1. Objectives of the project:

(i) Tracking the spread of molecular types of hybrid strains of *Vibrio cholerae* O1

(ii) Epidemiology and molecular characterization of *Campylobacter* spp in Kolkata, India

(iii) Phenotypic and genetic characterization of *Shigella* spp isolated from diarrheal patients

2. Study Design:

(i) Tracking the spread of hybrid strains of *Vibrio cholerae* O1

The epidemic and pandemic cholera is caused by toxigenic *Vibrio cholerae* is known for its dynamicity, as the organism often changes its biotypic features as well as molecular configuration. *V. cholerae* belonging to serotype O1 has two well established biotypes, namely, classical and El Tor, that are differentiated based on number of phenotypic traits like susceptibility to polymyxin B, chicken cell (erythrocytes) agglutination (CCA), hemolysis of sheep erythrocytes, Voges-proskauer (VP) test, which measures the production of acetylmethylcarbinol, and phage susceptibilities (1, 2). Biotype is a sub specific taxonomic classification of *V. cholerae* O1.

Comparative genetic analyses have revealed a high degree of conservation among diverse strains of *V. cholerae* but have also shown genes that differentiate classical biotype from El Tor biotype (3). Molecular biotyping of *V. cholerae* O1 using multiplex PCR targeting the *ctxA-tcpA* gene complex exploits the nucleotide sequence differences of the major subunit protein of the toxin co-regulated pilus (TCP) gene (*tcpA*) to differentiate between classical and El Tor biotypes (4). Only in toxigenic *V. cholerae* O1 El Tor and O139 strains, cholera toxin prophage region (CTXΦ) is often flanked by an element termed RS1 containing *rstC* gene (5). The only difference between RS1 and RS2 is the presence of *rstC* gene in RS1 alone (5, 6). Another virulence associated protein known as repeat in toxin (RTX) encoded by a cluster of genes of 10kb size, comprising four ORFs, *rtxABCD*, of which the *rtxC* gene has been observed only in El Tor biotype (7). Nucleotide sequence comparison of hemolysin encoding *hlyA* gene from classical and El Tor strains reveal the presence of an 11-base-pair deletion in classical

strains that results in a truncated protein product of 27 kilodaltons in classical strains rendering it non-hemolytic, whereas in El Tor strains the HlyA is intact 82-kilodalton and biologically active (8). On the basis of differences in the sequences of *hlyA* genes, a 19-base-pair oligodeoxynucleotide probe has been developed to distinguish between the two biotypes of *V. cholerae* serogroup O1 (9). This gene marker was found to be very useful to differentiate the biotypes than the other commonly used methods, which are less reliable and often difficult to interpret (9). Recently, comparative genomic studies using a *V. cholerae* DNA microarray on 11 epidemic isolates identified two regions, *Vibrio* seventh pandemic island I (VSP-I), encompassing VC0175 to VC0185 and VSP-II, encompassing VC0490 to VC0497, that were found exclusively among El Tor biotype isolates (3). Subsequently, it was shown that the VSP-II region actually encompassed a 26.9 kb region (VC0490–VC0516) in *V. cholerae* biotype El Tor and O139 serogroup isolates (10). Besides these phenotypic and genotypic differences, there are also dissimilarities in the infection pattern of disease caused by the two biotypes (11). Epidemiological studies proved occurrence of more asymptomatic carriers of El Tor strains that outnumber active cases by a ratio of up to 50:1 (12), better survival of El Tor strains in the environment and in the human host, and more efficient host-to-host transmission of El Tor strains than of classical strains (13).

Cholera toxin (CT), the primary toxin produced by *V. cholerae* O1 and O139, is responsible for most of the manifestations of the disease cholera. Based on the B subunit of CT, two immunologically related but not identical epitypes have been designated: CT1 is the prototype elaborated by classical biotype strains and by U.S. Gulf Coast strains, while CT2 is produced by the El Tor biotype and O139 strains (14). Another classification identifies three types of *ctxB* genes based on three non-random base changes resulting in changes in the deduced amino acid sequence. Genotype 1 is found in strains of the classical biotype worldwide and in US Gulf Coast, genotype 2 is found in El Tor biotype strains from Australia, and genotype 3 is found in El Tor biotype from the seventh pandemic and the Latin American epidemic strains (15). Thus, the *V. cholerae* O1 El tor biotype of the ongoing seventh pandemic produces CT of the CT2 epitype and genotype 3, while the classical biotype CT belongs to the CT1 epitype and genotype 1.

Although the classical biotype of *V. cholerae* O1 is extinct, even in southern Bangladesh, the last of the niches where this biotype prevailed, Nair *et al.* (2002) (16) identified new varieties of *V. cholerae* O1, of El Tor biotype with traits of classical biotype, from hospitalized patients with acute diarrhea in Bangladesh. These strains could not be biotyped and were, therefore, designated as "hybrid type". The impact of such hybrids was emphasized when *V. cholerae* O1 isolated from Mozambique during an epidemic of cholera in early 2004 were found to carry the classical type CTX prophage but otherwise was identical to El Tor biotype (17, 18). Recently, a collection of *V. cholerae* O1 strains isolated in Bangladesh during the past four and a half decades were examined using monoclonal antibodies specific for classical and El Tor CT and the nucleotide sequence of the B subunit of CT of representative strains to determine the deduced amino acid sequence. This study revealed that all *V. cholerae* O1 El Tor strains isolated since 2001 produced CT subtype of the classical biotype indicating a cryptic change in the seventh pandemic El Tor biotype strains of *V. cholerae* O1 has occurred (19). Therefore, the epitype and genotype of CT of the El Tor strains currently associated with cholera in Bangladesh has shifted from epitype CT2 to epitype CT1 and from genotype 3 to genotype 1. The presence of classical CT in El Tor biotype *per se* is not novel and has been reported (16, 17, 20). In fact, the US Gulf Coast clone of *V. cholerae* O1 is El Tor strains that possess classical CT (15). The fact that El Tor strain producing classical CT has completely replaced the prototype seventh pandemic El Tor strains that produced the El Tor CT in Bangladesh is interesting. More recently, retrospective analysis of *V. cholerae* O1 strains over a period of more than a decade established that hybrid CTX prophage with El Tor *rstR* and classical *ctxB* replaced El Tor type completely since 1995 in Kolkata, India (21).

