Infection, Genetics and Evolution 41 (2016) 153–159

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

# Major *tdh*$^+$ *Vibrio parahaemolyticus* serotype changes temporally in the Bay of Bengal estuary of Bangladesh

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ARTICLE INFO

*Article history:*
Received 1 February 2016
Received in revised form 2 April 2016
Accepted 5 April 2016
Available online 7 April 2016

*Keywords:*
*V. parahaemolyticus*
*tdh*
Serotype
O3:K6
Pandemic
O8:K21

ABSTRACT

*Vibrio parahaemolyticus* is responsible for seafood-related gastroenteritis worldwide. In Bangladesh, diarrhea is endemic and diarrheagenic *V. parahaemolyticus* serotypes occur naturally in the coastal and estuarine aquatic environment. *V. parahaemolyticus* strains, isolated from estuarine surface water of the Bay of Bengal villages of Bangladesh during 2006–2008, were tested for the presence of virulence and pandemic-marker genes, serodiversity, and phylogenetic relatedness. PCR analysis of *V. parahaemolyticus* (n = 175) showed 53 (30.3%) strains to possess *tdh*, the major virulence gene encoding thermostable direct hemolysin. Serotyping results revealed the *tdh*$^+$ *V. parahaemolyticus* strains to belong to 10 different serotypes, of which the O8:K21 (30.2%) and O3:K6 (24.5%) were predominantly non-pandemic and pandemic serotypes, respectively; while O5:K30 and O9:KUT were new. The pandemic markers, *orf8* and *toxRS*$^{variant}$, were present only in the pandemic serotype O3:K6 (n = 13) and its serovariant O4:K68 (n = 2). Temporal distribution of the *tdh*$^+$ serotypes revealed the O8:K21 to be predominant in 2006 and 2007, while O3:K6 was the predominant *tdh*$^+$ serotype in 2008. Pulsed-field gel electrophoresis (PFGE) of *Sfi*I-digested genomic DNA revealed high genetic diversity among the *V. parahaemolyticus* strains, while dendrogram constructed with the PFGE patterns formed two major clusters separating the *tdh*$^+$ O3:K6 and its pandemic serovariants from the *tdh*$^+$ non-pandemic (O8:K21) strains, suggesting different lineages for them. The potential health risk related to the prevalent *tdh*$^+$ strains, including the observed temporal change of the predominant *tdh*$^+$ serotype, from O8:K21 to the pandemic serotype O3:K6 in estuarine surface waters serving as the major source of drinking water suggests the need for routine environmental monitoring to prevent *V. parahaemolyticus* infection in Bangladesh.



## 1. Introduction

*Vibrio parahaemolyticus*, a natural inhabitant of marine and estuarine waters, is the causal agent of gastroenteritis, wound infection and septicemia related to the consumption of raw or undercooked seafood and untreated surface water (Martinez-Urtaza et al., 2004; Nair et al., 2007). The bacterium was first identified as the major cause of seafood-borne disease outbreak in 1950 in Japan (Fujino et al., 1951). *V. parahaemolyticus* gastroenteritis has cholera-like symptoms, e.g., diarrhea with low-grade fever, abdominal cramps, nausea, vomiting, watery stools with visible mucus, and dehydration (Klontz et al., 2012). Diarrhea outbreaks caused by water-borne pathogens including *V. parahaemolyticus* may be influenced by various climate factors, e.g., sea surface temperature, salinity, and turbidity (Johnson et al., 2012). An increasing trend in *V. parahaemolyticus*-related illnesses has been observed since 2000 in many parts of the world including the United States (Centers for Disease Control and Prevention, CDC, 2006). However, the evolutionary relationships between clinical and environmental strains and the mechanisms promoting the emergence of pathogenic strains of this bacterium are not well explored.

Pathogenicity of *V. parahaemolyticus* is strongly correlated with the production of either thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), or both (Honda and Iida, 1993; Nishibuchi and Kaper, 1995). *V. parahaemolyticus* strains isolated from coastal and estuarine environments are less likely to carry *tdh* and *trh* (<5%) (DePaola et al., 2003; Nishibuchi and Kaper, 1995), although these highly dispersed naturally occurring population carrying virulence genes are responsible for majority of the seafood borne diarrhea

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http://dx.doi.org/10.1016/j.meegid.2016.04.003
1567-1348/

outbreaks globally (Alam et al., 2009; Mala et al., 2016). In *V. parahaemolyticus* other virulence factors include the type III secretion system 1 and 2 (T3SS1 and T3SS2), which are responsible for cytotoxic activity and enterotoxicity, respectively (Morita et al., 2013; Noriea et al., 2010).

