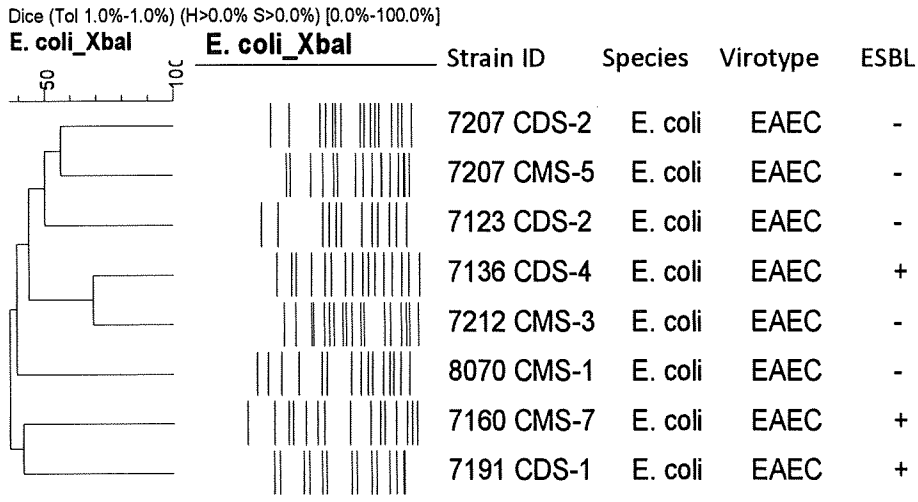

Figure 1: Dendrogram showing the PFGE patterns of EAEC strains isolated from diarrheal patients and non-diarrheal controls. The dendrogram was constructed with Bionumerics 4.5 software using the unweighted pair group method with arithmetic means (UPGMA) method. Thin lines were added by the program and show the location (peak of densitometry curve) of less-intense bands included in the analysis

Table 2: Summary of all characteristic patterns of all EPEC strains

Strain ID	Antibiotic Susceptibility Profile	Plasmid Pattern	PFGE pattern	ESBL
7175 CMS-5	$AMC^{I}SXT^{S}MEL^{S}NA^{S}CIP^{S}CRO^{S}$	P15	B	-
8001 CMS-5	$AMC^{I}SXT^{S}MEL^{S}NA^{S}CIP^{S}CRO^{S}$	P2	C	-
7012 CMS-13	$AMC^{R}SXT^{S}MEL^{R}NA^{R}CIP^{S}CRO^{S}$	P20	D	-
7176 CMS-7	$AMC^{I}SXT^{S}MEL^{I}NA^{R}CIP^{S}CRO^{S}$	P18	E	-
7108 CMS-9	$AMC^{I}SXT^{R}MEL^{S}NA^{R}CIP^{S}CRO^{S}$	P1	A1	-
7211 CMS-5	$AMC^{I}SXT^{R}MEL^{S}NA^{R}CIP^{S}CRO^{S}$	P1	A1	-
8027 CDS-1	$AMC^{I}SXT^{R}MEL^{I}NA^{R}CIP^{S}CRO^{S}$	P1	A1	-
7108 CDS-5	$AMC^{S}SXT^{S}MEL^{S}NA^{R}\ CIP^{S}CRO^{I}$	P16	A2	-
7166 CDS-5	$AMC^{R}SXT^{S}MEL^{R}NA^{R}CIP^{S}CRO^{S}$	P17	G	-
7072 CDS-5	$AMC^{R}SXT^{R}MEL^{R}NA^{R}CIP^{R}CRO^{R}$	P24	H	-
7121 CDS-6	$AMC^{I}SXT^{R}MEL^{R}NA^{R}CIP^{S}CRO^{S}$	P14	F	-

Key: I=Intermediate, R=Resistant, S=Sensitive

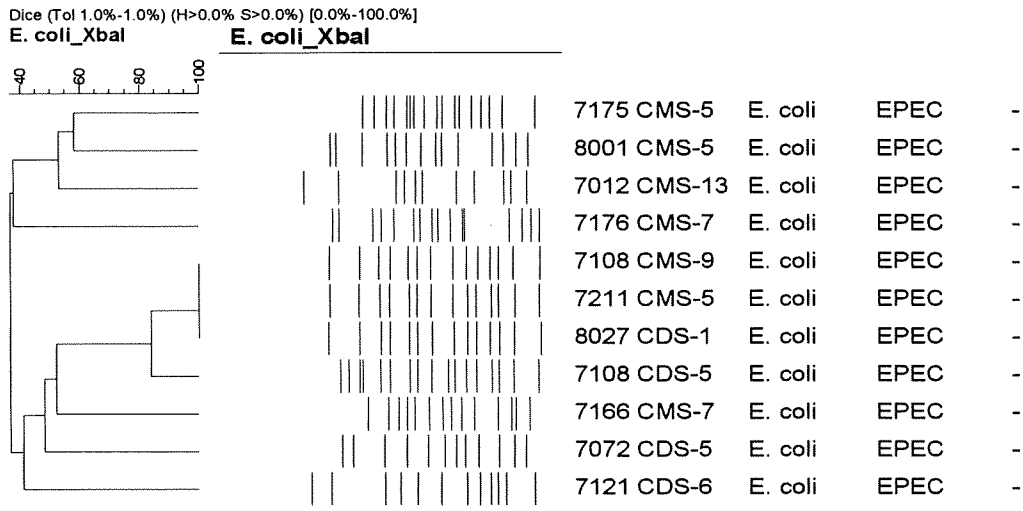

Figure 2: Dendrogram showing the PFGE patterns of EPEC strains isolated from diarrheal patients and non-diarrheal controls. The dendrogram was constructed with Bionumerics 4.5 software using the unweighted pair group method with arithmetic means (UPGMA) method. Thin lines were added by the program and show the location (peak of densitometry curve) of less-intense bands included in the analysis.

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Other activities:

Paper published:

1. Islam Z, van Belkum A, Wagenaar JA, Cody AJ, de Boer AG, Tabor H, Jacobs BC, **Talukder KA**, Endtz HP. Comparative genotyping of Campylobacter jejuni strains from patients with Guillain-Barré syndrome in Bangladesh. PLoS One. 2009 Sep 30; 4(9):e7257.

