Molecular epidemiologic Analysis of *V. cholerae* O1 isolates by Pulsed-Field Gel Electrophoresis in Vietnam from 2007 to 2009

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Summary:

In this study, we analyzed 94 of *V. cholerae* isolates which were collected from patients and 4 the environmental isolates in northern Vietnam during period 2007 to 2009. Of the 98 isolates, 23 were collected in 2007, 37 were collected in 2008 and 38 were collected in 2009. All of the 98 isolates were identified as being of the *V. cholerae* O1 Ogawa serotype. Multiplex PCR were used to identify the specific genes of *V. cholerae,* positive for V.O1,*ctx A, ToxA* genes.

The result of PFGE showed that all the 98 isolates during period 2007 to 2009 in Vietnam with very similar PFGE pattern (shared a coefficient of similarity at $\geq$ 93%) are clonally related or belong to a single clonal cluster, which includes isolates of human and enviromental origin.

Introduction

Cholera is an acute diarrhoea caused by *Vibrio cholerae* O1 and O139. People living under poor sanitary conditions are exposed to the threat of cholera infection after consuming contaminated food and water. Before 2005, only a few cases of cholera were reported in the northern part of Vietnam. However, by the end of 2007, major outbreaks of cholera were occurring in this region (8). From 24 October 2007 to 25 June 2009, there were more than 7,000 cases with acute severe, watery diarrhoea in 22 cities and provinces in Northern Vietnam. Molecular techniques for genomic comparisons of closely-related bacterial species or strains of the same species are extremely valuable for molecular epidemiological surveillance of a particular infectious disease as: Multilocus enzyme electrophoresis (MEE), restriction

fragment length polymorphism (RFLP), Ribotyping and Amplified fragment length polymorphism fingerprinting (AFLP). Among the diferent typing techniques, pulsed-field gel electrophoresis (PFGE) has proved to be highly dis criminatory for the analysis of *V. cholerae* O1 genetic diversity. This method has been successfully used for molecular epidemiology surveillance in monitoring the dissemination of this pathogen and the emergence of new epidemic clones (1,3,4,10, 11).

The molecular characteristics of the clinical isolates of *V. cholerae* and the molecular epidemiology of the disease caused by the bacteria in the country have never been investigated. In order to investigate the origin of strains in domestic cases, a total 98 strains of *V. cholerae* O1 isolated from patients and enviroment in period 2007 to 2009 were analysed by PFGE for genotyping. Our data should provide useful epidemiological baseline information with public-health implications, such as for epidemic tracing of indigenous strains and for identifying their genetic relationship with the strains that may emerge in the future (5,7).

In this study, we analyzed 94 of *V. cholerae* isolates which were collected from patients and 4 the environmental isolates in northern Vietnam during period 2007 to 2009. Of the 98 isolates, 23 were collected in 2007, 37 were collected in 2008 and 38 were collected in 2009. All of the 98 isolates were identified as being of the *V. cholerae* O1 Ogawa serotype.

Objective:

- To investigate relationships between the *V. cholerae*O1 isolates from cholera outbreaks in Vietnam from 2007 to 2009
- To compare molecular characterization of *V. cholerae* O1 isolated from patient and enviroment
- To identify the PFGE patterns of *V. cholerae* O1 isolates in Vietnam from 2007 to 2009

MATERIALS AND METHODS

Bacterial strains

Ninety eight of *V. cholerae* O1 isolates were included in this study. Ninety four strains were isolated from patients with cholera and 4 enviromental isolates from water, vegestable in period 2007 to 2009 in different provinces of Vietnam. All the isolates were of the Ogawa serotype. Rectal swabs of patients were individually placed in Cary-Blair transport medium and subsequently enriched in alkaline peptone water for four hours before plating onto TCBS (Oxoid) agar. Yellow colonies were subjected to appropriate biochemical testing (5). The *V. cholerae* isolates were tested by agglutination reaction with polyvalent O1 antiserum, and the strains that gave a positive agglutination were serotyped using monovalent Ogawa and Inaba antisera (Denka Seiken, Japan). The *V. cholerae* O1 isolates were stored in LB broth (Sigma) containing 15% glycerol at -70 °C until use.