Apart from classical and El Tor type biotypes, two new biotypes have been proposed, one possessing conventional phenotypic properties of both classical and El Tor thus designated as 'Hybrid biotype' and another which is similar to the El Tor biotype by conventional phenotypic traits, but produces classical type CT and thus designated as 'El Tor variant'. In the original publication (19), we had named these strains as altered El Tor but now renamed them as 'El Tor variant' (22). A recently developed mismatch amplification mutation assay (MAMA) PCR is useful in detecting El Tor or classical type

ctxB (23). We believe that this amendment is essential in view of the current thought that some of these hybrids might cause a more severe kind of cholera and the evidence to this effect (24) is becoming available. There is also indication that the hybrid and El Tor variant type of strains are spreading to other parts of the world (25).

Following the standard protocol of pulsed-field gel electrophoresis (PFGE) (26) it will be easy to identify the clonal spread of *V. cholerae* O1 in many regions of the world, as the each strain can be compared with the preexisting *Not*I PFGE profiles. At the NICED we have initiated banking the *V. cholerae* O1 PFGE profiles of Indian strains as well as bundle files received from different parts of the world as a part of the Global *Vibrio cholerae* data base (GVD).

Results

Identification of hybrid strains using MAMA-PCR

One hundred and fifty *V. cholerae* O1 strains isolated from different parts of India were included in this study collected from 2007 to 2010 were included in this study. In addition, we have tested 50 strains isolated from cholera cases from Zanzibar and Keya isolated in the year 2009-2010. The identity of *V. cholerae* strains was confirmed by several conventional tests (growth in thiosulphate-citrate-bile salts-sucrose agar, serogrouping using O1poly and mono-specific Ogawa, Inaba and O139 antisera). Serologically, all the strains belonged to O1 Ogawa serotype. MAMA-PCR assay confirmed that all the strains belonged to El Tor variant (23). In this study, all the strains were identified as El Tor variant having classical *ctxB*.

Banking of PFGE profiles in the GVD

PFGE of *Not*I digested genomic DNA was performed using a CHEF-Mapper (Bio-Rad Laboratories, Hercules, CA) according to the PulseNet standardized protocol for subtyping of *V. cholerae* O1. The PFGE image was captured using a Gel Doc XR system (Bio-Rad). The gel images were normalized by aligning the peaks of the *Salmonella braenderup* size standard and analyzed using the BioNumerics software Version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). Degree of band similarity was

determined by comparison of the Dice coefficient, and the clustering correlation coefficients were calculated by an unweighted pair-group method with arithmetic averages (UPGAMA). We have also stored all the information regarding the source of the each strain, isolation date, and antimicrobial susceptibility pattern.

Overall, we have identified 30 PFGE *Not*I profiles with all the 200 strains tested. *V. cholerae* strains from Kenya exhibited distinct profile in the PFGE with profile 18 and 19. *V. cholerae* strains from Zanzibar exhibited distinct profile in the PFGE with profile 20 and 21 (Fig. 1).