Paper submitted for publication:

2. M. Aminul Islam, Mondol AS, Ishrat Jahan Azmi, E. de Boer, R. R. Beumer, M. H. Zwietering, , A. E. Heuvelink and **Talukder KA**. Shiga toxin-producing *Escherichia coli* in raw meat, raw milk and street vended juices in Dhaka, Bangladesh. Submitted in Applied Microbiology.

Paper presented in scientific conferences:

1. **Talukder KA**, Ishrat J. Azmi, Z. Islam, D. K. Dutta, M. A. Islam, Abdus S. Mondol, Lorenz von Seidlein, **Alam Nur-E-Kamal,** G. B. Nair, D. A. Sack, Hubert P Endtz, and Alejandro Cravioto. Shigella flexneri 1c, a new variant of Shigella: prevalence and their characterization at the molecular and cellular level. In: 6th PulseNet Asia Pacific Planning Meeting held in Nonthaburi, Thailand between 15-17 December, 2009.

2. **Talukder KA**, Abdus. S. Mondol, M. Aminul Islam, Ishrat J. Azmi, Zhahirul Islam[1], Mohammad Aslam[1], Dilip K. Dutta[1], Mahmuda Akter[1], M. A. Hossain, Filliol I,Thomas Cheasty, Hubert P Endtz and Alejandro Cravioto. Identification and characterization of provisional serovar of *S. dysenteriae* from diarrhoeal patients in Bangladesh. 44th US-Japan Conference on Cholera and Other Bacterial Enteric Infections held October 12-14, 2009 in San Diego, California USA.

3. **Talukder KA,** Aslam M, Islam Z, Khajanchi BK, Azmi IJ, Kabir Y, Cravioto A and Endtz HP. Fluoroquinolone resistant *Campylobacter jejuni* from Bangladesh show high degree of heterogeneity and are associated with a single point mutation in *gyrA*. In: "The 15th International Workshop on *Campylobacter*, *Helicobacter*, and Related Organisms (CHRO)" held on September 2-5, 2009. Niigata, Japan.

4. M. Aminul Islam, Mondol AS, Ishrat Jahan Azmi, E. de Boer, R. R. Beumer, M. H. Zwietering, , A. E. Heuvelink and **Talukder KA**. Shiga toxin-producing *Escherichia coli* in raw meat, raw milk and street vended juices in Dhaka, Bangladesh. Presented in 12th ASCOD, p-70, May 25-27, 2009, Yogyakarta, Indonesia.

5. **Talukder KA**, D. K. Dutta, M. A. Islam, Z. Islam, I. J. Azmi, A. S. Mondol, M. Aslam, S. K. Niyogi, L. von Seidlein, I. Filliol, A. Nur-E-Kamal, T. Cheasty, G. B. Nair, D. A. Sack, H. P. Endtz, and A. Cravioto. New variants of *Shigella* species in Bangladesh and their molecular characterization. Presented in US-Japan cholera conference, April 06-10, 2009, Kolkata, India.

Participation in PIC group:

Participated in PulseNet Asia Pacific: Platform for Inter-laboratory Comparison Work Group (PIC WG) 2009-Phase I and II:

Phase I: Phase I organism include non-typhoidal *Salmonella* (SM7) &/or *Shigella sonnei* (SH3).

Phase II: Phase II organism include *Salmonella* Typhi, *Salmonella* Gr. B and *Salmonella* Gr. C1

2. *Vibrio parahaemolyticus*:

Prevalence, and diarrheal link, of *tdh*+ serogroups (O3:K6 and O8:K21) of *Vibrio parahaemolyticus* in Estuarine Ecosystem of Bangladesh

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Abstract.

Recurrent diarrheal diseases kill many people in coastal villages of Bangladesh, although the causal agents remain mostly unknown because of the lack of microbiological study. Following a recent study that showed the virulence potential of *Vibrio parahaemolyticus* (Vp) occurring in the estuarine ecosystem across the mangrove forest, Sunder ban, of Bangladesh, we visited two clinics in that area and also, collected estuarine water from the same region between 2006 and 2007 to isolate Vp. In this study, a total of 41 strains, isolated from diarrhea (n=16) and environmental sources (n=25), were analyzed for virulence, phenotypic, and molecular traits, and compared to determine their clonal lineage. According to serotyping data, O3:K6 was prevalent among the diarrheal strains, accounting for 50%, followed by O8:K21 (19%) strains. Sero-analysis revealed O8:K21 to be prevalent among the environmental strains, accounting for 48%, followed by O9: KUT (28%), although serogroup O3:K6 was not found among the environmental strains tested. PCR screening revealed all of the diarrheal strains, belonging to different serogroups, to harbor the major virulence gene encoding thermostable direct haemolysin (TDH), which was detected in 64% of the environmental strains, including all of the O8:K21 strains, although TDH-related haemolysin (*trh*) gene was not detected in any of the diarrheal or environmental strains tested. All of the Vp strains tested had the gene thermolabile hemolysin (*tlh*), whereas 19 (76%) of the environmental strains had the gene *toxR*. Only 3 (19%) of the tdh^+ clinical O3:K6 strains were KP-positive. Seven of total 8 O3:K6 strains, including the only O1:KUT strain of diarrheal origin, possessed the pandemic group-specific (GS) and ORF-8 marker genes; however, none of the tdh^+ serogroup O8:K21 strains had any such pandemic markers. The DNA fingerprinting analysis by BOX-PCR, arbitrarily primed (AP)-PCR, and pulsed-field gel electrophoresis (PFGE), and construction of dendograms with the gel images obtained with these three techniques revealed the *tdh*+ diarrheal and environmental strains (both O3:K6 and O8:K21) to fall under the same or closely-related clusters, indicating clonal relatedness, although high diversity was evident within the individual clusters. The prevalence of *tdh*+ serogroups occurring in natural surface water and their link with diarrhea suggest Vp as a potential cause of diarrhea that remains a killer disease for the coastal villagers of this region.