Based on the variations in somatic O and capsular K-antigens, *V. parahaemolyticus* can be classified into 13 O and 71 K serogroups (Iguchi et al., 1995; Nair et al., 2007). Epidemiologically, the O3:K6 serotype of *V. parahaemolyticus* is considered as the major pandemic pathogen, predominantly associated with worldwide clinical cases since 1996 (approximately 50–80% of the patients), although various other non-pandemic serotypes namely O8:K21, O9:KUT are also known to possess the *tdh* gene and associated with diarrheal infections (Alam et al., 2009; Nair et al., 2007). Contemporary epidemiological investigations have indicated that some serotypes, e.g., O4:K68, O1:K25, O1:K untypable (O1:KUT), O1:K56, O3:K75, O3:KUT, O4:K8, O5:KUT, associated with gastroenteritis outbreaks in Asia, Africa and America (Ansaruzzaman et al., 2005; Matsumoto et al., 2000), are genetically closely related to the pandemic serotype O3:K6 and considered to have been serovariants evolved from the ancestor O3:K6 through seroconversion (Chen et al., 2011). The rapid emergence of serovariants of O3:K6 suggests genetic recombination and natural evolution of the pandemic clone generating more than 20 serotypes that belong to the pandemic clonal complex of *V. parahaemolyticus* (Caburlotto et al., 2010; Nair et al., 2007). Irrespective of their serodiversity, the pandemic *V. parahaemolyticus* strains ubiquitously harbor two genetic markers, i.e., a unique open reading frame (*orf8*) of the *f237* filamentous phage, and a variant *toxRS* operon ($toxRS^{variant}$) possessing pandemic group-specific (GS) sequence, which are used to distinguish between the pandemic and non-pandemic traits (Matsumoto et al., 2000; Nasu et al., 2000). In general, a strain can be considered a pandemic group when *V. parahaemolyticus* harbors both *tdh* and the new type *toxRS* operon ($toxRS^{variant}$) (Okura et al., 2003).

In Bangladesh, recurrent diarrhea outbreaks result in many deaths, particularly in the remote coastal villages where safe drinking water is scarce and people use surface water for drinking and other household chores (Alam et al., 2006). Toxigenic *V. parahaemolyticus* ($tdh^+$ and $trh^+$) including the pandemic O3:K6 strains have been isolated from the hospitalized patients suffering from gastroenteritis in Dhaka city in the central part as well as the estuarine environment of Chittagong in the southern part of the country (Bhuiyan et al., 2002; Islam et al., 2004). A previous study carried out in diarrhea endemic coastal villages of Bangladesh from various districts during 2005–2006, reported the isolation of $tdh^+$ strains belonging to diverse serotypes from the environment, with O8:K21 as the predominant non-pandemic serotype associated with diarrheal cases (Alam et al., 2009). In this study, to understand local variability, we focused on a single coastal location (Kuakata) over three years (2006 to 2008). *V. parahaemolyticus* strains isolated from the surface water of estuarine environment of Bangladesh were investigated for the presence of major virulence and related marker genes, the serodiversity of potentially pathogenic strains, and their genetic relatedness. The aim was to assist intervention and preventive measure against the toxigenic *V. parahaemolyticus* serotypes occurring in the estuarine environment of Bangladesh, where thousands of people suffer from recurrent diarrhea and are at high risk of water-borne diseases due to saline water intrusion resulted from the changing climate (Cruz et al., 2007; Khan et al., 2011).

## 2. Material and methods

### 2.1. Isolation of bacterial strains and confirmation of V. parahaemolyticus

A total of 175 *V. parahaemolyticus* strains, isolated between November 2006 and December 2008 from the 9 selected brackish sites of Kuakata, an estuarine ecosystem of the Bay of Bengal, Bangladesh were analyzed for serogroup, virulence, and molecular characteristics in the present study. The study site is located in the south-western part of Bangladesh, near the world's largest mangrove forest, the Sundarban. The latitude and longitude of Kuakata is 21.82104 and 90.12142, respectively. *V. parahaemolyticus* strains were confirmed by microbiological and molecular methods, as described elsewhere (Alam et al., 2009). Briefly, the presumptive, green and mucoid *Vibrio*-like (bigger) colonies on thiosulfate-citrate-bile salts-sucrose (TCBS) agar were selected and confirmed by microbiological (APHA, 2005) and molecular methods (Alam et al., 2009). *V. parahaemolyticus* strains with the year of isolation are shown in Table 1.