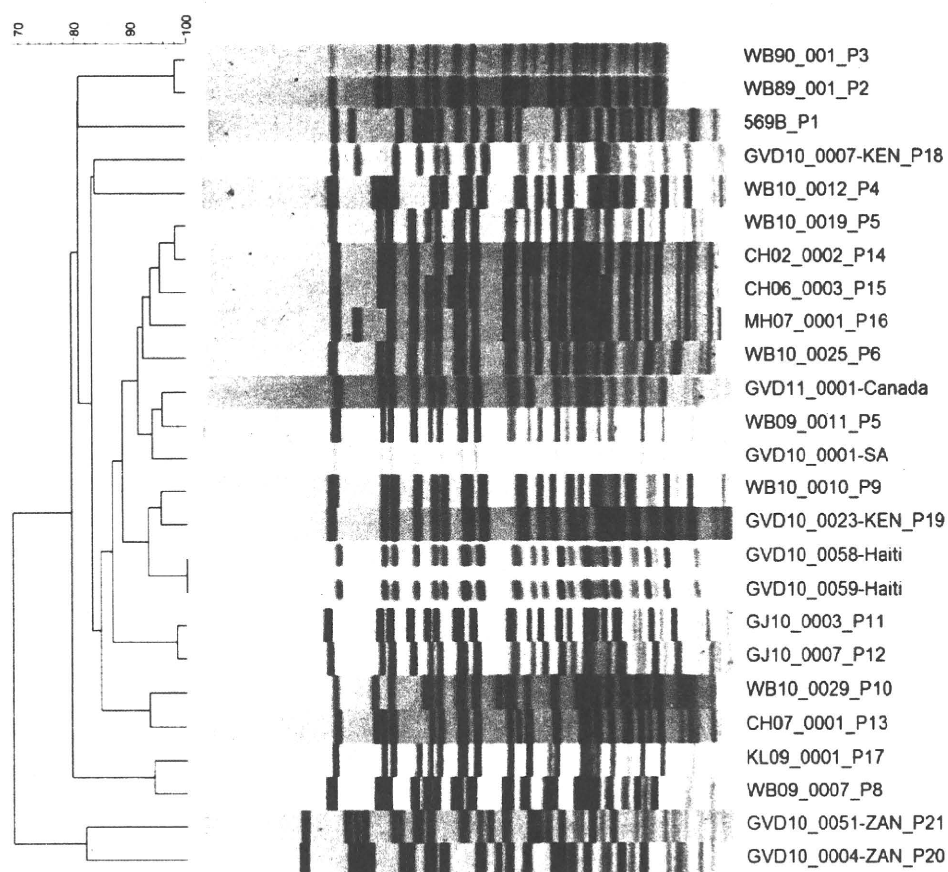


Fig. 1. Representative *Not*I PFGE profiles of *V. cholerae* O1 strains from different parts of India and Africa in comparison with the bundle file received from South Africa, Canada, Haiti and Venezuela.

Comparison of bundle files received from South Africa, Canada, Haiti and Venezuela

During 2010-2011 we have recived *Not*I PFGE profiles as bundle files from sporadic cholera cases from South Africa, Canada and Venezula and a large cholea outbrek from Haiti. These profiles were matched with the stored PFGE profiels from the GVD. Interestingly, the South African strain had identical match with the Indian strain that was isolated from Hyderabed cholera outbreak during 2009 (data not shown). When the Haiti profiles send by the CDC, Atlanta were compared, we found a close match with the West Bengal strains isolated after the cylcone Aila during May 2009 (Fig. 2). Bundle fiel recvied from Canada was also incldued in this stuy, which exhibited close match with the Haiti cholera outbrak strains. This patient had a history of visiting Haitii at the time of cholera outbreak. Recently, have also received the bundle file of *Not*I profiles of *V. cholerae* isolated from Venezula. These strains had identical match with the Haiti strains (Fig. 2). Cholera patientis from Venezula also had the travel history of visiting Haiti to attend a wedding in February 2011.

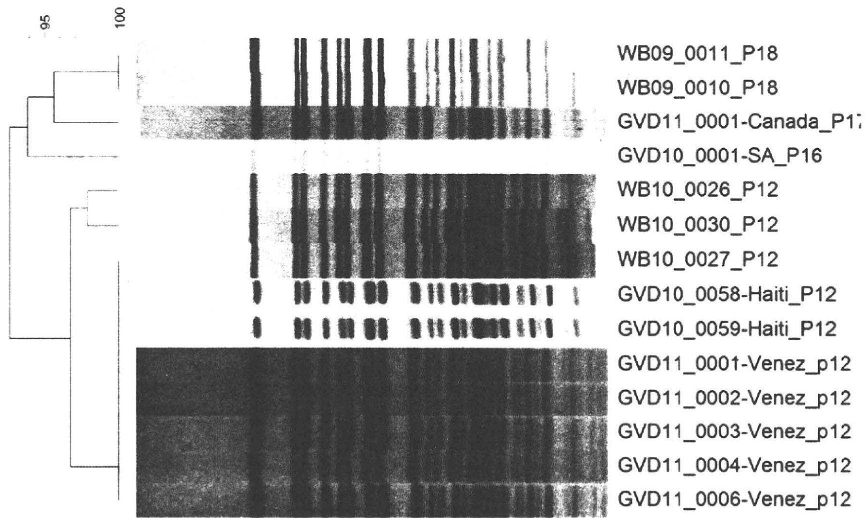


Fig. 2. Comparison of PFGE bundle files received from South Africa, Canada, Haiti and Venezuela.

(ii) Epidemiology and molecular characterization of *Campylobacter jejuni* in Kolkata, India

During the past three decades, *Campylobacter* spp. have been the focus of great attention because of the increasing frequency with which they have been isolated from infected man and animals, as well as contaminated food and water. After its successful isolation from stools in the 1970s, *Campylobacter* has rapidly become the most commonly recognized cause of bacterial gastroenteritis in man. Although several *Campylobacter* spp. (*C. jejuni, C. coli, C. upsaliensis, C. lari, C. concisus, C. fetus subsp. fetus, C. jejuni* subsp. *doylei, C. hyointestinalis*) have been associated with diarrhea, *C. jejuni* is by far the most frequent species isolated from humans. Among several species of campylobacters, *C. jejuni* is a well known to cause morbidity, in both industrialized and developing countries, and represents a considerable drain on economic and public health resources. In the industrialized world, acute self-limiting gastrointestinal illness, characterized by diarrhea, fever and abdominal cramps, is the most common presentation of *C. jejuni* infection, but symptoms and signs are not so distinctive that the physician can differentiate this infection from illness caused by other organisms. Campylobacter enteritis, and occurs most often in patients whose immune system is severely compromised.

The epidemiology of *Campylobacter* infection in developing countries differs markedly from that of the developed world. In developing countries, *C. jejuni* is isolated more frequently but also the rates of carriage in healthy populations are often high (27). There are also some reports from developing countries, where *C. jejuni* and *C. coli* have been isolated mostly from populations with diarrheal illness (27-29). Numerous studies from developed countries have demonstrated *C. jejuni* in 4—14% of patients with diarrhea and in fever than 1% of asymptomatic persons (29). Studies from the USA and other developed countries show that enteritis due to *Campylobacter* exceeds cases caused by *Salmonella* species, *Shigella* species or *Escherichia coli* O157:H7 (29, 30). It is estimated that true *Campylobacter* infection rates in the USA and UK are as high as 1% of the population per year (30).