Introduction

Vibrio parahaemolyticus, a halophilic gram-negative bacterium is widely distributed in coastal waters worldwide and associated with seafood-borne gastroenteritis, wound infections and septicemia in human (**Joseph *et. al.*, 1982; Bag *et al.*, 1999; DePaola *et al.*, 2003; Wong *et al.*, 2000; Yeung & Boor, 2004**). It was first identified as a causative agent of human disease after an outbreak of gastroenteritis in Japan in 1950 (**Fujino et. al., 1951**). This organism is associated with bacterial gastroenteritis not only in the United States and Europe but also have established as a pandemic paradigm worldwide (**Nair *et. al.*, 2007**). Virulence of *V. parahaemolyticus* is associated with the production of a thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH) (**Honda *et al.*, 1988; Nishibuchi *et al.*, 1985, 1989**) or both (**Honda and Iida, 1993; Nishibuchi and Kaper, 1995**). Manifestation of β-hemolysis on Wagatsuma blood agar, i.e., the Kanagawa phenomenon due to TDH production has been shown to be present in more than 90% of clinical and less than 1% of environmental strains (**DePaola *et al.*,1990, 2000; Earle and Crisley, 1975**). A separate gene, thermolabile hemolysin (*tlh*) has also been characterized which was shown to be present in all of the *V. parahaemolyticus* strains tested previously (**Taniguchi *et al.*, 1985, 1986**).

As least 75 different combinations of O and K serogroups of *V. parahaemolyticus* are recognized and known to be associated with gastroenteritis (**Ishibashi *et. al.*, 2000; Wong, 2003**). Normally, a wide variety of serovar are involved in outbreaks. After 1996, an increasing incidence of gastroenteritis in many parts of the world has been linked to pandemic O3:K6 serogroup (**Okuda *et. al.*, 1997; Bag *et. al.*, 1999**) which first appeared in 1996 in Calcutta, India (**Okuda *et. al.*, 1997**). This O3:K6 serogroup was associated with large outbreaks in the United States (**Daniels *et. al.*, 2000**) and across other continents in recent years, which suggests that this organism has pandemic potential (**Nair and Hormazabal 2005; Bag *et. al.*, 1999**). Recently isolated serogroups O4:K68, O1: KUT, O1:K25, and O1:K41 were related to the new O3:K6 clone by molecular techniques (**Ishibashi et.al.,2000; Matsumoto et.al.,2000**), suggesting that these strains may have diverged from the new O3:K6 clone by alteration of the genes associated with the O and K antigens (**Matsumoto *et.al.*,2000**) and currently 22 serogroups are reported to belong to the same genetic lineage of pandemic O3 : K6 and are believed to have emerged from a single origin (**Nair *et al.*, 2007, Serichantalergs, 2007**).

A recent study has shown virulence potential of *V. parahaemolyticus* occurring in the estuarine ecosystem across the mangrove forest, Sunder ban, of Bangladesh (**Alam, 2009**). Although recurrent diarrhea claims many lives in coastal villages, the causal agent remains mostly unknown because of the

lack of microbiological study. In the present study, which is a follow-up one on the serogroup, virulence, and molecular traits *V. parahaemolyticus*, we show the prevalence of *tdh*+ serogroup O8:K21 in natural surface water of the estuarine ecosystem where diarrhea was caused mainly by the pandemic serogroup O3:K6 together with the O8:K21 strains that pose potential health-risks for the coastal villagers of Bangladesh.

Methodology:

Isolation of *V. parahaemolyticus* strains. Brackish water samples from estuarine region close to the world's largest mangrove forest, Sunder ban, of the Bay of Bengal were collected between 2006 and 2007 employing methods described elsewhere **(Alam et. al., 2006).**

Rectal swabs from diarrhea patients admitted to the local clinics of Mathbaria and Bakerganj were also collected and transported in Carry-Blair medium, following methods recommended by the American Public Health Association **(APHA. 1970)**. The rectal swabs were plated after enrichment in APW onto TCBS agar as described previously **(Mukhopadhyay et. al., 1996)**.

Ca 2-3 loops-full of enriched APW broth were streaked onto thiosulfate–citrate-bile-salts-sucrose (TCBS) agar and incubated at 37°C for 18 to 24 h. Presumptive vibrio-like colonies were selected and confirmed by biochemical tests as described elsewhere **(Serichantalergs, 2007, Wong 2003)**.

Serogrouping. Serogrouping of the V. parahaemolyticus isolates was done using a commercially available V. parahaemolyticus antisera test kit (Denka Seiken, Tokyo, Japan), following manufacturer's instructions.

Hemolytic activity. V. parahaemolyticus isolates were grown on Wagatsuma agar medium and hemolytic assay performed following method, as described elsewhere **(Chun et. al., 1975)**.

Extraction and purification of chromosomal DNA. Chromosomal DNA was extracted using the Wizard® Genomic DNA purification Kit (Promega Corp., USA), according to manufacturer's instructions.

Polymerase Chain Reaction (PCR). PCR assays for the species-specific gene *toxR* and *tlh* and the two virulence genes *tdh* and *trh* were performed using *V. parahaemolyticus* genomic DNA as template, following methods described elsewhere (**Kim 1999**, **Suthienkul, 1995**).

Group specific (GS) and open reading frame 8 (ORF8)-PCR. PCR assays for amplification of the pandemic marker genes GS and ORF8 were performed as described elsewhere (**Kim 1999, Martinez-Urtaza 1999, Alam 2009**).

Arbitrarily primed (AP)-PCR. AP PCR was carried out using the 10-mer primer (**Wong, 2003**).

BOX-PCR. A highly conserved repeated DNA element has been identified in the chromosome of *V. parahaemolyticus* and given the name of the BOX repetitive element **(Hsueh *et al.*, 1998).**

Pulsed-field gel electrophoresis (PFGE) and Image analysis. The pulsed-field gel electrophoresis (PFGE) of *Sfi*I-digested DNA of *Vibrio parahaemolyticus* was performed using a standardized protocol, as described elsewhere (**Tenovar et al., 1995; Kam *et al.*, 2008; Alam et. al., 2009**). The fingerprint pattern in the gel was analyzed using a computer software package, Bionumeric Software Package (Applied Maths, Belgium).