### 2.2. Storage

*V. parahaemolyticus* strains confirmed by biochemical, serological, and molecular methods were subcultured on gelatin agar (GA) plates; and a single representative colony from the GA was aseptically inoculated into T1N1 broth (1% Trypticase, 1% NaCl; pH 7.6–7.8), incubated at 37 °C for 3 to 4 h, and stored at 80 °C with 15% glycerol until it was required for the experiment.

### 2.3. Extraction and purification of chromosomal DNA

For extraction of genomic DNA, a single colony of each of the bacterial strains were enriched overnight at 37 °C in 3 ml Luria-Bertani broth

**Table 1**
Serogroup, virulence, and molecular characteristics of *V. parahaemolyticus* strains isolated from environmental samples (2006–2008)[a].

| Year | Serotypes | No. of isolates (%) | Species-specific genes | | Virulence genes | | Pandemic markers | |
|---|---|---|---|---|---|---|---|---|
| | | | *toxR* | *tlh* | *tdh* | *trh* | *ORF8* | $toxRS^{variant}$ |
| 2006 | O8:K21 | 4 (12.1) | + | + | + | − | − | − |
| (Nov–Dec) | O5:KUT | 1 (3.0) | + | + | + | − | − | − |
| | ND | 28 (84.8) | + | + | − | − | − | − |
| 2007 | O8:K21 | 12 (19.7) | + | + | + | − | − | − |
| (Feb–Mar) | O9:KUT | 7 (11.5) | + | + | + | − | − | − |
| | O4:K46 | 3 (4.9) | + | + | − | + | − | − |
| | ND | 39 (63.9) | + | + | − | − | − | − |
| 2008 | O3:K6 | 13 (16.0) | + | + | + | − | + | + |
| (Nov–Dec) | OUT:KUT | 4 (4.9) | + | + | + | − | − | − |
| | O8:KUT | 3 (3.7) | + | + | + | − | − | − |
| | O1:KUT | 2 (2.5) | + | + | + | − | − | − |
| | O4:K68 | 2 (2.5) | + | + | + | − | + | + |
| | O5:KUT | 2 (2.5) | + | + | + | − | − | − |
| | O4:KUT | 1 (1.2) | + | + | + | − | − | − |
| | O5:K30 | 1 (1.2) | + | + | + | − | − | − |
| | O9:KUT | 1 (1.2) | + | + | + | − | − | − |
| | ND | 52 (64.2) | + | + | − | − | − | − |

[a] ND, Not done; OUT, 'O'-untypable, KUT, 'K'-untypable

(Difco, USA). The enriched cells were harvested and subjected to alkaline lysis by 10% SDS in the presence of TE buffer (10 mM Tris–HCl; 1 mM 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), followed by treatment with 1.0% CTAB/NaCl (cetyl trimethyl ammonium bromide in 0.7 M NaCl) for 10 min at 65 °C. RNA was removed by treating with RNase (final concentration 100 μg/ml) at 37 °C for 1 h. 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) at 260 and 280 nm.

### 2.4. Polymerase chain reaction (PCR)

Simplex PCR assays were performed to detect the species-specific gene *toxR* and *tlh* by following the methods, as described elsewhere (Bej et al., 1999; Kim et al., 1999). The presence of the two major virulence genes i.e., *tdh* and *trh*, was examined using a multiplex PCR assay with the genomic DNA of the *V. parhaemolyticus* strains following a previously described method (Bej et al., 1999).

### 2.5. 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 (Matsumoto et al., 2000; Nasu et al., 2000).

### 2.6. Serotyping

A commercially available *V. parahaemolyticus* antisera test kit (Denka Seiken, Tokyo, Japan) was used for serotyping of the toxigenic and pandemic *V. parahaemolyticus* strains according to the manufacturer's instructions. Briefly, the strains were first grown on LB agar (Difco) containing 3% NaCl. Following overnight incubation at 37 °C, a loop full of bacterial inoculum was mixed with 1 ml normal saline and an aliquot of this cell suspension was boiled for 2 h for use in the O-antigen based serogrouping. The remaining cell suspension (not boiled) was used for serogrouping based on the K-antigen.