The strains were used as references: *V. cholerae* O1 - H218 and *V. cholerae* O139 - AI 4450 for positive controls and *Salmonella* Braenderup H9812 for molecular mass marker

Polymerase Chain Reaction (PCR)

The specific gens for *V. cholerae* O1: V.O1, V.O139, ctx A, ToxA were detected by multiplex-PCR. PCR was carried out in a 0.2 ml microcentrifuge tubes with 24 µl of the PCR mixture containing 1□ µl each of forward and reverse primers (20 µl and 1 µl (ca. 0.1 µg) of template DNA by using Go-Taq® Green Master Mix (Promega, Madison, Wis.). The solution was mixed, centrifuged briefly, and placed in an automated Eppendorf PCR Thermal Cycler (Hamburg, Germany). PCR amplification conditions were as follows: initial denaturation at 94°C for 2 min, and 30 cycles of 1min-denaturation at 94°C, 1min-annealing at 60°C, and 1 min-extension at 72□°C with a final extension step at 72°C for 7 min at the end of 30 cycles, followed by maintenance at -4°C. PCR products were separated by 2% agarose gel electrophoresis in 1xTAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0), The molecular masses of the amplicons were determined by comparison with

molecular mass markers of Ladder 100bp. Amplification products were stained with ethidium bromide, and visualized under UV light. The primers used in this study are shown in Table 1.

Table 1. Primers used in the multiplex PCR

Primers	Sequences(5` to 3`)	Target gene	Amplicon size (bp)
VCO1 F2-1 VCO1R2-2	5'GTT TCA CTG AAC AGA TGG G 3' 5'GGT CAT CTG TAA GAT CAA C 3'	O1	192
VCO139F2 VCO139 R2	5' AGC CTC TTT ATT ACG GGT GG 3' 5'GTC AAA CCC GAT CGT AAA GG 3'	O139	449
AX2 AX3	5' CGG GCA GAT TCT AGA CCT CCT G 3' 5' CGA TGA TCT TGG AGC ATT CCC AC 3'	ctxA	564
101F 837R	5'CCT TCG ATC CCC TAA GCA ATA C 3' 5'AGG GTT AGC AAC GAT GCG TAA G 3'	ToxA	779

III. *Pulsed-Field Gel Electrophoresis (PFGE)*

Pulsed-field gel electrophoresis was performed according to the standardized PulseNet PFGE protocol (9) . After embedded in the agarose blocks, bacteria were lysed in the agarose plugs and incubated overnight. The next day, the plugs were rinsed and sliced to 2.0-2.5 mm thickness . Slices of agarose plug were digested with 20 units of *Not*I restriction endonuclease (Promega, Madison, WI, USA). PGFE was carried out using a CHEF-DRIII system (Bio-Rad, Laboratories, Richmond, CA, USA) through 1.2% agarose with ramp times of 7-12 sec for 11.5 h, followed by 9-12 sec for 10.5 h for *Not*I. Electrophoresis was performed at 14°C at 6 volts/cm with a 120° reorienting angle. *Salmonella* Braenderup H9812 was used as the molecular mass marker. Gels were then stained with ethidium bromide (0.5 μg/mL) for 30 min, rinsed 3 times for a total of 30 min, and visualized by UV transillumination. Photographs were taken and filed.

The images of DNA band patterns were analyzed for cluster analysis using Molecular Fingerprinting Analyst (Bio-Rad, USA) sofware based on the Dice similarity coefficient and unweighted pair-group method with arithmetic averages.

Analysis of PFGE patterns

V. cholerae O1 chromosomal restriction PFGE patterns were classified according to Tenover *et al.* (11, 12) and Arakawa *et al.* (1). When four or more DNA bands in the PFGE patterns were different from each other, we assigned them as different patterns by Arabic numerals, i.e. patterns 1 to 17. Patterns with less than a four-band difference were considered subtypes, i.e. 2a, 2b, 2c, 2d, 2e, 8a, 8b, 8c, 11a, 11b, 11c, and 11d. The DNA restriction PFGE patterns obtained were also saved as TIFF files for use with Bio-profil (Vilber Lourmat, Marne-La-Vallée, France). For the latter, normalization was done according to the molecular size standards of each gel, with one molecular weight standard being used for 3-4 samples. Construction of similarity matrices was carried out by comparison of Dice coefficients (6). The band-based Dice coefficient is based on a comparison of designated band positions by dividing the number of matching bands between patterns by the total number of bands, thereby emphasizing the matching bands. In all the cases, an un-weighted pair group matching band average (UPGMA) at a 1.3% tolerance window was used for clustering the pulsed-field gel electrophoresed (PF) patterns.

RESULTS AND DISCUSSION

PCR analysis

Multiplex PCR were used to identify the specific genes of *V. cholerae* : V.O1,V.O139, *ctx A, ToxA.* The data showed that the 94 of clinical isolates and 4 of enviromental isolates tested were all positive for V.O1,*ctx A, ToxA* genes.