Results and Discussion

The serological typing, which is performed by targeting the somatic antigen O and the capsular antigen K for the identification and characterization of *V. parahaemolyticus* strains (**Shinoda *et. al.*, 1983**), revealed that the isolated strains were diverse serologically. Of the known typeable 13 O and 71 K types recognized to date (**Ishibashi, 2000**), 4 different combination of O:K serogroup were found among 25 environmental isolates and 7 in the clinical isolates where O8:K21 was predominant in environmental strains followed by O9:KUT. A previous study conducted in the estuarine ecosystem of Bangladesh has also reported O8:K21 as the toxigenic serogroup which continues to prevail as one of the two major serogroups causing diarrhea in this region (**Alam *et. al.*, 2009**). The pandemic serogroup O3: K6, reported previously in diarrhoeal outbreaks in Bangladesh and India (**Ansaruzzaman, 2005, Bhuiyan, 2002**), was not detected among the 25 environmental *V. parahaemolyticus* strains tested in this study. Absence of O3: K6 among the 25 strains tested in the present study does not essentially rule out their existence in this region because O3:K6 and O1: KUT have been reported as a significant cause of gastroenteritis in Bangladesh (**Bhuiyan *et. al.*, 2002, Islam *et. al.*, 2004**) and India (**Matsumoto *et.al.*, 2000; Okuda *et.al.*, 2000**). Besides, the abundance of the serogroup O8:K21 in natural estuarine water and its link with

clinical cases of diarrhea indicates its involvement with gastroenteritis cases that claim many lives in the coastal villages.

The *toxR* gene was first discovered as the regulatory gene of the cholera toxin operon, but it also has regulatory function in *V. parahaemolyticus* (**Lin *et. al.*, 1993**). So presence of this gene can be used for the specific identification *V. parahaemolyticus* (**Kim *et. al.*,** 1999). Although *toxR* appears to be well conserved among *Vibrio* species, five strains did not seemed to have *toxR*. But all the isolates conferred their species level identification being positive for the presence of *tlh* (**Taniguchi *et. al.*, 1985, 1986**) and that all five strains reacted to O and K antisera, confirming their *V. parahaemolyticus* identity. Again, among the *toxR* negatives strains, two belonging to serogroup O9:KUT were carrying the major virulence gene *tdh*. Based on these results, it is presumed that the *toxRS* operon of these strains may have undergone mutation which likely altered or deleted the primer annealing sites.

The frequency of the detection of *tdh* or *trh* in environmental samples and seafood was reported to be very low compared to clinical isolates (**Kiiyukia *et. al.*, 1989; Ogawa *et. al.*, 1989; Cook *et al.*, 2002**). Strains that produce *tdh* and/or *trh* genes are considered virulent. Also, deletion of the Vp-TDH gene results in loss of enterotoxic activity in laboratory models (Using chamber and rabbit ileal loop assays) (**Nishibuchi *et. al.*, 1992**). For the clinical isolates, all carried the *tdh* gene but not *trh*, as was the case for the tdh^+ environmental isolates in the present study. So, *tdh* might be playing a major role in the gastroenteritis caused by *V. parahaemolyticus* occurring in this region.

Thermostable direct hemolysin (Vp-TDH) is responsible for the beta-hemolysis observed when the organisms are plated on a modified blood agar known as Wagatsuma agar (**Chun *et. al.*, 1999, Joseph *et. al.*, 1983**) known as the "Kanagawa phenomenon" (KP) and this is one of the important virulence markers for *V. parahaemolyticus*. The KP^+ strains have been shown to cause diarrhea in volunteers (**Sanyal and Sen, 1973**). In contrast, a high dose of KP^- strains failed to cause diarrhea (**Bhuiyan, 2002, Sanyal and Sen, 1973**). In the present study, majority of the 32 tdh^+ strains, including 16 diarrheal and pandemic serogroup O3:K6, were KP^-, suggestion that KP may no longer be a reliable marker for the virulence of *V. parahaemolyticus*.

Most *V. parahaemolyticus* strains isolated from clinical specimens manifest KP but most environmental strains do not (**Sakazaki *et al.*, 1968; Miyamoto et al., 1969**). Therefore, KP-positive *V. parahaemolyticus* strains have been considered virulent strains. KP-positive strains contain two chromosomal *tdh* genes named *tdh1* and *tdh2* (**Nishibuchi and Kaper, 1990**). KP-negative but *tdh* gene-positive strains usually carried one chromosomal *tdh* gene. The sizes of all *tdh* coding sequences so far examined were identical (567 bp) (**Nishibuchi and Kaper, 1995**). The mature form of *tdh* gene product

is composed of 165 amino acid residues and is biologically active when it forms a dimmer (**Takeda *et al.*, 1983; Nishibuchi and Kaper, 1995**). In a study it was demonstrated that the haemolytic activity of a KP-positive strain was caused by TDH produced from the *tdh2* gene but not the *tdh1* gene (**Nishibuchi et al., 1991**). These results indicated that the difference in the level of *tdh* expression is mainly responsible for the difference in the haemolytic activity of the gene-positive strains. The very high-level *tdh2* expression was shown to be due in part to transcriptional modulation by a regulatory protein, ToxR, which is present in all strains of *V. parahaemolyticus* (**Lin *et al.*, 1993**). Base difference at a certain position of the promoter sequences of various *tdh* genes can largely account for the difference in the level of *tdh* expression (**Okuda and Nishibuchi, 1998**). In this study, the primers used to detect *tdh* (**Kim et. al., 1999**) were not specific for the *tdh1* or *tdh2*. So, it is difficult to predict as to what was actually the case, because the presence of *tdh* does not essentially ensure its expression, as we have observed in the present study.