### 2.7. Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) of *Sfi*I-digested DNA of *V. parahaemolyticus* was performed using a standardized protocol, as described elsewhere (Kam et al., 2008). *Xba*I-digested *Salmonella enterica* serovar Braenderup DNA was used as molecular size markers. Following electrophoresis, gels were stained with ethidium bromide and images were captured using a GelDoc system (BioRad, Hercules, CA, USA).

### 2.8. Image analysis

The PFGE gel images were analyzed using BioNumeric® (Applied Math, Belgium) computer software package to determine their fingerprint patterns. After background subtraction and gel normalization, the fingerprint patterns were subjected to typing on the basis of banding similarity and dissimilarity using Dicesimilarity coefficient and unweighted-pair group method using average linkages (UPGMA) clustering methods, as recommended by the manufacturer; these were graphically represented as dendrograms.

## 3. Results and discussion

### 3.1. High prevalence of toxigenic V. parahaemolyticus in the Bay of Bengal estuary

*V. parahaemolyticus* strains (n = 175), isolated from water samples collected from the Bay of Bengal estuary during 2006 (n = 33), 2007 (n = 61) and 2008 (n = 81), were confirmed by a combination of culture and molecular methods as described elsewhere (Alam et al., 2009). PCR assays were performed to screen the strains for the presence of virulence and related marker genes. All of the isolated strains harbored the *tlh* gene encoding a thermolabile hemolysin, and the virulence regulatory gene *toxR* confirming all to be *V. parahaemolyticus* (Bej et al., 1999; Kim et al., 1999). As shown in Table 1, 56 (32.0%) of the *V. parahaemolyticus* strains tested in the present study displayed pathogenic potential, being positive for the toxin genes *tdh* or *trh*. This result appears concordant with the results reported by Johnson et al. (2012), but contrary to some past studies reporting very low prevalence of these virulence genes in *V. parahaemolyticus* strains occurring in natural coastal and estuarine waters (DePaola et al., 2003; Nishibuchi and Kaper, 1995). Nonetheless, in the present study, the occurrence of *V. parahaemolyticus* strains carrying major toxigenic gene, *tdh*, varied from year to year with 15.1% (5 out of 33) in 2006, 31.2% (19 out of 61) in 2007, and 35.8% (29 out of 81) in 2008 (Fig. 1). *V. parahaemolyticus* carrying *trh* gene, encoding TDH-related hemolysin was also observed, but in a low frequency, accounting for 4.9% (3 of 61) of the strains isolated in 2007. None of the tested *V. parahaemolyticus* strains in the present study carried both *tdh* and *trh*, although both genes can occur in the same strain in relatively low frequency (~5%), as reported from studies carried out in various coastal regions of Asia, Europe, and USA (DePaola et al., 2003; Nishibuchi and Kaper, 1995; Ottaviani et al., 2013).

### 3.2. High serotypic diversity among toxigenic strains in a single geographic location

Serotyping results of the strains isolated in the estuarine waters surrounding Kuakata, Bangladesh, revealed that the *V. parahemolyticus* strains carrying virulence genes, *tdh*$^+$ or *trh*$^+$, belonged to an array of serotypes; 11 different combinations of O and K antigens were identified from among 56 toxigenic strains, including pandemic serotypes, i.e., O3:K6 and O4:K68 (Table 1). The predominant O serogroup was found to be O8 (n = 19, 33.9%), while the main K serotype was K21, which accounted for 16 (28.6%) of the toxigenic strains (Table 1). However, four (7.1%) and 21 (37.5%) of the *tdh*$^+$ *V. parahaemolyticus* strains did not react to the existing O or K antisera, respectively; while four

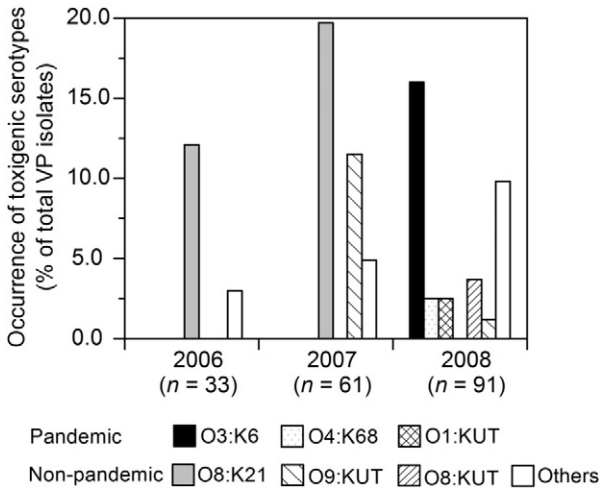


**Fig. 1.** Year-wise (2006–2008) occurrence (%) of *tdh*$^+$ *V. parahaemolyticus* (Vp) serotypes in the coastal and estuarine surface water of Bangladesh.