Year of isolation	Number of isolates	Presence of gene			
		ctxA	V.O1	V.O139	ToxA
2007	23	+	+	−	+
2008	37	+	+	−	+
2009	38	+	+	−	+
	Total: 98				

PFGE analysis with NotI restriction enzyme

98 of *V. cholerae* O1 isolates collected from different provinces during various times exhibited very similar, although not entirely identical, PFGE banding patterns which suggest that they are clonal. The numerical similarities between isolates, as defined by Dice coefficients, were in the ranges of 0.93-1.0 by using *Not*I. The high Dice coefficients represented the high similarities among chromosomal restriction fragment patterns. These isolates are epidemiological related isolates. The banding pattern of clinical isolates in PFGE were similar from those of the enviromental isolates.

The result of PFGE showed that all the ctxA positive strains of *V. cholerae* O1 with very similar PFGE pattern are clonally related or belong to a single clonal cluster, which includes isolates of human and enviromental origin.

PFGE was found to be a useful method for investigating the source, transmission, and spread of cholera infection.

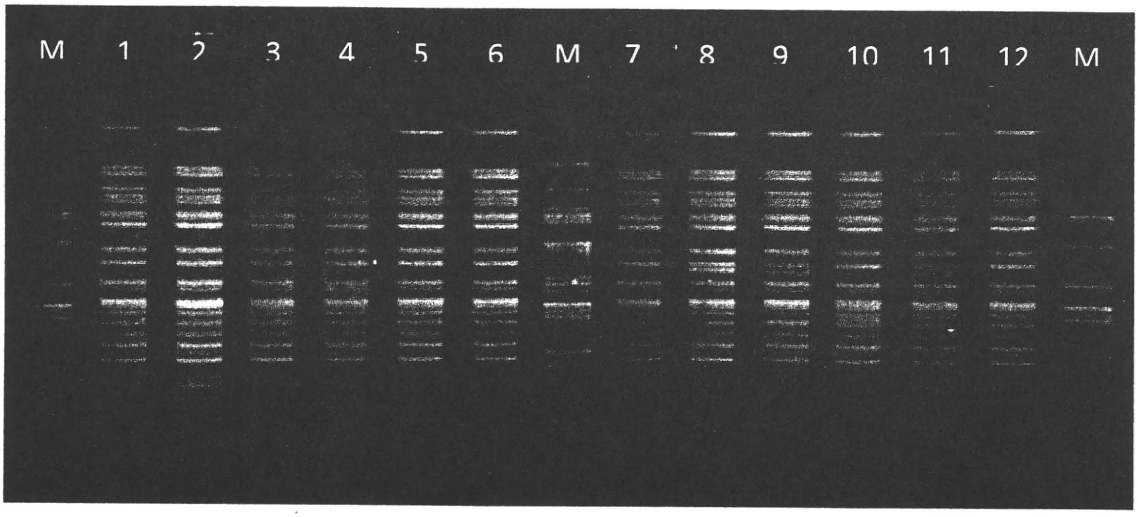


FIG.1. Representatives PFGE patterns of *V. cholerae* O1 strains cleaved with restriction *Not*I. Lanes 1-7 and 10, 12 (VC70-07, 75-07, 229-08, 310-08, 167-09, 7: 99-09, 316-07, 211-09) show representatives PFGE patterns of type. Lanes 8-9 and 11 (48-07, 51-07, 324-07) show representatives PFGE another patterns of type.
Lane M: Molecular mass Marker: *Salmonella* Braenderup H9812 .

