1	H9812	XbaI	40U	Salmonella braenderup	
2	VPS04	SfiI	1998	40U	O4:K68
3	VPS10	SfiI	2005	40U	O4:K8
4	VPS14	SfiI	2001	40U	O3:K6
5	H9812	XbaI	40U	Salmonella braenderup	
6	VPS19	SfiI	2004	40U	O1: KUT
7	VPS27	SfiI	2005	40U	O2:K28
8	VPS31	SfiI	1997	40U	O4:K12
9	VPS35	SfiI	2003	40U	O6:K18
10	H9812	XbaI	40U	Salmonella braenderup	

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.

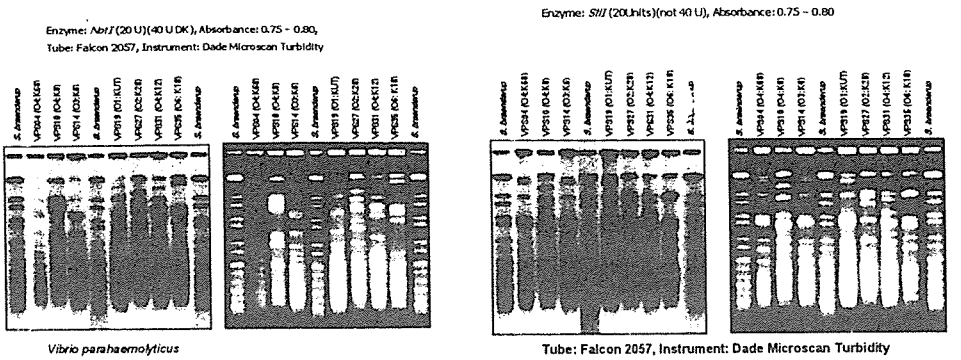

Fig. 1. PFGE images of *NotI* and *SfiI*-digested genomic DNA that was analyzed before optimization and adoption of PulseNet Asia Pacific-proposed PFGE protocol for *V. parahaemolyticus* in our laboratory conditions.

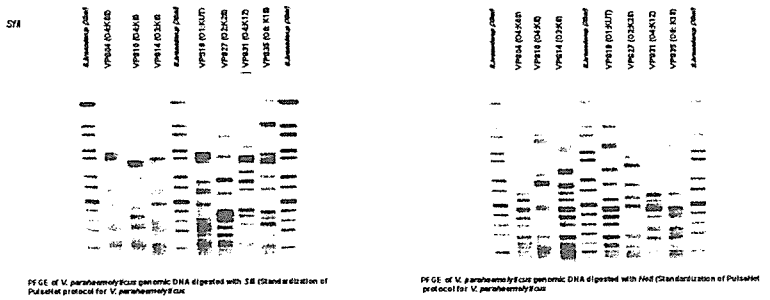

Fig. 2. PFGE images of *SfiI* and *NotI*-digested genomic DNA after optimization and adoption of PulseNet Asia Pacific-proposed PFGE protocol for *V. parahaemolyticus* in our laboratory conditions.

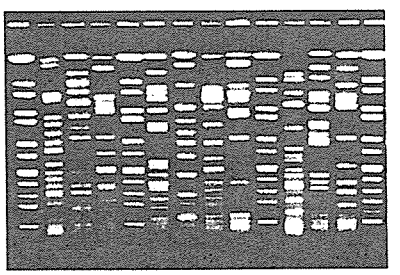

Fig. 3. PFGE images of *NotI*-digested genomic DNA of selected *V. parahaemolyticus* strains (PulseNet adopted PFGE protocol) isolated from coastal ecosystem of Bangladesh

The *NotI*–digested PFGE profiles of the *V. parahaemolyticus* strains isolated from coastal areas of Bay of Bengal 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.