(7.1%) strains did not react to any of the existing O or K antisera (OUT:KUT), suggesting new serotype(s). All of the three *trh*$^+$ *V. parahemolyticus* were isolated in 2007, and belonged to the serotype O4:K46. Among the *tdh*$^+$ strains, O8:K21 was found to be the predominant serotype during 2006–2007, as reported earlier in 2005 from the estuarine aquatic environments of Bangladesh (Alam et al., 2009). In 2007, this predominant *tdh*$^+$ serotype O8:K21 (63.2%; 12 of 19) was accompanied by O9:KUT, which accounted for the remainder, 36.8% (7 of 19), of the *tdh*$^+$ strains (Fig. 1).

### 3.3. Major shift of prevalence towards the major V. parahaemolyticus pandemic serotype and its serovariants in a single year

In 2008, a major shift in the incidence of the toxigenic serotypes occurred, as the *tdh*$^+$ serotype found in the three previous years, O8:K21, was not detected among the isolated strains. Instead, the pandemic O3:K6 serotype was found to be the predominant among *tdh*$^+$ strains (45%; 13 out of 29 strains) occurring in the estuarine environment near Kuakata, Bangladesh (Table 1).This change was remarkable as the major *tdh*$^+$ serotype O3:K6 was accompanied by a *tdh*$^+$ pandemic serovariant O4:K68 that was also absent in the preceding three years at that site (Table 1) (Alam et al., 2009). Interestingly, in 2008, the major *tdh*$^+$ serotype O8:K21 was not found, but the pandemic serotype, O3:K6, and its serovariants were accompanied by a new *tdh*$^+$ serotype O8:KUT. These results suggest a major genetic reshuffling in the year 2008 of *V. parahemolyticus* population in the Kuakata estuarine region. The emergence in 2008 of the non-pandemic *tdh*$^+$ serotype O8:KUT is suggestive of sero-conversion (Bhoopong et al., 2007; Chen et al., 2011), presumably from the *tdh*$^+$ O8:K21 serotype prevalent in the preceding years, 2006–2007, as shown in 2005 (Alam et al., 2009).

*V. parahemolyticus* pandemic serotype O3:K6 was found associated with diarrhea outbreaks in India in 1996 (Okuda et al., 1997), and subsequently in Bangladesh (Bhuiyan et al., 2002) where it was isolated from natural surface water (Islam et al., 2004), suggesting aquatic reservoir for the bacterium. In the present study, O3:K6 serotype was not detected among the *V. parahemolyticus* strains isolated from the estuarine aquatic environment of Bangladesh during 2006 and 2007, as nor was it in 2005 from a previous study (Alam et al., 2009). The absence of O3:K6 among the strains tested in 2006–2007 does not rule out the possibility that the pandemic clone and its serovariants existed, but remained undetected presumably due to low number related to seasonal variation, as environmental determinants have been proposed for the occurrence and distribution of *V. parahaemolyticus* (Martinez-Urtaza et al., 2004). The other possibility is that the appearance of the pandemic serotype O3:K6 and its serovariant O4:K68, in Bangladesh was due to seroconversion of a previously established strain (Bhoopong et al., 2007; Chen et al., 2011), as reported for *V. cholerae* (Blokesch and Schoolnik, 2007; Stroeher et al., 1992). Nonetheless, the observed temporal fluctuation of major *tdh*$^+$ serotypes on an annual scale, O8:K21 and O3:K6 in the estuarine aquatic environment of Kuakata appeared in congruence with a recent study showing rise and fall of the pandemic *V. parahaemolyticus* serotypes O3:K6 in southern Chile (García et al., 2013).