Efforts have recently focused on determining important risk factors for *Campylobacter* infection to guide interventions aimed at reducing disease burden. Such risk factors are commonly determined for other pathogens through investigations of outbreaks; however, despite the large number of *Campylobacter* notifications, outbreaks are rarely detected. Case control studies to determine risk factors for infection have identified consumption of chicken, exposure to animals, and consumption of contaminated water as significant. A meaningful typing system that could be applied to *Campylobacter* isolates as they arrive in the public health laboratory could aid outbreak detection and help identify common sources of infection.

Campylobacteriosis is considered to be a very potential zoonotic disease (animal to man infection and vice versa), which can causes significant morbidity and even mortality in adult and children particularly in developing countries like India. Recent surveys showed that people, in rural areas, who are mostly vulnerable to this disease due to their close association with farm animals and less hygienic precautions, do not have any knowledge and awareness regarding this common disease. In this scenario, molecular epidemiology of the *Campylobacter* species needs to be investigated in and around Kolkata which is most densely populated metro city in India, with special reference to the distribution of virulence genes. Investigation of molecular epidemiology of *Campylobacter* species using different typing tools might be very much useful for – 1) Understanding routes of infection, 2) Identification of pathogenic strains, 3) Correlation between strains, separated by hosts and locations and 4) Suitable chemotherapy for *Campylobacter* mediated infections.

Studies conducted in Kolkata showed that *C. jejuni* is typically associated with predominantly watery diarrhea and isolated in frequencies varying from 9 to 13% (2, 12). Specific properties involved in adhesion, colonization, invasion, and toxin production appear necessary in the process of infection. In this study, *flaA*, *cadF*, *racR*, and *dnaJ* were selected as pathogenic genes responsible for the expression of adherence and colonization; *virB11*, *ciaB*, and *pldA* were selected as pathogenic genes responsible for the expression of invasion; *cdtA*, *cdtB*, and *cdtC* were selected as pathogenic genes responsible for the expression of toxin production; and *wlaN* was selected as a gene that

is presumably involved in the expression of ganglioside mimics in Guillian-Barre′ syndrome. Another putative virulence gene, *ceuE*, encoding a lipoprotein (a component of a protein-binding-dependent transport system for the siderophore enterochelin) of *C. jejuni* was also included in this study. We have followed the published PCR assay methods to detect the above mentioned virulence genes (31).

The objective of this aspect are subdivided as follows

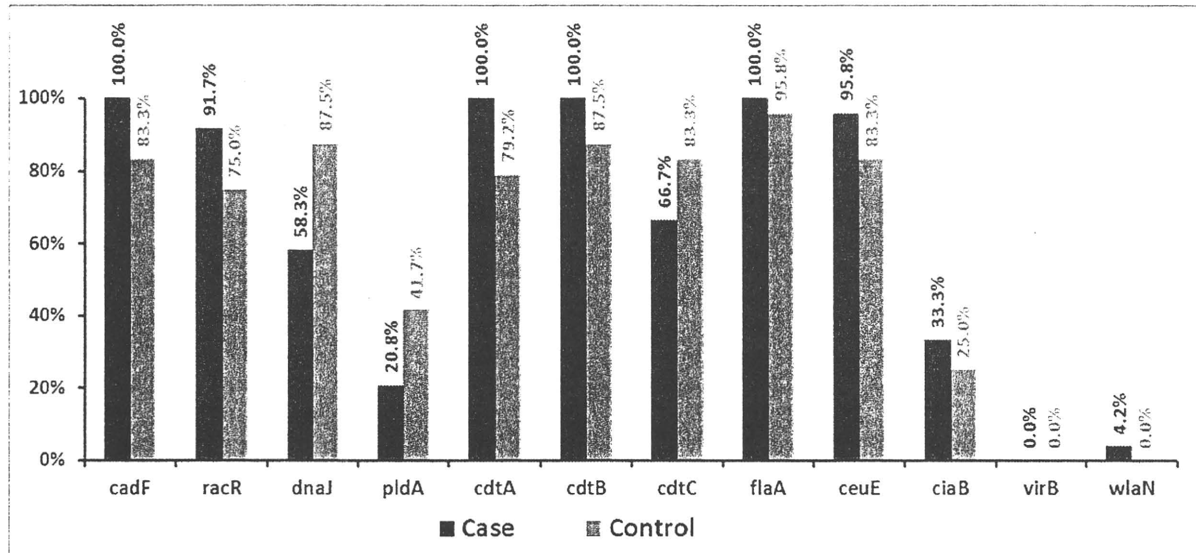
a) Identification and speciation of campylobacters isolated from diarrheal and normal children of less than 5 year age group in urban slums of Kolkata.
b) Virulence gene profiling of *Campylobacter jejuni* isolated as sole pathogens from diarrheal patients

Results:

C. jejuni was isolated using standard procedures form children below five years of age with diarrhea (13.6%, n=1571) and without diarrhea (9.4%, n=2019). This pathogen was isolated as a single etiological agent in 2.5% and 3.5% of the case and controls, respectively. We have considered only the *C. jejuni* strains that were isolated as a single organism from representative case and controls (24 each). After initial isolation using selective media, the suspected isolates were identified by staining and standard biochemical testes. Speciation was made using a PCR assay, targeting the *cdtB* gene that identifies *C. jejuni, C. coli* and *C. fetus*. *C. jejuni*.

Virulence gene profile analysis indicated that except for *dnaJ, pldA,* and *cdtC*, the rest of the genes (*cadF, racR, cadtA, cdtB, ceuE* and *ciaB*) were high diarrheal cases than controls at various proportions (Fig. 3). One the virulence gene that is responsible for adherence and colonization (*virB*) was not detected in this study. The gene *wlaN* that is presumably involved in the expression of ganglioside mimics in Guillian-Barre′ syndrome was detected only in diarrheal children though in less number of cases (4.2%).