The pandemic O3:K6 (and its variants) could be distinguished from other strains by possession of the *tdh* gene but not the *trh* gene, by unique profiles in an arbitrarily primed PCR (AP-PCR) analysis, and by a new PCR method which targeted to the *toxRS* operon, encoding a transcriptional regulator. Thus, exploiting the polymorphism of the *toxRS* operon, this PCR method was named GS-PCR for group-specific PCR (**Matsumoto *et. al.*, 2000**). Similarly, open reading frame 8 (ORF8) of the filamentous phage f237 lysogenizes pandemic *V. parahaemolyticus* serogroup O3 : K6 and provides another unique molecular marker for pandemic *V. parahaemolyticus* (**Blackstone, G, 2001, Iguchi, 2005, Nasu et. al., 2000**). However, changes in the pandemic serogroup has been reported to occur over time since GS-PCR positive pandemic clones have been shown to be tdh^- and $orf\text{-}8^-$ (**Bej, 1999, Kim et. al. 1999, Serichantalergs, 2007**) and an increasing number of non-pandemic serogroups, such as O4 : K68, O1 : K25, O1 : KUT, O1 : K41, O4 : K12, and O3 : K46, carry pandemic marker genes (**Bhuiyan, 2002, Chowdhury, 2004, Serichantalergs, 2007, Wong, 2000**). A recent study in Thailand reported O3 : K46 to be a new, emergent serovar having pandemic traits, while dominance of other pandemic serogroups, such as O 3 : K6, O1 : K25 and O1 : KUT was demonstrated in diarrhoeal cases (**Serichantalergs, 2007**). Although non-O3: K6 serogroups are presumed to have evolved over time via alteration of the O and K antigens and by sero-marker transformation of O3: K6 strains (**Chowdhury, 2004; Ansaruzzaman, 2005**). surface water isolates of *V. parahaemolyticus* (n=25) in this study did not included any pandemic serogroup reported previously from diarrhoeal cases in Bangladesh (**Bhuiyan, 2002**). Furthermore, none of the environmental *V. parahaemolyticus* strains, possessed the pandemic marker genes, indicating no relationship with pandemic clones, only the eight clinical pandemic serogroup strains O3:K6 and one O1:KUT were GS^+ and $orf8^+$.

The DNA based typing of isolates is of great importance for tracking the clonal relatedness among the pandemic and non-pandemic strains. The results of AP-PCR fingerprinting with the appropriate primers revealed that some of the patterns obtained could be related to the origin of the strains, which indicate its potential use in epidemiological studies of this organism (**Maluping, et. al., 2005; Matsumoto et. al., 2000; Zahid et al., 2006**). In this study, the results of AP PCR fingerprinting with the appropriate primers revealed that some of the patterns obtained could be related to the origin of the strains, which indicate its potential use in epidemiological studies of this organism. AP-PCR bands ranged between 10 – 12 and molecular weight of the bands varied between 0.15 kb - 4.6 kb. Analysis of the similarity of the AP-PCR patterns using the Dice coefficient and UPGMA has revealed the high genetic variation among strains of both environmental and clinical origin. As shown in the dendogram (Fig.....), only few strains clustered with a similarity of 90–99%. Strains from environment and from clinical samples have been clustered separately and thus, have varied origin. Strains with similarities below 90% were considered genetically unrelated (**Szczuka and Kaznowski 2004**). Such results were somehow significant because if we go through the dendogram some strains having similar serogroup (*viz.* O8:K21 or O9: KUT) are placed together irrespective of their source which means the clinical strains might have environmental ancestors and the infection pathway is clear. Only two strains of environmental origin belonging to serogroup OUT:K33, that have been clustered with clinical strains showed an interesting pattern because they neither shared serogroup compatibility, nor they possessed any virulence gene. Still they remain potentially hazardous. Based on similarity using the Dice coefficient, our *V. parahaemolyticus* strains are genetically diverse wherein majority have similarity below 90%. This result agreed with other studies confirming the genetic diversity among *V. parahaemolyticus* strains (**Goarant *et al.* 1999; Sudheesh *et al.*, 2002**). It is interesting to point out that, regardless of the AP-PCR profiles observed, all strains showed some common bands. These fragments would be favorable traits for the development of genetic amplification and hybridization assays for diagnostic purpose (**Dalla Valle *et al.*, 2002**), which can be important to verify *V. parahaemolyticus* strains that are relatively difficult to identify.

BOX-PCR of genomic DNA from various *V. parahaemolyticus* strains resulted in amplification of multiple fragments (4–16 amplified bands) of DNA that ranged from 0.29 to 6.0 kb long. Using BOX-PCR as a fingerprinting tool enabled us to clearly discern that the clinical strains were of environmental origin. As was the case for AP-PCR results, BOX-PCR also placed *V. parahaemolyticus* strains into different clusters with similarity ranging between 90-100% within one group. The PCR profile was diverse and highly heterogeneous. BOX-PCR could differentiate the pandemic clones (O3:K6 and O1:KUT) into same group with only single band differing at 0.8 kb region. Unlike AP PCR, strains in the BOX-PCR have shown little competence in banding pattern. Thus this technique enabled us to unveil the

clonal origin of the clinical and environmental isolates irrespective of serogroups. The two environmental isolates (EKP015, EKP016) of serogroup OUT:K33 were again as like for AP PCR found to be closely related and genetically indistinguishable with virulent clinical O3:K6 strains.