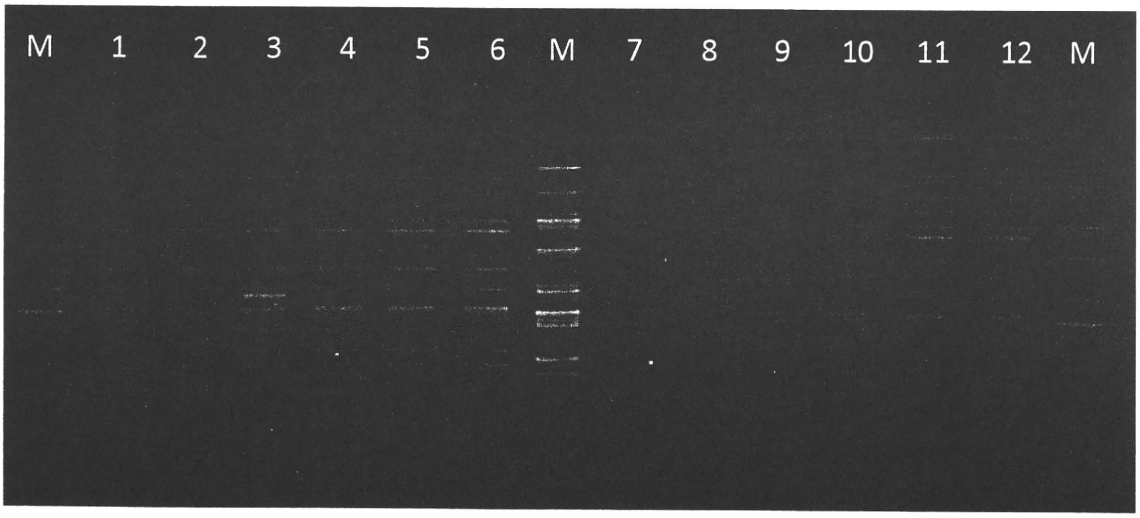
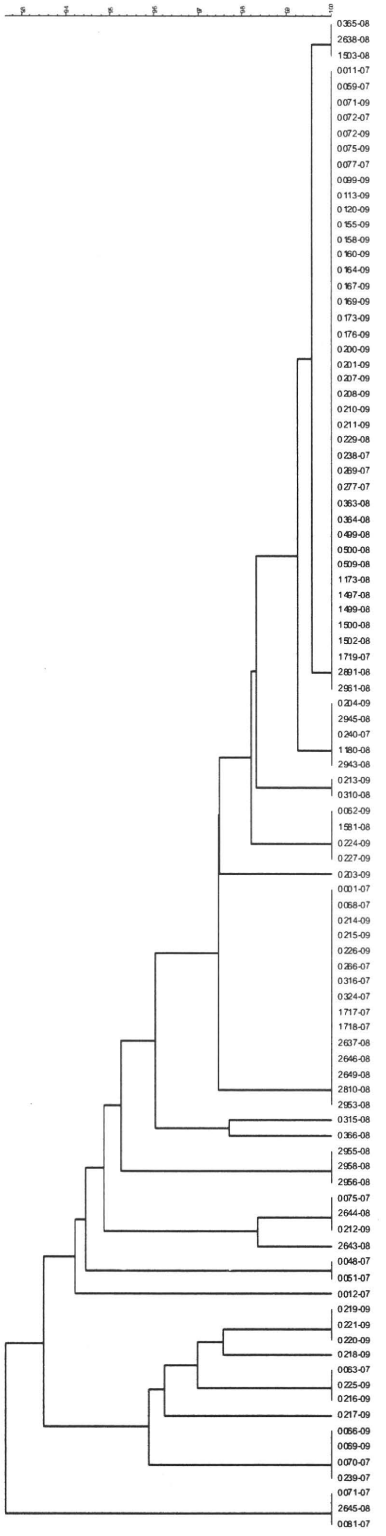


FIG.2. Comparison of Representatives PFGE patterns of *V. cholerae* O1 clinical strains and enviromental strains. Lanes 1-4: *V. cholerae* O1 enviromental strains (VCE11-07, 12-07, 81-07, 217-07).
Lanes 5-12: *V. cholerae* O1 clinical strains (VC0225-09, 0227-09, 59-07, 269-07, 363-08, 2691-08, 169-09, 213-09) show representatives PFGE another patterns of type.
Lane M: Molecular mass Marker: *Salmonella* Braenderup H9812 .

FIG.3.. Dendrogramme and PFGE pattern related following the year of isolates



Acknowledgement

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Report

STUDY TITLE: **Application of PCR-based genotyping to *Campylobacter jejuni.***

STUDY FACILITY: Institute of Environmental Science & Research
Christchurch Science Centre, 27 Creyke Road
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STUDY DIRECTOR: **Dr Brent Gilpin**

1) Objective:

A rapid, epidemiologically relevant tool for genotyping *Campylobacter jejuni.*

Background

Canadian collaborator Eduardo Taboada (Laboratory for Foodborne Zoonoses Lethbridge Unit, Public Health Agency of Canada, Lethbridge, Alberta, Canada) has developed a series of multiplex PCR assays, designed on the basis of large-scale comparative genomics meta-analysis of *Campylobacter jejuni* isolates. He has 8 multiplex assays, each detecting 5 different genomic targets, for a total of 40 informative data points. These have with Canadian isolates a high correlation with MLST typing, and have the potential to provide a high throughput assay.

Methods and Results

We established 5 of the multiplex assays targeting 25 PCR assays (Table 1). This assay was termed CGF25. We detected these products using a Shimadzu MultiNA.

One *C. lari* isolate was typed, which only amplified with one of the assays. This assay may not be suitable for *C. lari*, and subsequent analysis was restricted to 193 *C. jejuni*

and 17 *C. coli* isolates. These were from human (127), chicken (42), ovine (31), bovine (5), turkey (3) and wildfowl (2) sources. One CGF25 marker amplified from all 210 isolates, while the remainder amplified from between 18 and 208 isolates (average 127 isolates). 83 different CGF25 genotypes were identified. The top 10 genotypes accounted for 49% of isolates, with 16 of the isolates having the same type. These isolates were also genotyped by PFGE using *Sma*I and *Kpn*I, and MLST. Comparison (Table 2) of these typing methods suggests that CGF25 genotyping is less discriminatory than PFGE, and perhaps of equivalent discrimination to MLST.