Fig. 3. Comparison of virulence gene profiles of *C. jejuni* isolated from children below five years with diarrheal and normal controls.



(iii) Phenotypic and genetic characterization of *Shigella* spp isolated from diarrheal patients

Of the bacterial causes of dysentery, shigellosis continues to remain a considerable public health problem in the many parts of the world. Surveys conducted during 1990-2009 indicated that approximately 125 million *Shigella* infected cases occur annually in Asia, of which ~14,000 cases were fatal (32). The distribution of serogroups of *Shigella* spp differs from country to country. Serogroups such as *S. flexneri*, *S. sonnei* and *S. boydii* are predominant in the developing countries, while *S. sonnei* is frequently reported in the industrialized countries. Prevalence of *S. dysenteriae* is mostly reported from South Asia and sub-Saharian Africa (33). Unlike other acute diarrheal illness that required adequate fluid replacement by either oral or intravenous rehydration, shigellosis requires antimicrobial therapy to reduce the duration of the illness and to prevent transmission to close contacts. In this study, we analyzed the distribution of virulence genes and clonality of diverse serogroups of *Shigella* strains isolated from hospitalized children and adult patients with acute diarrhea.

During November 2007-October 2009, a total of 212 (6.5%) *Shigella* strains were isolated from 3262 enrolled diarrheal cases admitted in the Infectious Diseases Hospital, Beliaghata, Kolkata. Virulence genes such as *set* (ShET-1), *sen* gene (ShET-2), *ipaH, ial, sat* and *virF* were detected by simplex PCR assay using published primer pairs (34, 35). PFGE of *Not*I and *XbaI* digested genomic DNA was performed using a CHEF-Mapper (Bio-Rad Laboratories, Hercules, CA) according to the PulseNet standardized protocol for subtyping of *Shigella* species (36). The PFGE image was captured using a Gel Doc XR system (Bio-Rad). The gel images were normalized by aligning the peaks of the *Salmonella braenderup* size standard and analyzed using the BioNumerics software Version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). Degree of band similarity was determined by comparison of the Dice coefficient, and the clustering correlation coefficients were calculated by an unweighted pair-group method with arithmetic averages (UPGAMA).

Results:

During this study, 212 (6.5%) *Shigella* strains were identified from 3262 diarrheal patients. The proportion of different *Shigella* serogroups were: 160 (75.5%) *S. flexneri*, 33 (15.6%) *S. sonnei*, 14 (6.6%) *S. boydii* and 5 (2.3%) *S. dysenteriae*. Throughout the study period, *S. flexneri* was the most common serogroup with predominance of serotype 2a during 2009. Prevalence of *S. flexneri* 3a was also high in this study, which was next to 2a. Interestingly, 19 (11.8%) *S. flexneri* strains agglutinated only with the polyvalent type B antiserum and complete serotyping of these strains was not accomplished with commercially available *Shigella* antisera and none of them were reacted with the provisional *S. flexneri* monoclonal antiserum (# 88-893). Hence, these strains were designated as untypable. *S. dysenteriae* type 1, type 3 (one strain each) and 3 strains of type 2 were isolated in this study. Among 14 *S. boydii* strains, type 12 was common (4 strains) and the others were represented by the serotypes 1, 11 and 15 (2 strains each) and 9, 18 and 10 (1 strain each). One strain agglutinated with only polyvalent *S. boydii* antiserum but not with any of the monovalent antisera.

More than 75% *Shigella* positive cases were infected with *S. flexneri* followed by *S. sonnei* (14.4%). About 31% and 69% *Shigella* infected cases were children under five years and greater than five years of age group, respectively. Polymicrobial etiology was detected in 51% of the patients from whom the *Shigella* strains were isolated, especially with the *S. flexneri* serogroup. Sixty per cent of *Shigella* positive cases in under five-year age were found with polymicrobial infection. The distribution of *Shigella* serogroups and co-occurrence of *S. flexneri* infection was significantly associated with viral pathogens (*P*=0.29) compared to other enteric bacterial or parasitic pathogens.

All the tested strains were positive for invasive plasmid antigen H (*ipaH*) gene and 75% of *Shigella* strains harbored virulence plasmid (*virF*). Secreted autotransporter toxin Sat, encoded by the *sat* gene harbored by 71.2% of the *Shigella* strains (151of 212), [3 of 5 (60%) *S. dysenteriae*, 146 of 160 (91.2%) *S. flexneri* and 7 of 14 (50%) *S. boydii* strains]. None of the *S. sonnei* strains harbored this gene. The *set* gene that encodes for *Shigella* enterotoxin 1 (ShET1) that contains two subunits A and B were exclusively present in the all strains of *S. flexneri* serotype 2a. One *S. flexneri* serotype variant Y harbored only the ShET1 subunit A gene. The *sen* gene encoding the ShET2 was present in 196 of 212 (92.4%) *Shigella* strains, which was present in 144 of 160 (90.0%) *S. flexneri*, 32 of 33 (96.9%) *S. sonnei*, 13 of 14 (92.8%) *S. boydii* and all the *S. dysenteriae* (100%). Invasion associated locus that encodes *ial* gene was present in 164 of 212 (77.3%) *Shigella* strains, which was predominant among *S. flexneri* (62.2%), followed by *S. sonnei* (8.4%), *S. boydii* (4.2%) and *S. dysenteriae* (2.3%). One *S. dysenteriae* type 1 strain isolated in this study harbored the *stx1* gene encoding for Shiga toxin.