Pulsed-field gel electrophoresis (PFGE) is widely used as a state of the art molecular tool to determine genetic relatedness among bacteria (**Tenover et. al., 1995; Laohaprertthisan, 2005**). The PFGE profiles of 25 environmental and 16 clinical *V. parahaemolyticus* strains obtained with *Sfi I* showed a variety of patterns. Isolates from clinical samples belonging to serogroup O3:K6 and O1: KUT showed a high degree of genetic relatedness which was consistent with previous studies (**Chowdhury *et. al.*, 2000; Matsumoto *et. al.*, 2000**) and supports the hypothesis of a related origin of these three new serogroups (**Matsumoto *et. al.*, 2000**). All the environmental and clinical strains belonging to serogroup O8:K21 showed slightly different but closely related PFGE pattern. They posed a similarity range within 80-100% indicating that the origin of infection relating to this serogroup might be the environment. Interestingly this was the only serogroup that was evenly distributed among environmental and clinical samples of that region (**Alam *et. al.*, unpublished**). But the other prevalent environmental serogroup O9:KUT did not seem to have any clinical clone, neither they shared any similar PFGE pattern with other clinical pandemic or non-pandemic isolates. Such findings might indicate that these strains are yet to cause any detectable human infection although some were *tdh*$^+$. Clinical strains belonging to serogroup other than pandemic like O3:K5 and O8:K22 showed varied banding pattern with the pandemic group strains. They share only 1 or 2 bands in common that implies their genetic distance form the virulent pandemic group, but still they remain pathogenic. Strain from the serogroup O8:K22 has been clustered with pandemic clones when analyzed by BOX-PCR and when AP-PCR was done they were found to be closely related to environmental isolates. All the other non prevalent serogroups from clinical strains like O1:K56 and O4:K55 have shown closely related banding pattern with the pandemic group strain O3:K6 which means they have same origin and such pattern is common for all virulent strains (**Alam *et. al.*, unpublished**). Such non pandemic strains have been found to be related to pandemic strains in other geographic regions also (**Gil *et. al.*, 2007**). These serogroups were unique for clinical strains of our study region. 2 other non prevalent environmental serogroup namely OUT: K33 and O4:K46 were giving identical restriction pattern with clinical pandemic O3:K6, which was significant. These exotic strains might appear potentially virulent because they were genetically related and also carrying virulent genes like *tdh*. So these strains should be studied more extensively for the presence of *toxRS/new* sequence (**Okura *et. al.*, 2003**) or determine sequence type (ST) through MLST in order to be grouped as pandemic "Clonal Complex" (**Gonzalez-Escolana *et. al.*, 2008**).

The overall PFGE and cluster analysis data, coupled with results of other investigators, show conclusively that DNA signatures are reliable for identifying pandemic serogroup strains (**Serichantalergs, 2007**) that expand, with respect to O : K sero-markers (**Chowdhury, 2000a, Laohaprertthisan, 2005, Matsumoto, 2000**) So the Pulsed Field Gel Electrophoresis provides a scope for phylogenetic analysis with a better and sophisticated approach. In this study we could distinguish among the clinical and environmental isolates with these techniques and enabled ourselves to investigate their clonal origin. Our results indicate some genetic diversity among isolates classified into different serogroups. Most recently the first MLST scheme for *V. parahaemolyticus* using sequences of internal fragments of seven HK genes has been reported which indicates that *V.parahaemolyticus* is genetically diverse with a semi-clonal population structure and that frequent recombination events seem to play an important role in the first steps of clonal diversification **(Gonzalez-Escolana *et. al.*, 2008).**

This study in fact is the continuation of our first ever findings that showed that a significantly higher portion of *V. parahaemolyticus* strains occurring in the surface waters of coastal villages of Bangladseh is potentially virulent **(Alam *et. al.*, unpublished)**. The previously reported pandemic serogroup of *V. parahaemolyticus* could be recognized among clinical strains tested in this study and its existence in this region is indicated by past records **(Nair et. al., 2007)**. Again the prevalence of newly emerged virulent serogroup O8:K21 in environment and clinical samples strongly indicates prolongation of the infection process from surface water and thus jeopardizing human health. Existence of pandemic clones in patients stool and failure to isolate them from environment does not rule out the probability of their existence in nature. Additionally, serogroup conversion and emergence of serovariants of the pandemic serogroups have been shown in other regions **(Bhoopong et. al., 2007)**. Thus carry-over study is on demand for pursuing pandemic serogroup strains existing in aquatic ecosystem of this coastal area which might be mimicked by its serovariants or non pandemic counterparts.

The divergence demonstrated by Bengal strains of *V. parahaemolyticus* is in agreement with many studies reporting similar results for other regions (**Matsumoto et. al., 2000; Wong and Liu, 1999**). The divergence exhibited in molecular signature by *V. parahaemolyticus* suggests that Horizontal gene transfer (HGT) in vivo and in ecological niche may play a significant role in the ecology and evolution of *V. parahaemolyticus*. The search for strains with pandemic marker genes may pose persistent challenge if HGT is as extensive in *V. parahaemolyticus* as it is in *V. cholerae*.

In conclusion, the *V. parahaemolyticus* serogroup O3:K6, O8:K21, O9:KUT and others strains recognized as toxigenic are genetically closely related, be it in the environment or in human system causing disease. Their presence in drinking water sources of coastal villages of Bangladesh poses a great

health risk. Since diarrhoeal diseases are endemic in Bangladesh (Alam et. al., 2006), where morbidity and mortality due to recurrent waterborne diseases remain a longstanding problem, it is concluded that morbidity and mortality in Bangladesh resulting each year from diarrhea, particularly in coastal villages, may be attributed, in part, to water born toxigenic and pathogenic *V. parahaemolyticus* that prevail in the aquatic ecosystem of this region.

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Table 1. Virulence properties of *V. parahaemolyticus* isolated from diarrhoea and surface water of estuarine rgion of Bangladesh (2006 - 2007)

Source	Serogroup	No. of Strains	PCR						KP
			toxR	*tlh*	*tdh*	*trh*	*orf-8*	*GS*	
Environmental	O8:K21	12	+	+	+	-	-	-	-
	O9:KUT	4	-	+	-	-	-	-	-
	O9:KUT	3	+	+	-	-	-	-	-
	O4:K46	3	+	+	-	-	-	-	-
	OUT:K33	3	+	+	-	-	-	-	-
Clinical	O8:K21	3	+	+	+	-	-	-	-
	O3:K6	5	+	+	+	-	+	+	-
	O3:K6	2	+	+	+	-	+	+	+
	O3:K6	1	+	+	+	-	-	-	+
	O1:KUT	1	+	+	+	-	+	+	-
	O4:K55	1	+	+	+	-	-	-	-
	O1:K56	1	+	+	+	-	-	-	-
	O8:K22	1	+	+	+	-	-	-	-
	O3:K5	1	+	+	+	-	-	-	-