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

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4. Summary:

In our previous study we have shown that cholera caused by *Vibrio cholerae* O1, Inaba has spread to many parts of India in the form of outbreaks as well as sporadic infection. Molecular studies revealed the prevalence of several clones of Inaba and majority of then belongs to pulsotype H1 and ribotype IV. Analysis of *V. cholerae* O1 isolates in Kolkata during 2005 has shown that prevalence of other new ribotypes and pulsotype N in this region. In addition, few new ribotypes of *V. cholerae* O1 were also identified among Inaba serotype. Recently, cholera due to *V. cholerae* serogroup O139 is seldom reported in India. However, between 2001 and 2006, there were 42 cases of cholera were reported in Delhi caused by *V. cholerae* serogroup O139. Most of the O139 isolates (71.4%) harboured *rstR* El Tor and Calcutta alleles, while 21.4% were with El Tor allele alone. Interestingly, 3 isolates (7.1%) isolated during 2001 were positive for all the *rstR* alleles tested in this study. Most of the *V. cholerae* O139 isolated during 2004-2006 belonged to ribotype B2 and the 2001 isolates exhibited new pattern. In the pulsed-field gel electrophoresis, the 2001 isolates were placed in a separate cluster, while the 2004 and 2006 isolates were similar. Further molecular characterization of these *V. cholerae* O139 isolates are under progress.

Compared to early 2000s, the incidence pattern of *V. cholerae* non-O1, non-O139 serogroups is increasing during 2000 to 2006 in Kolkata. In this study, we have employed a newly designed PCR method for the specious-specific identification of *Vibrio fluvialis* and used in the retrospective analysis with all the vibrios isolated from the acute diarrhea patients. *V. fluvialis* was detected among 12-50% of the *V. cholerae* non-O1, non-O139 isolates and the incidence among diarrheal cases varied between 0.74 and 2.85%, which was not recorded before. Incidence of *V. cholerae* non-O1, non-O139 among diarrheal patients on the other hand varied from 0.74 to 3.34%.

Among hospitalized diarrheal patients, the incidence of *V. parahaemolyticus* remained low (1.32%) during 2005 to 2006 in Kolkata. The serovars O3:K6 and O1:K25 were the predominant and majority of the isolates harbored *tdh* and HUα encoding genes and was also

positive in the GS-PCR. Serovars such as OUT:K3, O4:K8, OUT:K25, OUT:K8, which have the pandemic strain markers were newly recorded in this study. We have encountered 6 *V. parahaemolyticus* isolates showing positive only in the GS-PCR assay and belonged to different serovars. On the other hand, 4 isolates were positive for the HUα encoding gene, but were negative in the GS-PCR. Such changes in the marker genes for the pandemic strains of *V. parahaemolyticus* indicate that the genetic instability of these isolates. PFGE of these isolates might give some clue on the epidemiological relationship between the isolates.

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. Many 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 in order 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:

Incidence of *Vibrio cholerae* was monitored through a surveillance program of the National Institute of Cholera and Enteric Diseases on cholera. Analysis for this study was made for a period of two years from January 2004 to December 2005. Stool specimens were obtained from patients admitted to the Infectious Diseases Hospital, Kolkata, with 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 diarrheal 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. *Vibrio parahaemolyticus* isolates were serotyped using commercial

antisera kit (Denka Seiken, Tokyo, Japan). The Clinical and Laboratory Standards Institute's (formerly NCCLS) antimicrobial susceptibility test (1) was adapted for testing the bacteria isolates with commercially available discs (Becton Dickinson Co, Sparks, MD).

Uniplex PCR was performed for the amplification of *rstR* gene alleles encoding regulation of the lysogeny of CTX phage (2) in a standard PCR reaction mixture. For ribotyping, *Bgl*I digested chromosomal DNA from the representative isolates were transferred to Hybond N^+ membrane (Amersham International PLC, Buckimhamshire, England) and hybridization was made with the 7.5-kb *BamH*I fragment as a probe from plasmid pKK3535 (3) (ECL Nucleic Acid Detection System (Amersham). Pulsed-field gel electrophoresis (PFGE) was performed as described previously (4).