PFGE analysis of representative serotypes of *S. flexneri* after digestion with *NotI* revealed serotype specific clusters with 80 to 100% similarities within each serotype (Fig. 4). When *XbaI* digested geneomic DNAs of recent *S. dysenteriae* type 1 and epidemic strains isolated previously in this region were compared, the recent strain was identified as a different clone. *S. dysenteriae* type 2 strains were identified as closely related (Fig. 5) and the representative *S. sonnei* strains were observed to be clonally related (Fig. 6).

Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE-NotI
PFGE-NotI

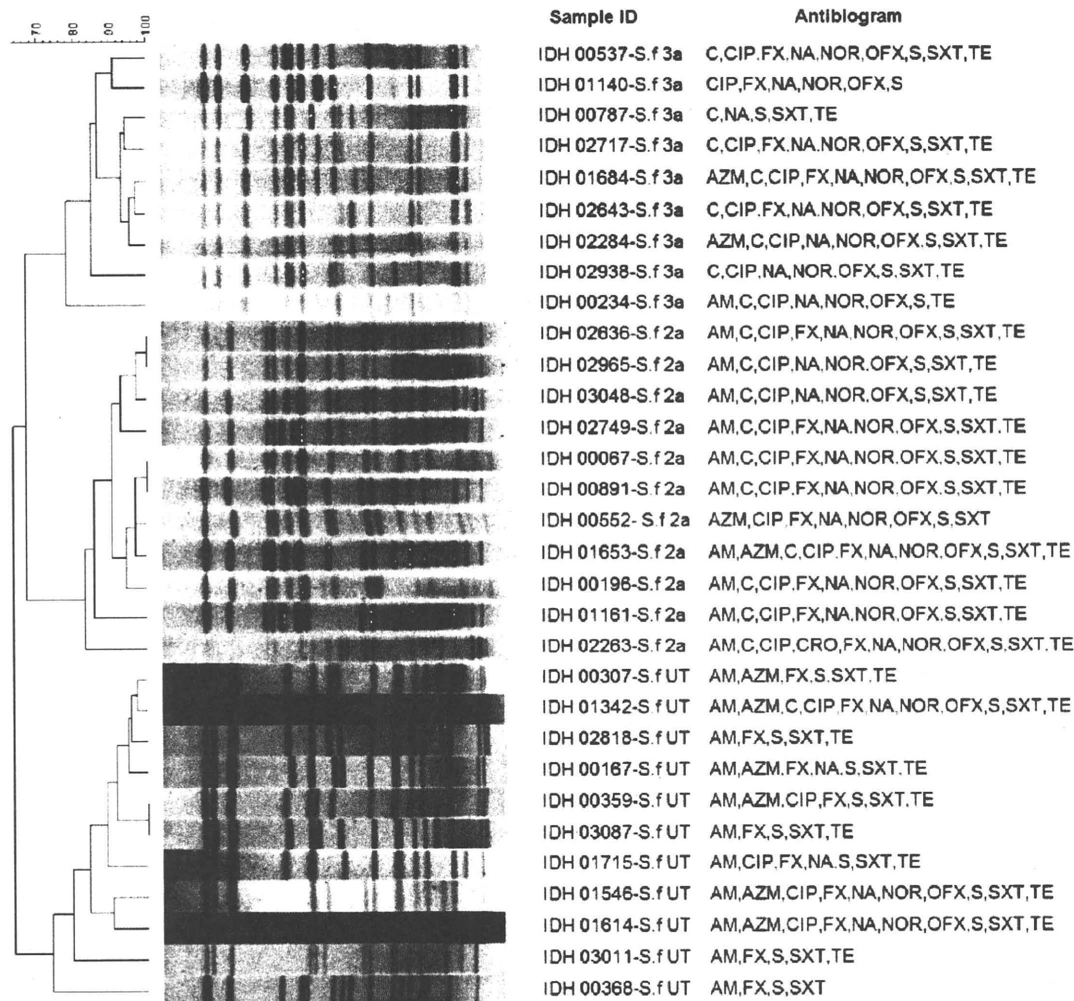


Fig. 4. *NotI* profile *of S. flexneri* strains and dendrogram with percentage similarity.

Dice (Opt 1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]

PFGE-XbaI **PFGE-XbaI**

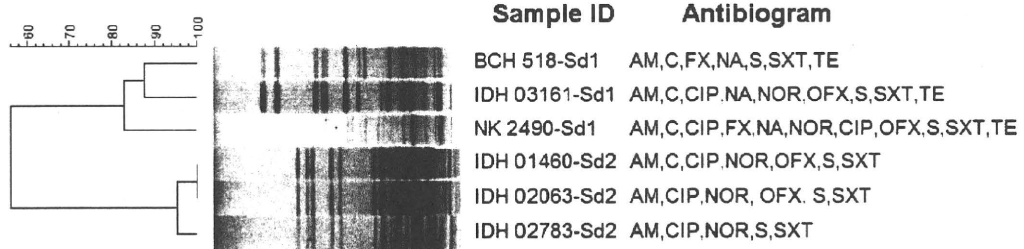


Fig. 5. *XbaI* profile *of S. dysenteriae* strains and dendrogram with percentage similarity.