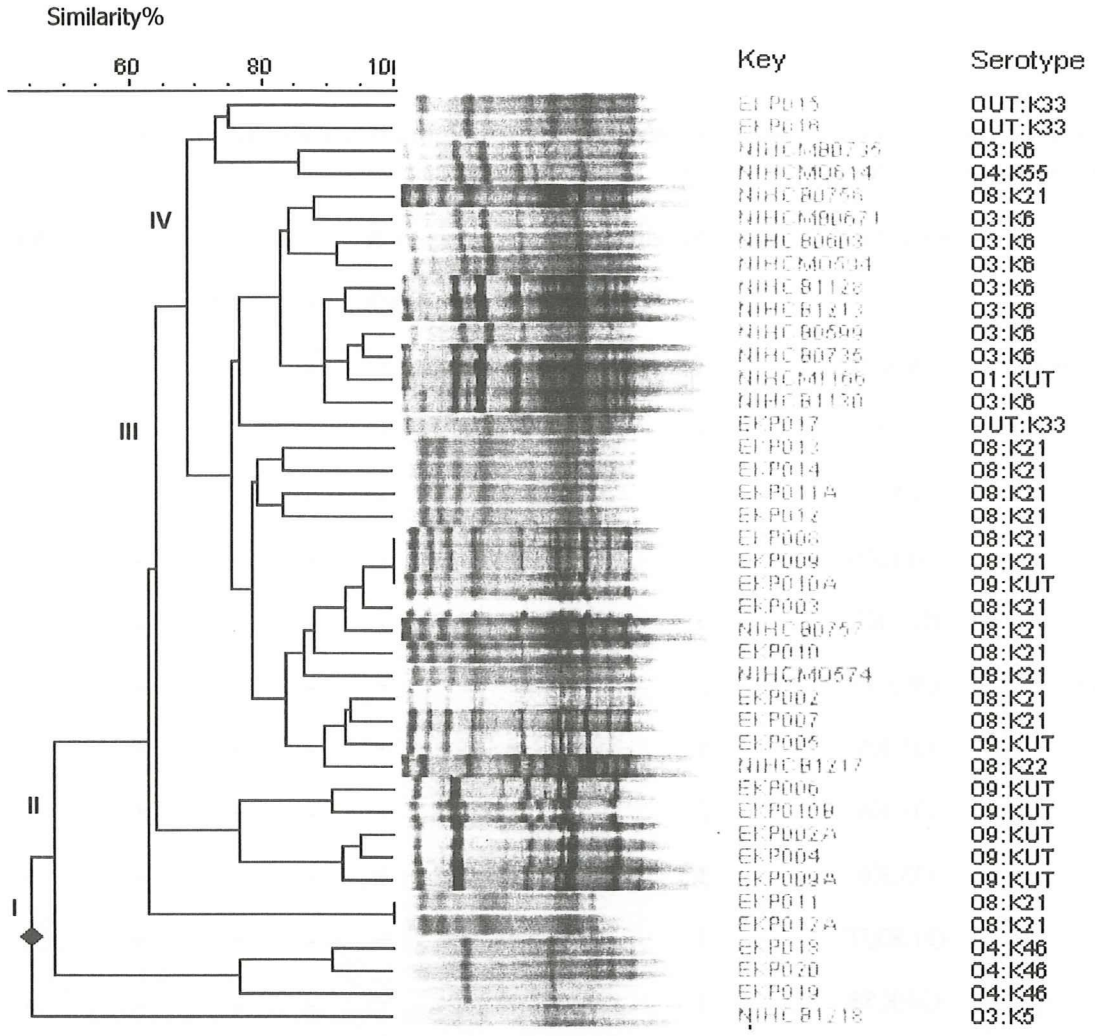

Fig 1. Arbitrarily primed (AP)-PCR was carried out for *Vibrio parahaemolyticus* strains (n=41) using the 10-mer primer as described elsewhere **(Wong, 2003).** The amplified bands were visualized after staining with ethidium bromide (0.5mg/ml) and photographed under UV light. The DNA fingerprinting patterns were analyzed..... Strain identification number and sero-markers are indicated. The dendrogram was constructed by Bionumeric Software Package (Applied Maths) using the Dice similarity coefficient and UPGMA of ERIC-PCR profiles of the *V. parahaemolyticus* strains tested in the present study.