PCR assay for the species-specific detection of *Vibrio fluvialis*

One ml of the test isolate culture grown in Luria broth (Difco, USA) was centrifuged at 8000 rpm for 10 min. After centrifugation, the supernatant was discarded and the pellet was suspended in 300 μl of sterile distilled water, boiled for 10 min and then placed immediately on ice for 10 min. Centrifugation was again done at 12000 rpm for 10 min at 4°C to remove the cell debris and the supernatant was collected in a sterile microfuge. Amplification was carried out using bacterial cell lysate as the source of template DNA. PCR amplification was carried out in a thermal cycler (Applied Biosystem) using 200 μl PCR tubes with a reaction mixture volume of 25 μl. Each of the reaction mixtures contained 5.3 μl of the template, 2.5 μl of 10X buffer containing 15 mM $MgCl_2$ (Takara, Shuzo, Otsu, Japan), 0.20 μl of Taq Polymerase (Takara), 2.0 μl of 2.5mM deoxynucleoside triphosphate, 2.5 μl of each primer (10 pmol/μl) and 10μl of sterile distilled water. The amplification conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. The forward and reverse primers designed for this study includes 5'-TTGATCGCGACCGTCGAAAGCG-3' and 5'-TGGCGTGCTGACAATCACA TCG-3', respectively. PCR products were electrophoresed through 2% agarose gel to resolve 321 bp amplicon and visualized under UV light in a gel documentation system (Gel-Doc 2000, BioRad, Hercules, Calif.) after ethidium bromide staining.

PCR for the detection of virulence and other marker genes among *Vibrio parahaemolyticus*

PCR-assay was performed to determine the species-specific *toxR* of *V. parahaemolyticus* (5) and *tdh* and *trh* virulence genes (6). The group specific (GS) PCR was also performed with *V. parahaemolyticus* isolates using the methods described previously (7). PCR amplification was done with 2.5 µl of 10X amplification buffer (500 mM KCl, 100 mM Tris HCl, 15 mM $MgCl_2$, pH 8.3), 2.5 mM each of dNTP mixture, 10 pmole each of the primers, 1.25 unit of rTaq DNA polymerase (Takara) and 5 µl of template. The reaction volume was adjusted to 25 µl using sterile triple distilled water. PCR was performed in an automated thermocycler (Applied Biosystems) for 30 cycles.

Pulsed-field gel electrophoresis (PFGE)

PFGE of *V. cholerae* was performed as described previously (4). *Not*I (Takara) digested inserts of *V. cholerae* were applied to contour-clamped homogenous electric fields in a CHEF Mapper system (Bio-Rad, Hercules, California) using 1% PFGE grade agarose in 0.5X TBE (44.5 mM Tris HCl, 44.5 mM boric acid, 1.0 mM EDTA, pH 8.0) for 40 h 24 min at 14°C. Run conditions were generated by the auto-algorithm mode of the CHEF Mapper, PFGE system using a size ranges of 20 to 300-kb. For *V. fluvialis*, and *V. cholerae* O139 isolates, the one-day protocol of PulseNet program was followed. After electrophoresis, the gels were stained with ethidium bromide (1.0 µg per ml) in distilled water for 30 min, destained for 15 min and photographed under UV light using the Gel Doc 2000 documentation system (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.

7. Results:

7.1 Phenotyping and genotypic characteristic of epidemic *V. cholerae* isolated from acute diarrheal patients

A total of 3902 patients hospitalized with acute secretary diarrhoea during 2004-05 were examined for the presence of various enteric pathogens. *V. cholerae* isolates belonging to O1, Inaba serotype was first recorded among few cholera cases during July 2004 and by March 2005, this serotype became completely dominant over Ogawa. There was a total replacement of Ogawa serotype by the Inaba from June 2005.