Dice (Opt 1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]

PFGE-Xbal PFGE-Xbal

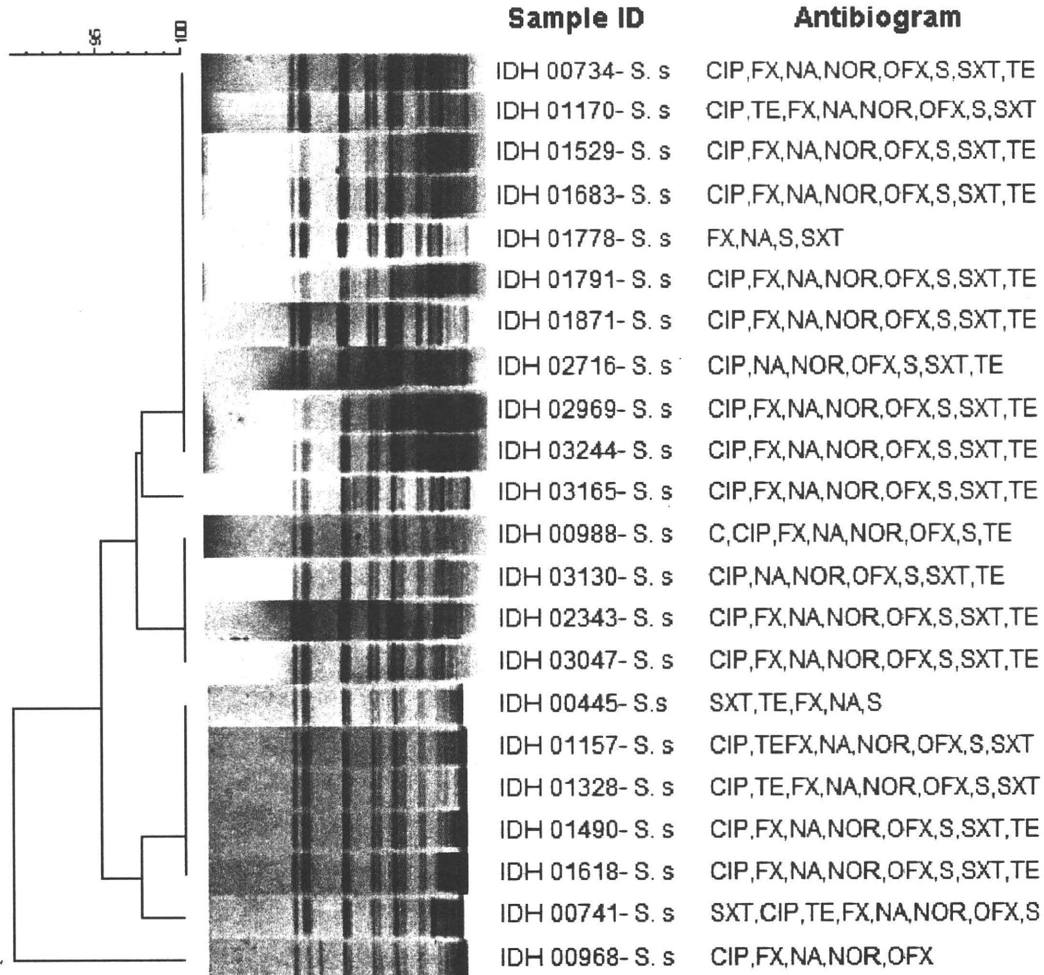


Fig. 6. *XbaI* profile *of S. sonnei* strains and dendrogram with percentage similarity.

REFERENCES

1. Faruque SM, Albert MJ, Mekalanos JJ. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae. Microbiol Mol Biol* Rev 1998; 62:1301-14. Review.

2. Kaper JB, Morris JG Jr, Levine MM. Cholera. *Clin Microbiol* Rev 1995; *8* : 48-86.

3. Dziejman M, Balon E, Boyd D, Fraser CM, Heidelberg JF, Mekalanos JJ. Comparative genomic analysis of *Vibrio cholerae* genes that correlate with cholera endemic and pandemic disease. *Proc Natl Acad Sci USA* 2002; 99 : 1556-61.

4. Keasler SP, Hall RH. Detecting and biotyping *Vibrio cholerae* O1 with multiplex polymerase chain reaction. *Lancet* 1993; *34* : 1661.

5. Waldor MK, Rubin EJ, Pearson GDN, Kimsey H, Mekalanos JJ. Regulation, replication, and integration functions of the *Vibrio cholerae* CTXΦ are encoded by region RS2. *Mol Microbiol* 1997; *24*: 917–26.

6. Davis MB, Moyer KE, Boyd EF, Waldor MK. CTX prophages in classical biotype *Vibrio cholerae*: functional phage genes but dysfunctional phage genomes. *J Bacteriol* 2000; *182*: 6992–8.

7. Lin W, Fulner KJ, Clayton R, Sexton JA, Rogers MB, Calia KE, *et al.* Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. *Proc Natl Acad Sci USA* 1999; *96*: 1071-6.

8. Rader AE, Murphy JR. Nucleotide sequences and comparison of the hemolysin determinants of *Vibrio cholerae* El Tor RV79(Hly+) and RV79(Hly-) and classical 569B(Hly-). *Infect Immun* 1988; *56*: 1414-9.

9. Alm RA, Manning PA. Biotype-specific probe for *Vibrio cholerae* serogroup O1. *J Clin Microbiol* 1990; *28*: 823-4.

10. O'Shea WA, Finnan S, Reen FJ, Morrissey JP, O'Gara F, Boyd EF. The Vibrio seventh pandemic island-II is a 26.9 kb genomic island present in *Vibrio cholerae* El Tor and O139 serogroup isolates that shows homology to a 43.4 kb genomic island in *V. vulnificus*. *Microbiol* 2004; *150*: 4053-63.

11. Nair GB, Mukhopadhyay AK, Safa A, Takeda Y. Emerging hybrid variants of *Vibrio cholerae* O1. In: S. M. Faruque and G.B. Nair, editors. *Vibrio cholerae*: Genomics and Molecular biology. Norwich (UK): Horizon Scientific Press; In press 2008. p.179-90.