The CGF25 types correlated with the MLST clonal complexes in many cases (ie those isolates with the same CFG25 type, had the same MLST clonal complex). However frequently they had a range of MLST sequence types.

Comparison with Canadian isolates found a number of indistinguishable genotypes, even among the limited number of isolates compared. The most common CGF25 pattern (475721460) are all ST61 complex, as are the four Canadian isolates of the same type. They are however 6 different ST types within that set of isolates. This pattern was repeated with other common genotypes. The *C. coli* isolates clustered together.

Conclusions: A PCR based typing method offers a number of advantages. An isolate on a plate can be analysed rapidly (results 4-8 hours later), and relatively cheaply (at least a quarter of the price of PFGE). For this CGF25 assay, the discrimination ability is lower than PFGE, and equivalent to MLST. However a direct correlation between typing results is unlikely as these assays target different genomic regions. Results were reported to PulseNet Asia Pacific meeting in Hong Kong in December 2010. A paper will be prepared for publication.

Table 1. CGF25 typing of NCTC1168, and RM1221.

Isolate	cj0057 (175bp)_MP05	cj0860 (282bp)_MP01	cj1431c (307bp)_MP04	cj0733 (441bp)_MP04	cj1427c (613bp)_MP06	cj0297c (300bp)_MP03	cj1727c (369bp)_MP08	cj0264 (406bp)_MP03	cj0008 (524bp)_MP01	cj1585 (630bp)_MP08	cj1550c (188bp)_MP01	cj1329 (307bp)_MP07	cj0177 (399bp)_MP04	cj1334 (462bp)_MP05	cj0566 (558bp)_MP07	cj0421c (100bp)_MP01	cj0033 (206bp)_MP08	cj0486 (301bp)_MP02	cj0569 (399bp)_MP02	cj0625 (498bp)_MP03	cj0755 (101bp)_MP02	cj0763 (205bp)_MP07	cj0967 (301bp)_MP05	cj1141 (413bp)_MP01	cj1136 (510bp)_MP04
NCTC 1168	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RM 1221	1	1	0	1	0	1	1	0	0	1	0	1	1	1	0	1	0	1	0	1	1	1	1	0	0

Table 2. Comparison of genotyping results between CGF25, PFGE and MLST.

	CGF25	PFGE			MLST	
		*Sma*I	*Kpn*I	*Sma*I:*Kpn*I	ST[1]	CC[2]
Isolates	210	210	210	210	210	210
Genotypes	83	99	120	135	75	20*
Single genotypes	57	60	81	98	48	3
Isolates with the most common type	16	13	12	7	22	58
Isolates in the most common 10 genotypes	103	78	62	50	120	191*
Types in the most common 25% of isolates	4	6	8	11	4	1
Types in the most common 50% of isolates	11	18	25	34	9	3*
Types in the most common 75% of isolates	31	46	63	83	25	6*

[1]Sequence type, [2]Clonal Complex

* includes 24 isolates with unassigned clonal complexes (counted as one genotype)

Serotypic shift of *tdh*$^{+}$ *V. parahaemolyticus* occurring in estuarine ecosystem of Bangladesh, 2006 – 2008

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ICDDR,B

Introduction

Vibrio parahaemolyticus-related gastroenteritis was thought to be far less common in Bangladesh, where raw seafood consumption is rare, and diarrhoeal diseases are mainly caused by *V. cholerae*. However, its importance as a severe pathogen in other parts of the world displays a different scenario. It was first identified as the major cause of an outbreak in 1950 in Japan (Fujino *et. al.*, 1951). Since 1996, gastroenteritis caused by *V. parahaemolyticus* serogroup O3:K6 strains (designated as pandemic O3:K6) was reported from Southeast East Asian countries (Wong *et al.*, 2000), including Bangladesh and India. This serogroup was then found widespread in North America (Khan *et al.*, 2002), South America (Goodridge *et al.*, 2003), Europe (Martinez-Urtaza *et al.*, 2005), and Africa (Ansaruzzaman *et al.*, 2005). *V. parahaemolyticus* is a common gram-negative food-borne enteric pathogen in Taiwan, Japan, and other Asian Pacific countries (Lan *et al.*, 2009). But for its worldwide since1996, this bacterium was regarded as the pandemic pathogen (Okuda *et al.*, 1997).