Following the ribotyping scheme of Sharma et al (3), 18 representative isolates of both serotypes were tested after *BglI* digestion of chromosomal DNA. With this analysis, new ribotype RIV was detected among most of the recently emerged Inaba isolates. Some of the Inaba isolates exhibited RIII ribotype pattern, which was common among Ogawa isolates. The ribotyping pattern of Ogawa strains during the same period is mainly RIII type, but two isolates showed different profiles (Fig. 1). Nine Inaba and 9 Ogawa were tested following the PFGE typing scheme established by Yamasaki et al. (4). Among the Inaba isolates, 7 showed "H1" pulsotype while one isolate exhibited "H" type and another Inaba was identified as "H2" type (Fig. 2). On the other hand, majority of the Ogawa isolates displayed H pulsotype, which prevailed among O1 strains for the last 10 years, while one strain exhibited H2 type and another one was identified as "N" type (data not shown).

The incidence pattern of *V. cholerae* and its serogroups during 2005 to 2006 are depicted in Table 1. Over all, the isolation status of the serogroup O1 was high (18.8 and 7.0%, respectively during 2005 and 2006) among the diarrheal patients admitted in the Infectious Diseases Hospital and B. C. Roy Memorial Hospital for children in Kolkata. Among the serogroup O1, the isolation status of the Inaba serotype was high compared to Ogawa. Like previous years, the incidence of the serogroup O139 was very less in Kolkata (less than 1%). The isolation status of *V. cholerae* non-O1, non-O139 serogroups are increasing (Table 1) among diarrheal patients.

Antimicrobial susceptibility assay with the clinical isolates of *V. cholerae* has shown the different resistant patters between 2005 and 2006 (data not shown). Generally, most of the O1 isolates were found to be resistance to ampicillin, chloramphenicol, co-trimoxazole, furazolidone, nalidixic acid, and streptomycin. Ciprofloxacin resistance was constantly high during this study period (18 and 11%, respectively during 2005 and 2006). Resistance to tetracycline was high (18.3%) among the O1 isolates during 2005. However, this trend was reverted during 2006 (2.2%). Among *V. cholerae* non-O1, non-O139 isolates, resistance was seen with many antimicrobials such as co-trimoxazole, furazolidone, nalidixic acid and streptomycin. During 2005, increased resistance was seen with ampicillin, ciprofloxacin, neomycin, and norfloxacin. All the non-O1, non-O139 isolates were sensitive for chloramphenicol, neomycin and norfloxacin during 2006.

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 is still existing. We have included all the 42 *V. cholerae* O139 isolated from the Infectious Hospital in Delhi for molecular characterization in this study. The *rstR* gene alleles are used as a marker to study the epidemiological difference in the *V. cholerae* isolated over a period of time and location. Of the 12 *V. cholerae* serogroup O139 isolated during 2001 8 (19%) harbored only the El Tor allele of *rstR* and one had the new allele, Calcutta (Table 2). Interestingly, three 2001 isolates had all the three alleles of *rstR* including classical type, which was not reported till date among the O139 isolates (Table 2). During 2004 to 2006, majority of the isolates (69%) had El Tor and Calcutta alleles of *rstR*.

Fourteen representative isolates of *V. cholerae* serogroup O139 from the Delhi covering all the four years were selected for ribotyping. The ribotype B2 was detected among 8 isolates isolated during 2004 (3 isolates), 2005 (one isolate) and 2006 (4 isolates) (Fig. 3). Ribotype B1 was identified with only 2 isolates (2001 and 2006 isolates). Compared to five established ribotypes in the *V. cholerae* O139 serogroup, 2 new ribotypes were identified in this study, which includes isolates 37, 46, and 103 (isolated during 2001) and 3722 (isolated during 2004) (Fig. 3). The *Not*I digest of chromosomal DNA of *V. cholerae* O139 isolates were resolved in the PFGE (Fig 4a) and the DNA banding patters were analyzed using Diversity data base software (BioRad, USA) (Fig. 4b). In this analysis, two groups were detected with isolates isolated during 2001 (group A) and 2004 and 2006 (group B). The isolate 5037 was placed separately from these two groups (Fig 4b).

