in

strain NK2788, $bla_{oxa-1}$, $bla_{TEM-1}$ and *tet*A genes were detected (Table 5). Group 9 CTX-M beta-lactamase has been reported in *S. boydii*. In this study, $bla_{CTX-M-3}$ was found in a *S. boydii* type 1 strain (G24371) and to our knowledge; this is the first report of this enzyme in this serotype. The majority of *S. sonnei* strains harboured *str*A (88%) and *tet*A (76%) genes rather than *aadA*1 and *tet*B (6% each). Genes encoding resistance for kanamycin (*aph1a*), gentamicin (*aad*B) and tetracycline (*tet*C, *tet*D, *tet*E and *tetY*) were not found (data not shown). The chromosomal multi-antibiotic resistance locus of *S. flexneri* type 2a (Rajakumar *et al.*, 1997) was identified supporting its common occurrence among several serotypes of *S. flexneri* (Casalino *et al.*, 1994; Thong *et al.*, 2002). We found 97% and 3 % of ampicillin resistant *Shigella* strains harbouring $bla_{oxa-1}$ and $bla_{TEM-1}$ genes, respectively. The predominance of $bla_{oxa-1}$ in *Shigella* has been reported from many countries (Maraki *et al.*, 1998; Siu *et al.*, 2000; Huang *et al.*, 2005). Similar to the reports from Mexico and Brazil (Martinez-salazar *et al.*, 1986; Peirano *et al.*, 2005), we found a high frequency of *tet(B)* among *S. flexneri* strains and in common with some South American *Shigella* strains (Peirano *et al.*, 2005), the presence of the chloramphenicol resistance gene *catAI* that encodes for chloramphenicol o-acetyl-transferase, was confirmed in *S. flexneri* strains either with *str*A or *aadA*1 genes or both (Table 5).

**PFGE analysis**

It was necessary to show whether the frequency of antimicrobial resistance and its determinants was due to the widespread occurrence of specific clones. Two *S. dysenteriae* type 5 (NK2440 and NK2454) had identical *Xba*I restriction patterns by PFGE, but were different from serotype 1 strains which had similar patterns (Fig. 1), which are homologous to the previously reported PFGE profile (Pazhani *et al.*, 2004). Sixteen strains representing different serotypes of *S. flexneri* showed extensive variation in PFGE profile (Fig 2) but six strains of type 2a wre identical and clustered closest to two strains of serotype 2b. The remainders of the *S. flexneri* serotypes were distinct in DNA profile. Of the seven *S. boydii* strains, 3 belonging to serotype 12 were closely related in DNA profile while the remainders were distinct (Fig. 3). Eleven of the 17 *S. sonnei* strains were identical in DNA pattern and two strains clustered closely to this group being different by two to three bands (Fig. 4). This underlines the findings by others of the clonal nature of *S. sonnei* (Alcoba-Florez *et al.*, 2005; Mammina *et al.*, 2005).

Although PFGE has proved to be a powerful tool for the discrimination of strains and identification of clonal lineages in several bacterial species, in some *S. boydii* serotypes it may not be as indicative of absolute strain relatedness. Woodward et al. (2005) reported that strains of *S. boydii* serotypes 1, 18, 19 and 20 from Canada gave highly similar *Xba*I macrorestriction which suggests that some strains which express different lipopolysaccharide antigen epitopes share a common genetic background. Other serotypes were genetically heterogeneous. The population structure of this species therefore warrants further investigation with complementary molecular tools such as multilocus sequence typing.

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**Table 1. Incidence of different serogroups of *V. cholerae* in Kolkata**

| Year (No. of Sample) | Serogroup | | |
|---|---|---|---|
| | **O1** | **O139** | **Non-O1, non-O139** |
| 1997 (1451) | 88 | 136 | 30 |
| 1998 (1513) | 237 | 59 | 52 |
| 1999 (1336) | 126 | 70 | 46 |
| 2000 (2028) | 165 | 33 | 30 |
| 2001 (2131) | 139 | 16 | 42 |
| 2002 (2285) | 90 | 71 | 49 |
| 2003 (1673) | 135 | 3 | 53 |
| 2004 (2430) | 466 | 2 | 31 |
| 2005 (1472) | 183 | 2 | 38 |
| 2006 (930) | 83 | 3 | 19 |

**Table 2. Serotyping results of *V. cholerae* non-O1, non-O139 strains**

| Year | Serogroups (no of strains) |
|---|---|
| 2004 | O3, O6, O11, O12, O20(4), O27, O36, O62(2), O97(3), O122, O107, O109, O137, O183, O185, ONT(9) |
| 2005 | O6, O11, O17, O18, O26, O37, O39, O43(2), O59(2), O70, O97(5), O107, O110, O128, O137, O185, ONT(14) |
| 2006 | O2, O6 (2), O7, O10, O11, O12, O42, O44 (2), O45, O46, O94 (3), O176, ONT (3) |