Infections caused by *V. parahaemolyticus* are usually associated with diverse serogroups, with as many as 75 different recognized combinations of O and K serogroups related to gastroenteritis (Ishibashi *et al.*, 2000; Wong, 2003). However, not all strains are considered to be pathogenic. Pathogenic *V. parahaemolyticus* has been known to produce either thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) or both (Honda & Iida, 1993; Nishibuchi & Kaper, 1995). TDH and TRH encoded by *tdh* and *trh* genes, respectively, are now recognized as major virulence factors of *V. parahaemolyticus* (Honda & Iida, 1993; Nishibuchi & Kaper, 1995) which are present in most clinical isolates and have

been detected with low frequencies in the environmental strains (DePaola *et al.*, 2003; Dileep *et al.*, 2003). It has been shown that the group-specific(GS) sequence of the *toxRS* operon and the presence of ORF8 in the filamentous phage f237 are specific for pandemic O3:K6 clones, and these genetic markers are used to distinguish between pandemic and non pandemic traits(Matsumoto *et al.*,2000;Nasu *et al.*,2000). Since 1996, it has been reported that the appearance of the pandemic group belonged to O3:K6 and its derivates O4:K68, O1:K25, O1:KUT serotypes, which spread to and caused an increased incidence of rapid outbreaks in Asia, Africa and America (Nair *et al.*, 2007). Molecular biology-based analyses of these additional serotypes indicated that they may have diverged from the pandemic O3:K6 strains by alteration of their O: K antigens (Chowdhury *et al.*, 2000, Matsumoto *et al.*, 2000), and currently 24 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 *et al.*, 2007, Caburlotto *et al.*, 2010).

In Bangladesh, deaths due to *V. parahaemolyticus* infections are rarely reported; this may be due to lack of routine surveillance, although there are reports of severe illnesses that results many deaths in the remote coastal villages, where people do not have access to pure drinking water, and thus they are compelled to use surface water for drinking and their daily activities (Alam et al., 2007). The pandemic serogroups of *V. parahaemolyticus* (O3:K6 and O4:K68) were reported from the hospitalized gastroenteritis patients in Dhaka in early 2000s (Bhuiyan *et al.*, 2002). A recent study in the coastal villages of Bay of Bengal reported data on the phenotypic, genotypic and virulence traits of *V. parahaemolyticus* strains isolated from coastal aquatic ecosystem, where *tdh*+ *V. parahaemolyticus* strains were reported from diarrheal cases and aquatic ecosystem (Alam *et al.*, 2009). Alam *et al.*, (2009) in their earlier study showed the serogroup distributions pattern in this southern coastal region, where tdh^+ O8:K21 was found to be the most prevalent serogroup in aquatic ecosystem, showing that O8:K21 was also linked to diarrheal cases in the same region, suggesting its potential to cause epidemic. Our subsequent observation, which was from the analyses of stool specimens of an ongoing cholera epidemiological surveillance carried out in the coastal villages, was that *V. parahaemolyticus* strains were often linked to severe diarrhea that was clinically recognized as cholera. The serogroup analysis of these *V. parahaemolyticus* strains revealed the pandemic serogroup O3:K6, followed by O8:21 to be the prevalent serogroups, respectively Alam *et al.*, (Unpublished data). In the current study, which is not a routine one but a follow-up carried out subsequently between late 2007 and early 2008 in the same

coastal area, we present data showing how the *tdh*+ *V. parahaemolyticus* strains present in the coastal aquatic ecosystem undergo temporal shift in their serogroups, suggesting that this environment serves as an important breeding ground where pandemic pathogen *V. parahaemolyticus* O3:K6 was first reported in 1996.

Materials and Methods

Sample collection, processing, and the identification of *V. parahaemolyticus*. Seawater and brackish water samples were collected from the Bay of Bengal and its estuaries at 20 different sites located within Kuakata, a coastal area in the Barishal division during the period from 2007-2008 employing established methods (Alam *et al.*, 2009). Stool specimens or rectal swabs of diarrhea patients admitted to hospitals at Mathbaria and Bakerganj, adjacent areas within this coastal belt were collected between Feb 2006 and May 2007 employing methods recommended by the American Public Health Association (APHA. 1970). Stool samples were plated directly, while rectal swabs were plated after enrichment in alkaline peptone water (APW) onto Thiosulfate Citrate Bile salts Sucrose (TCBS) agar, as described by Mukhopadhyay *et al.* (Mukhopadhyay *et al.*, 1996).

Storage of strains. *V. parahaemolyticus* strains confirmed by biochemical methods were subcultured on TCBS agar, and a single representative colony from gelatinase agar was aseptically inoculated into T_1N_1 broth (1% Trypticase and 1% NaCl), incubated at 37°C for 3 to 4 h, and stored at -80°C after adding 15% glycerol.

Serogrouping. Serogrouping of the *V. parahaemolyticus* isolates was done using a commercially available *V. parahaemolyticus* antisera test kit (Denka Seiken, Tokyo, Japan) according to manufacturer's instructions. A loopful of inoculum was mixed with 1 ml normal saline and an aliquot of the cell suspension in normal saline was boiled for 2 hours for use in serotyping, based on the O antigen. The remaining cell suspension (not boiled) was used for serotyping based on the K antigen.