7. 2 Detection of *Vibrio fluvialis* among non-O1 and non-O139 strains isolated from diarrheal patients in Kolkata

In this retrospective analysis, we have included 362 *V. cholerae* non-O1, non-O139 isolates collected after screening 12,949 diarrheal patients in Kolkata. These isolates were not agglutinated with antisera specific for O1 and O139 serogroups and were tentatively identified as *V. cholerae* non-O1, non-O139 serogroups. For species-specific identification by PCR assay, primers targeted to the *toxR* gene of *V. fluvialis* were first tested with the strains that were previously identified as *V. fluvialis* by 16S rDNA sequencing. The primers were designed from the transcriptional activation domain and the membrane "tether" region of the *toxR* gene of *V.*

fluvialis, respectively (Accession No. AF170885). The membrane tether region is highly variable, but unique for each *Vibrio* species.

The primers designed in our previous study did not give the expected amplicon with two *V. fluvialis* strains belonging to serogroups O8 and O13. We have received newly designed primers from Dr. H. Izumiya, National Institute of Infectious Diseases, Tokyo, Japan for this study. All the *V. cholerae* non-O1, non-O139 isolates were tested with *ompW* (8) and VF-*toxR* for the species-specific identification of *V. cholerae* and *V. fluvialis*, respectively. The isolates positive in the VF-*toxR* PCR were negative for *V. cholerae*-specific *ompW* PCR. Based on these results, the incidence of *V. fluvialis* varied from 0.48 to 1.3% and the trend has increased during 2005 and 2006 (Table 3). Most interestingly, about 12 to 50% of the isolates were detected among presumptively identified *V. cholerae* non-O1, non-O139 isolates (Table 3).

With a set of 9 *V. fluvialis* we did the PFGE to detect the genetic relationship among these isolates following the protocol recommended for *V. cholerae*. The results indicate that there is no clonal relationship with the tested isolates (Fig. 5). PFGE studies are in progress to with additional isolates and also serotyping of them with the *V. cholerae* non-O1 and non-O139 antisera.

7.3 Incidence and molecular characterization of *Vibrio parahaemolyticus* isolated from acute diarrheal patients in Kolkata

Of the 2653 diarrheal stool specimens tested, the incidence rate of *V. parahaemolyticus* remained low (1.32%) during 2005 to 2006. As shown in the Table 4, O3:K6 and O1:K25 were the predominant serovars during the study period. Thirty-four isolates of *V. parahaemolyticus* were screened for the virulence genes such as *tdh* and *trh*, and marker genes specific for the pandemic strains. Majority (70.6%) of the *V. parahaemolyticus* isolates harbored *tdh* and HUα encoding genes and was positive in the GS-PCR (Table 4). Serovars such as OUT:K3, O4:K8, OUT:K25, OUT:K8, which have the pandemic strain markers were not found during our early studies. With most of the *V. parahaemolyticus* isolates, there was a good correlation between results of GS and HUα PCRs. However, we have encountered 6 *V. parahaemolyticus* isolates, showing positive only in the GS-PCR and all the isolates were belongs to different serovars (Table 4). On the other hand, 4 isolates were positive for the HUα encoding gene but were negative in the GS-PCR.

8. Discussion:

Emergence of *V. cholerae* O1 Inaba in Kolkata and subsequent total replacement of O1 Ogawa serotype for a brief period provided us impetus to characterize the representative strains at molecular level. The epidemiological influences due to the rapid changes in the prevalence of serotypes have been reported for other pathogens (9-11). In many findings, *V. cholerae* O1 Inaba was found to coexist with the Ogawa serotype (12-14). The Inaba serotype was first identified in a cholera outbreak in Delhi in 2004 and subsequently this serotype has caused several outbreaks in several States (15). In this study we have identified newer clones of *V. cholerae* O1 Inaba in Kolkata and its spread to other parts of the country had to be monitored carefully.