12. Sack DA, Sack RB, Nair GB, Siddique AK. Cholera. *Lancet* 2005; *63*: 223–33.

13. Finkelstein, RA. Cholera, *Vibrio cholerae* O1 and O139, and other pathogenic vibrios. [cited 2006 February 25]. Available from: http://gsbs.utmb.edu /microbook/ch024.htm.

14. Finkelstein RA, Burks F, Zupan A, Dallas WS, Jacob CO, Ludwig DS. Epitopes of the cholera family of enterotoxins. *Rev Infect Dis* 1987; *9*: 544–61.

15. Olsvik O, Wahlberg J, Petterson B, Uhlen M, Popovic T, Wachsmuth IK, *et al.* Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. *J Clin Microbiol* 1993; *31*: 22–25.

16. Nair GB, Faruque SM, Bhuiyan NA, Kamruzzaman M, Siddique AK, Sack DA. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *J Clin Microbiol* 2002; *40*: 3296–9.

17. Ansaruzzaman M, Bhuiyan NA, Nair GB, Sack DA, Lucas M, Deen JL, *et al*; the Mozambique Cholera Vaccine Demonstration Project Coordination Group. Cholera in Mozambique, variant of *Vibrio cholerae*. *Emerg Infect Dis* 2004; *10*: 2057–9.

18. Faruque SM, Tam VC, Chowdhury N, Diraphat P, Dziejman M, Heidelberg JF, *et al.* Genomic analysis of the Mozambique strain of *Vibrio cholerae* O1 reveals the origin of El Tor strains carrying classical CTX prophage. *Proc Natl Acad Sci USA* 2007; 104 : 5151-6.

19. Nair GB, Qadri F, Holmgren J, Svennerholm AM, Safa A, Bhuiyan NA, *et al.* Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. *J Clin Microbiol* 2006; *44*: 4211-3.

20. Tamplin ML, Jalali R, Ahmed MK, Colwell RR. Variation in epitopes of the B subunit of *Vibrio cholerae* non-O1 and *Vibrio mimicus* cholera toxins. *Can J Microbiol* 1990; *36* : 409–13.

21. Raychoudhuri A, Patra T, Ghosh K, Ramamurthy T, Nandy RK, Takeda Y, Balakrish-Nair G, Mukhopadhyay AK. Classical ctxB in *Vibrio cholerae* O1, Kolkata, India. Emerg Infect Dis. 2009;15:131-132.

22. Raychoudhuri A, Mukhopadhyay AK, Ramamurthy T, Nandy RK, Takeda Y, Nair GB. Biotyping of *Vibrio cholerae* O1: time to redefine the scheme. Indian J Med Res. 2008; 128:695-698.

23. Morita M, Ohnishi M, Bhuiyan NA, Nusrin S, Alam M, Siddique AK, *et al.* Development and validation of a mismatch amplification mutation assay PCR to distinguish between the cholera toxin B subunit of classical and El Tor biotypes of *Vibrio cholerae* O1. *Microbiol Immunol*; 2008; 52:314-317.

24. World Health Organization. Cholera 2006. *Wkly Epidemiol Rec* 2007; *82*: 273-84.

25. Safa A, Sultana J, Cam PD, Mwansa JC, Kong RYC. Classical cholera toxin producing *Vibrio cholerae* O1 hybrid El Tor strains in Asia and Africa. *Emerg Infect Dis* 2008; *14*:987-8.

26. Cooper KL, Luey CK, Bird M, Terajima J, Nair GB, Kam KM, Arakawa E, Safa A, Cheung DT, Law CP, Watanabe H, Kubota K, Swaminathan B, Ribot EM. Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae*. Foodborne Pathog Dis. 2006;3:51-8.

27. Blaser MJ, Wells JG, Feldman RA, Pollard RA, Allen JR. *Campylobacter* enteritis in the United States: a multicentre study. Ann Intern Med 1983; 98:360-5.

28. De Mol P, Brasseur D, Hemelhof W, Kalala T., Butzler JP, Vis HL. Enteropathogenic agents in children with acute diarrhoea in rural Zaire. Lancet i, 1983; 516-18.

29. Linton, D., Lawson, A.J., Owen, R.J., Stanley, J., 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. J. Clin. Microbiol. 35, 2568-2572.

30. Oberhelman RA, Tayor DN. *Campylobacter* infections in developing countries. In: Nachamkin I, Blaser JM (Eds.), *Campylobacter*, second ed. ASM Press, Washington, DC, 2000; pp. 139-154.

31. Kaisar A. T., Aslam, M., Islam, Z., Ishrat J.A., Dutta, DK et al. Prevalence of virulence genes and cytolethal distending toxin production in *Campylobacter jejuni* isolates from diarrheal patients in Bangladesh. J. Clin. Microbiol. 2008; 46:1485-1488.

32. Bardhan P, Faruque AS, Naheed A, Sack DA. Decrease in shigellosis-related deaths without *Shigella* spp.-specific interventions, Asia. Emerg Infect Dis. 2010;16:1718-23.

33. Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ, et al. Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. Bull World Health Organ. 1999;77:651-66.

34. Frankel G, Giron JA, Valmassoi J, Schoolnik GK. Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool. Mol Microbiol. 1989;3:1729-34.

35. Thong KL, Hoe SL, Puthucheary SD, Yasin RM. Detection of virulence genes in Malaysian *Shigella* species by multiplex PCR assay. BMC Infect Dis. 2005;5:8.

36. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog Dis. 2006;3:59-67.