Isolation of Genomic DNA. For extraction of genomic DNA, cells were incubated overnight in 3 ml Luria-Bertani broth (DIFCO, USA). The harvested cells were subjected to alkaline lysis by 10% SDS in the presence of TE buffer (10mM Tris-HCl; 1mM EDTA, pH 8.0). The cells were then treated at 37°C for 1 h with freshly prepared Proteinase K (final concentration 100 µg/ml in 0.5% SDS). Then 1.0% CTAB/NaCl (cetyl trimethyl ammonium bromide in 0.7M NaCl) was added followed by incubation for 10 min at 65°C. RNA was removed by treating with RNase (final concentration 100µg/ml) at 37°C for 1h. This was followed by phenol chloroform extraction and precipitation of the nucleic acid in the presence of isopropanol (Chowdhury *et al.*, 2000). Excess salt was removed by 70% alcohol wash and the nucleic acid was air dried, resuspended in sterile TE buffer and stored at -20°C for subsequent PCR analysis. The purity of the DNA was assayed using a spectrophotometer (Gene Quant, England) that self calculates the ratio of optical densities at 260 and 280 nm.

Polymerase Chain Reaction (PCR). Simplex PCR assay were performed to detect *toxR* gene (Chowdhury *et al.*, 2004). For species specific gene *tlh* and the two virulence genes *tdh* and *trh*, multiplex PCR was employed using *V. parahaemolyticus* genomic DNA as template, following methods described by Bej *et al.* (Bej *et al.*, 1999).

Group specific (GS) and open reading frame 8 (ORF8)-PCR. PCR assays for amplification of the pandemic marker genes GS and ORF8 were performed using specific primers previously reported to detect *toxRS* sequences unique to the pandemic O3: K6 clone of *V. parahaemolyticus* and the orf8 sequence of phage f237 respectively (Laohaprertthisan *et al.*, 2003, Matsumoto *et al.*, 2000, Nasu *et al.*, 2000).

Pulsed-field gel electrophoresis (PFGE). The PFGE of the isolated environmental and clinical strains was performed using a standardized protocol for sub-typing *V. parahaemolyticus* by methods followed in some previous studies (Kam *et al.*, 2008; Alam *et al.*, 2009). *Salmonella branderup* was used as the control strain. Following electrophoresis, gels were stained with ethidium bromide (10 mg/ml) and photographed under UV transilluminator.

Image analysis. The fingerprint pattern in the gel was analyzed using the BioNumeric® (Applied Math, Belgium) computer software package. After background subtraction and gel normalization, the fingerprint patterns were subjected to typing based on banding similarity

and dissimilarity. Dendrograms were computed using the Dice similarity coefficient, and UPGMA for AP-PCR, BOX-PCR and PFGE profiles of *V. parahaemolyticus* strains. Two methods for measuring similarity were compared, one based on binary data of occurrence of the band (band-based), calculated using the Dice coefficient, and the other on the overall densitometry profile (curve-based) of the banding pattern, calculated using Pearson's product moment correlation.

RESULTS

Clinical data: Our clinical data came from the analyses of stool specimens of an ongoing cholera epidemiological surveillance carried out between 2004 and 2007 and clinically typical cholera stool samples were collected biweekly from hospitalized patients in clinics located at the coastal villages. Therefore, although our major aim was to isolate and detect *V. cholerae*, interestingly *V. parahaemolyticus* strains were often found linked to severe diarrhea that was clinically recognized as being cholera.

The serogroup analysis of these *V. parahaemolyticus* strains revealed the pandemic serogroup O3:K6, followed by O8:21 to be the prevalent serogroups, respectively Alam *et al.*, (Unpublished data).

Environmental data: In the current study, which is not a routine one but a follow-up carried out in two subsequent sampling carried out between late 2007 and early 2008 in the same coastal area, *V. parahaemolyticus* strains isolated from aquatic ecosystem showed the presence of *tdh*+ O8:K21 among other tdh+ serogroups, namely O9:KUT, O4:K46, and OUT:K33, but no pandemic serogroups namely O3:K6 was isolated during 2007. But in the subsequent sampling in the same coastal region in early 2008, pandemic pathogen O3:K6 was found to be the prevalent serogroup present with the other pandemic seogroups O4:K68 and O1:KUT. The other *tdh*+ serogroups present in the aquatic ecosystem were OUT:KUT, O8:KUT, O3:KUT, O5:KUT, and O4:KUT.

Genetic characteristics of *V. parahaemolyticus*: Sixty *V. parahaemolyticus* characterized in the present study included 48 environmental and 16 clinical strains that were isolated from estuarine ecosystem of Bangladesh between 2006 and 2008. *V. parahaemolyticus* strains were identified by employing established methods, as described earlier (Alam et al., 2009).