Till 1993, three ribotypes (RI through RIII) were identified among *V. cholerae* O1 serogroup (3). The RII and RIII ribotypes were identified after the emergence of *V. cholerae* O139 (3) and these two types were not recorded in the previous scheme (16). N the present finding, ribotype IV was exclusively present among *V. cholerae* Inaba isolates. The PFGE identified new clones of the *V. cholerae* O1 El Tor that were different from the isolates before the advent of the O139 serogroup in Kolkata (4). The *V. cholerae* O1 "H" pulsotype continues to dominate since its first appearance in July 1993. But majority of the Inaba isolates encountered in this study belonged to the "H1" pulsotype. Interestingly, all the Inaba strains exhibiting RIV ribotype patterns displayed H1 pulsotype, which was detected in many parts of India (15). On the other hand, all the O1 strains (both Ogawa and Inaba) exhibiting RIII ribotype patterns displayed either "H" or "H2" patterns. This study showed that there was a good correlation between the two molecular typing methods used for analyzing the *V. cholerae* isolates.

The cholera toxin-encoding gene (*ctx*) is encoded in a filamentous phage that integrates into the *V. cholerae* chromosome to form stable lysogens. In *ctx* lysogens, gene expression originating from the *rstA* phage promotor is repressed by the phage-encoding repressor RstR. In many of the *V. cholerae* isolates, the location of the *rstR* various in the chromosome and there at least three different alleles are existing. The *rstR* region is classified into $rstR^{class}$, $rstR^{ET}$ and $rstR^{Calc}$, respectively for classical, El Tor and O139 (17). When emerged during 1992, the *V. cholerae* serogroup O139 had $rstR^{ET}$ allele. The isolates emerged during 1996 carried two distinct CTX prophages intergrated in tandem CTX^{ET}, the prophage previously characterized within El Tor strains, and a new CTX Calcutta prophage (CTX^{Calc}) (17). The functional differences between the nucleotide sequences of $CTX^{Calc}\phi$ and $CTX^{ET}\phi$ are located within the

phages' repressor genes (*rstR*^{Calc} and *rstR*^{ET} respectively) and their RstR operators (18). RstR^{Calc} is an allele-specific repressor that regulates replication of CTX$^{Calc}\phi$ by inhibiting the activity of *rstA*^{Calc} promotor. However, this novel repressor has no inhibitory effect upon both classical and El Tor *rstA* promotors (which are regulated by cognate RstRs). In addition, production of RstR^{Calc} renders a CTX^{Calc} lysogen immune to super infection by CTX$^{Calc}\phi$ but susceptible (heteroimune) to infection by CTX$^{ET}\phi$ (18). In this respect, detection of all these three alleles in 3 *V. cholerae* O139 isolates from Delhi is important and further detailed studies are needed to confirm the variation in the other genes including the *ctx* and its flanking regions.

When the epidemic isolates of *V. cholerae* O139 isolated from Bangladesh and India were analyzed by ribotyping, Faruque *et al.* (19, 20) have found that all the isolates belonged to a single ribotype, suggesting the clonal nature of the isolates. Since its emergence, 5 different ribotypes (BI through BV) were reported among the serogroup O139. In this study, we have identified two new ribotype patterns with Delhi isolates. Interestingly, the three isolates harbouring all the three-*restR* alleles were belonged to one new ribotype (isolates 37, 46, 103 from 2001) and hence can be considered as a new clone. The PFGE results also supported the ribotyping results indication that the isolates harboring *rstR*^{cla} allele is of clonal origin.

Vibrio fluvialis, has been reported to cause sporadic infections and outbreaks of diarrhoea in humans (21-24). However, significance of this pathogen in public health is not studied in detail due to the lack of simple and reliable diagnostic tests. Although the bacterium is known to produce several potent toxins, their role in pathogenesis is not well established (25-27). Information regarding virulence genes, which is responsible for the pathogenesis and standard genetic markers for the identification of this organism, are not fully exploited. Recently, commercial bacterial identification systems are extensively used in many laboratories. Despite the use of an array of biochemical tests, proper identification of *V. fluvialis* still remains a problem due to its phenotypic similarity with *Aeromonas* species (28).