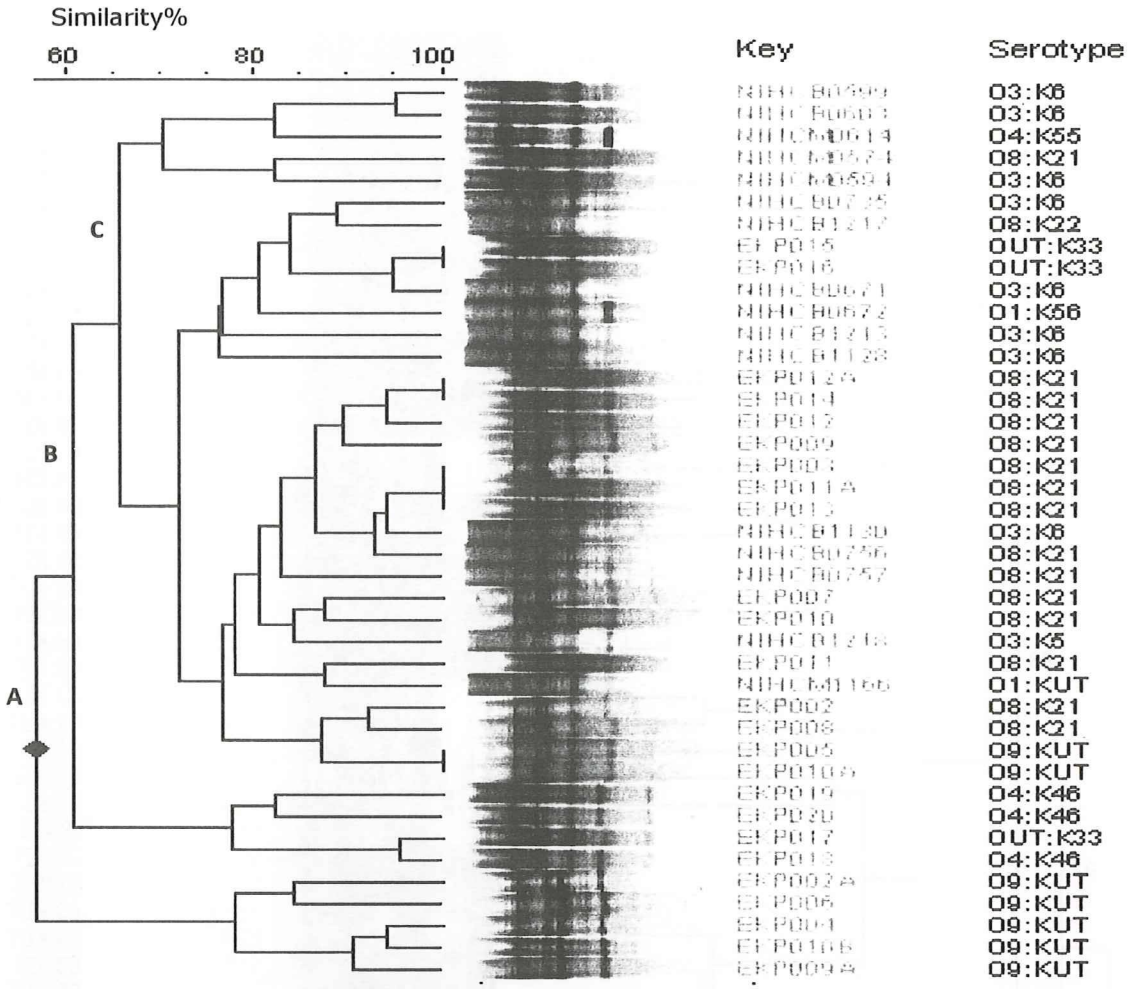

Fig 2. BOX-PCR fingerprinting patterns obtained for isolated strains of *Vibrio parahaemolyticus* (n=41). Strain identification number and sero-markers are indicated. The dendrogram was established by the Bionumeric Software Package (Applied Maths) using the Dice similarity coefficient and UPGMA of ERIC-PCR profiles of the *V. parahaemolyticus* strains tested in the present study.

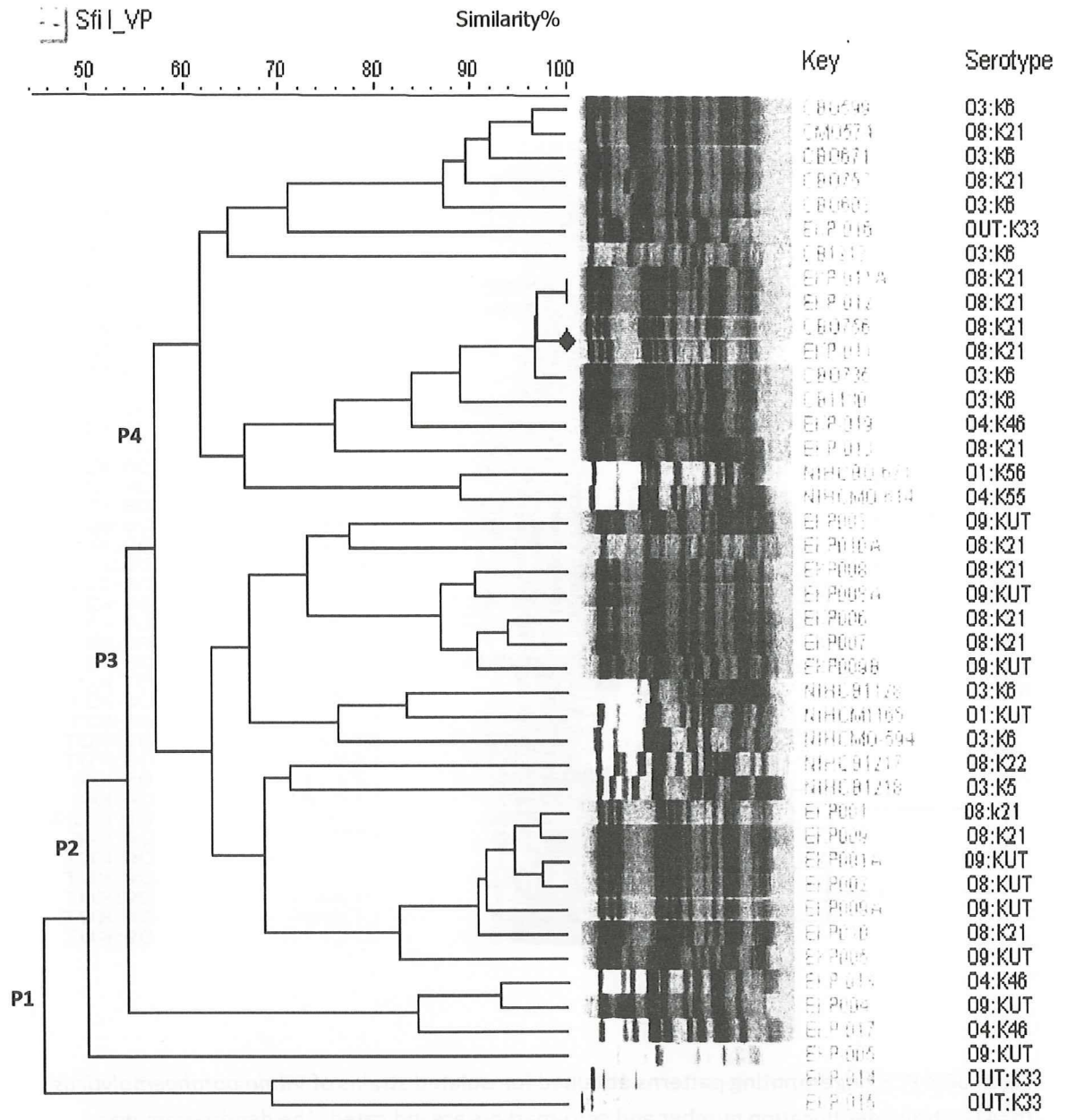

Fig 3. PFGE patterns of *Sfi* I-digested genomic DNA of isolated (n=41) *V. parahaemolyticus* isolates from the estuarine ecosystem of Bangladesh. Strain identification number and sero-marker of the strains are indicated. The dendrogram was established by the Bionumeric Software Package (Applied Maths) using the Dice similarity coefficient and UPGMA of the PFGE profiles of the *V. parahaemolyticus* strains tested.