Table 1 shows the source, serotype, virulence genes and genetic characteristics of the isolates investigated.

Serotyping A highly diverse array of serotypes was found among the sixty-four strains of *V.parahemolyticus*. Thirteen different combinations of O and K antigens were found among the forty-eight environmental strains which included two previously recognized pandemic serogroups O3:K6 and O4:K68 accounting for 33% (n=16) and 4% (n=2), respectively. While 4 different 'O' and 7 different 'K' types could be detected in 16 clinical *V. parahaemolyticus* strains (Table 1). The pandemic serogroup O3:K6 was also predominant among the clinical isolates (n=8) and only single strain that didn't react to any K antisera was revealed to belong to pandemic group O1: KUT (Nair *et al.*, 2007). The predominant O serogroup for 19 out of the forty-eight environmental strains appeared to be O3 followed by O8 (n=6), O4 (n=6), O9 (n=5) and O5 (n=3). In case of "K" antigen, 16 of the forty-eight strains were recognized by K6 antisera which was the prevalent K serogroup followed by K21, K46 and K33 accounting for 3 strains each as well as K68 and K30 for two and one strains, respectively. Among these strains seven strains were not recognized by specific O antisera and twenty strains not recognized by specific K antisera, four strains did not react to O:K antisera suggesting the presence of new variants. A total of 6 different array of K serogroups could be found in 16 clinical strains where K6 also appeared to be the most abundant among clinical strains which accounted for 8 strains followed by K21 (n=3) and K5, K22, K55, K56 (all represented by lone strain).

Serotype analysis revealed that 42% of the isolates collected in this study belonged to the pandemic serotypes O3:K6, O1:KUT and O4:K68. The same pandemic clones were present among clinical isolates in a previous study carried out on hospitalized gastroenteritis patients in Dhaka during the period of 1998–2000 (Bhuiyan *et al.*, 2002). Among these pandemic serogroups O3:K6 was predominant (n=24) and this serogroup was found to be evenly distributed among environmental and clinical strains followed by the non pandemic serogroup O8:K21 that was the second prevailing serogroup (n=6) among both environmental and clinical strains also reported in the same region in 2006 (Alam *et al.*, 2009).

Detection of genes by PCR. *V. parahaemolyticus* strains isolated from the environmental and clinical strains were screened for species specific and virulence genes, *toxR, tlh, tdh* and

trh by PCR. As shown in Table 1, gene *toxR* were confirmed to be present in all the environmental strains (n=25) except 3, which failed to provide 368 bp amplicon specific for *toxR* region. But all the clinical strains were found to carry the *toxR* gene. The species specific gene *tlh* was uniformly present in all the 64 environmental and clinical isolates when tested by multiplex PCR, while 39 (81%) of the 48 environmental strains had the major virulence gene *tdh* and all the clinical isolates possessed *tdh*. However, *trh* was not present in any of the isolates tested (Table 1).

Detection of pandemic markers. All the experimental isolates were subjected to group-specific (GS) and open reading frame 8 (ORF8) PCR that are pandemic markers for *V. parahaemolyticus*. Eighteen (37%) of the 48 environmental strains were found to be positive for GS-PCR and ORF8-PCR where 16 belonged to the serogroup O3:K6 and two belonged to O4: K68, which two are members of the pandemic clonal complex. Among the clinical strains nine clinical strains (57%) belonging to serogroup O3:K6 (n=8) and O1: KUT (n=1) were positive by GS and ORF8 PCR indicating that they belong to the pandemic clone.

Conclusions

Deaths due to diarrheal diseases are very common in the developing world including Bangladesh and India (7, 30). Although no systematic surveillance for *V. parahaemolyticus* is done in these countries, *V. parahaemolyticus* is often reported from hospitalized patients with high frequency. However, very little is known about the serogroup distribution, virulence potential, or molecular characteristics of *V. parahaemolyticus* occurring in the estuarine ecosystem of the Bay of Bengal, where the pandemic serogroup, O3 : K6, was first isolated and reported from this region (34). The clinical link and predominance of *V. parahaemolyticus tdh*+ serogroup 08:K21 strains were first shown in the estuarine ecosystem of the Bay of Bengal between 2005 and 2006 (Alam et al., 2009). Subsequent clinical data collected from an epidemiological surveillance targeted for cholera, carried out in coastal villages between 2006 and 2007, showed the presence of *V. parahaemolyticus* in clinically typical cholera stool samples collected biweekly from hospitalized patients in Bangladesh. The serogroup analysis of these clinical *V. parahaemolyticus* strains revealed the pandemic pathogen O3:K6 to be the prevalent serogroup, although the presence of *tdh*+ serogroup