Before initiating in-depth studies on the pathogenesis of *V. fluvialis* isolated among diarrheal patients in India, we have felt it is crucial to identify the strains first using a highly reliable technique. In our previous report we have evaluated our VF-*toxR* PCR assay and with biochemical and 16S-rRNA sequence methods and found the PCR assay was dependable for the identification of *V. fluvialis* (29). Retrospective analysis of *V. cholerae* non-O1, non-O139 isolates collected from 12, 949 diarrheal patients during 2000 to 2006 has shown that the overall

incidence status of *V. fluvialis* was 0.83%. However, during 2005-06 there was slight increase in the incidence was seen. VF-*toxR* is a useful tool in differentiating *V. cholerae* non-O1, non-O139 and *V. fluvialis* isolates and helped identifying 12 to 50% of the isolates as *V. fluvialis* among the non-O1, non-O139 vibrios. Infection caused by the *V. fluvialis* is gaining importance as its involvement is increasingly reported (27, 30). Currently, we are analyzing the clinical profiles of the *V. fluvialis* infected patients to understand the epidemiological importance of this pathogen.

From 1996, the incidence scenario of *V. parahaemolyticus* has changed as the serovar O3:K6 was associated with several diarrheal outbreaks all over the world (31). The emergence of pandemic strains of *V. parahaemolyticus* first reported in Kolkata, India (32) and this serovar has been reported during following years in many countries where the seafood consumption was high (31). In addition to O3:K6, the other serovars such as O4:K6, O1:KUT, O1:K25 were also reported to be associated with several diarrheal cases in South east Asian countries (31). These serovars have defined sequence in the *toxRS* gene and can be detected by GS-PCR (7). In addition, other findings suggested that the a unique sequence difference in the C-terminal region of the gene encoding for histone-like DNA binding protein, HUα in these serovars (33, 34). Based on the PCR results, considerable numbers of *V. parahaemolyticus* isolated in Kolkata did not fall into the pandemic strain category i.e positive in GS and HUα PCRs. Identification of *V. parahaemolyticus* isolates harboring *trh* gene and exhibiting pandemic strain features were observed for the first time in this study. Detailed molecular studies are under progress to understand such change.

9. Reference List:

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10. Publication list for this work:

1. Chakraborty, R., S. Sinha, A. K. Mukhopadhyay, M. Asakura, S. Yamasaki, S. K. Bhattacharya, G. B. Nair, and T. Ramamurthy. 2006. Species-specific identification of *Vibrio fluvialis* by PCR targeted to the conserved transcriptional activation and variable membrane tether regions of *toxR* gene. J. Med. Microbiol. 55: 805-808.

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5. Raychowdhuri A., S. Chatterjee, G. P. Pazhani, R. K. Nandy, M. K. Bhattacharya, S. K. Bhattacharya, T. Ramamurthy and A. K. Mukhopadhyay. 2007. Molecular characterization of recent Vibrio cholerae O1, El Tor, Inaba strains isolated from hospitalized patients in Kolkata, India during 2004-05. Communicated to Emerging Infectious Diseases.

Table 1. Incidence pattern of *V. cholerae* associated with diarrhea in Kolkata

Year	No of patient	*Vibrio cholerae* serogroup (%)		
		O1	O139	Non-O1, non-O139
2005	1472	189 (12.8)	2 (0.13)	59 (4.0)
2006	1181	83 (7.0)	3 (0.25)	33 (2.8)

Table 2. *rstR* alleles of *Vibrio cholerae* O139 isolated from cases of cholera in Delhi

Year	No of isolate (%)	$rstR^{El}$	$rstR^{cla}$	$rstR^{cal}$
2001	8 (19)	+	-	-
2001	3 (7.1)	+	+	+
2001	1(2.4)	-	-	+
2004	20 (48)	+	-	+
2004	1 (2.4)	+	-	-
2005	1 (2.4)	+	-	+
2006	8 (19)	+	-	+