### 3.4. Antigen variability and horizontal gene transfer of virulence determinants

In the present study, the pandemic *tdh*$^+$ serotype O3:K6 was found together with a wide range of *tdh*$^+$ serotypes, which included pandemic serovariant O4:K68, and non-pandemic strains such as O8:K21, O4:KUT, O5:K30, O5:KUT, O8:KUT, O9:KUT, and OUT:KUT (Table 1). The overall serotyping results suggest the O-antigen to be relatively more stable, as compared to the K-antigen of the potentially pathogenic *V. parahemolyticus* (Bhoopong et al., 2007). The observed temporal distribution patterns of *tdh* gene in at least 10 of the 75 reported *V. parahaemolyticus* serotypes suggest a high frequency of horizontal gene transfer (HGT) for this gene among the potentially toxigenic *V. parahaemolyticus* in the estuarine environments of Bangladesh (Kamruzzaman and Nishibuchi, 2008; Morita et al., 2013). This supposition is supported by recent studies showing the T3SS2, a component of the *V. parahaemolyticus* pathogenicity island (VPaI-7) harboring *tdh* gene, to be horizontally transferred not only to different *V. parahaemolyticus* serotypes but also to other *Vibrio* spp., namely *V. cholerae* and *V. mimicus* (Okada et al., 2010; Morita et al., 2013). This could be the source of the *tdh* gene identified in the O9:KUT and O5:K30 strains isolated in this study, two serotypes that have not been previously found to carry *tdh*, although *trh*$^+$ serotype O5:K30 has been reported from Japan (Fukushima, 2007).

*V. parahaemolyticus* strains carrying the major virulence markers, i.e., *tdh* or *trh*, were additionally screened for the presence of pandemic markers. Two genes typical to the pandemic group such as *orf8* and *toxRS*$^{variant}$ were present in all of the isolated strains belonging to the serotype O3:K6 and its serovariants namely O4:K68, but not in O1:KUT or O5:KUT despite being recognized as a pandemic serovariants (Table 1). Retrospective analysis of *V. parahaemolyticus* strains also revealed that the serotype O1:KUT strains previously isolated from the Bay of Bengal estuary in 2005 (Alam et al., 2009) lacked the major pandemic marker genes such as *orf8* and *toxRS*$^{variant}$. Also, none of the *tdh*$^+$ non-pandemic strains, including the isolate found to carry *trh*, harbored any of the two markers usually found in the serotype O3:K6 and its pandemic serovariants (Table 1 & Fig. 1). The occurrence in 2008 of O1:KUT and OUT:KUT (Nair et al., 2007) lacking the pandemic markers *orf8* and/or *toxRS*$^{variant}$ is intriguing. Either these strains are derived from the progenitor pandemic serotype, O3:K6 or its serovariants and lost virulence determinants or they represent local strains which acquired a novel serotype by HGT (Bhoopong et al., 2007; Chen et al., 2011).These two possibilities can be resolved by looking at the genetic relatedness of the isolates.

### 3.5. Genetic diversity of the V. parahaemolyticus population in estuarine Bangladesh

*V. parahemolyticus* strains carrying the major virulence genes such as *tdh* and *trh* were also subjected to pulsed-field gel electrophoresis (PFGE). Cluster analysis was also performed with the different PFGE patterns to understand the genetic relatedness and evolutionary trend of the toxigenic *V. parahemolyticus* strains isolated from the estuarine environments of Bangladesh. The PFGE patterns of the environmental *V. parahaemolyticus* strains were compared with the contemporary strains belonging to respective serogroups associated with diarrhea cases reporting to the hospitals in neighboring coastal region, to determine their clonal relatedness. The results revealed that the *Sfi*I-digested genomic DNA of toxigenic *V. parahemolyticus* strains yielded 12 to 19 fragments ranging from 20 kb to 350 kb (Fig. 2). A total of 37 different PFGE patterns and 19 major pulsotypes (P1 to P19) were obtained from 48 representative *V. parahemolyticus* strains tested in the present study. Results demonstrated that the naturally occurring toxigenic strains exhibited high genetic diversity in the coastal and estuarine environment of Bangladesh, which has been an established habitat for *V. parahemolyticus* (Alam et al., 2009; Islam et al., 2004), especially *V. cholerae* responsible for Asiatic cholera (Alam et al., 2006).

### 3.6. V. parahaemolyticus strains causing infections originate from local surface waters

Cluster analysis by dendrogram revealed two major clusters, separating the toxigenic non-pandemic O8:K21 serotype strains from the pandemic O3:K6 and its serovariant strains (Fig. 2). The pandemic and non-pandemic strains belonging to the two major clusters were further subdivided into multiple small sub-clusters comprising genetically closely related strains (Fig. 2). These small sub-clusters comprised heterogeneous but clonally closely related strains forming ‘clonal

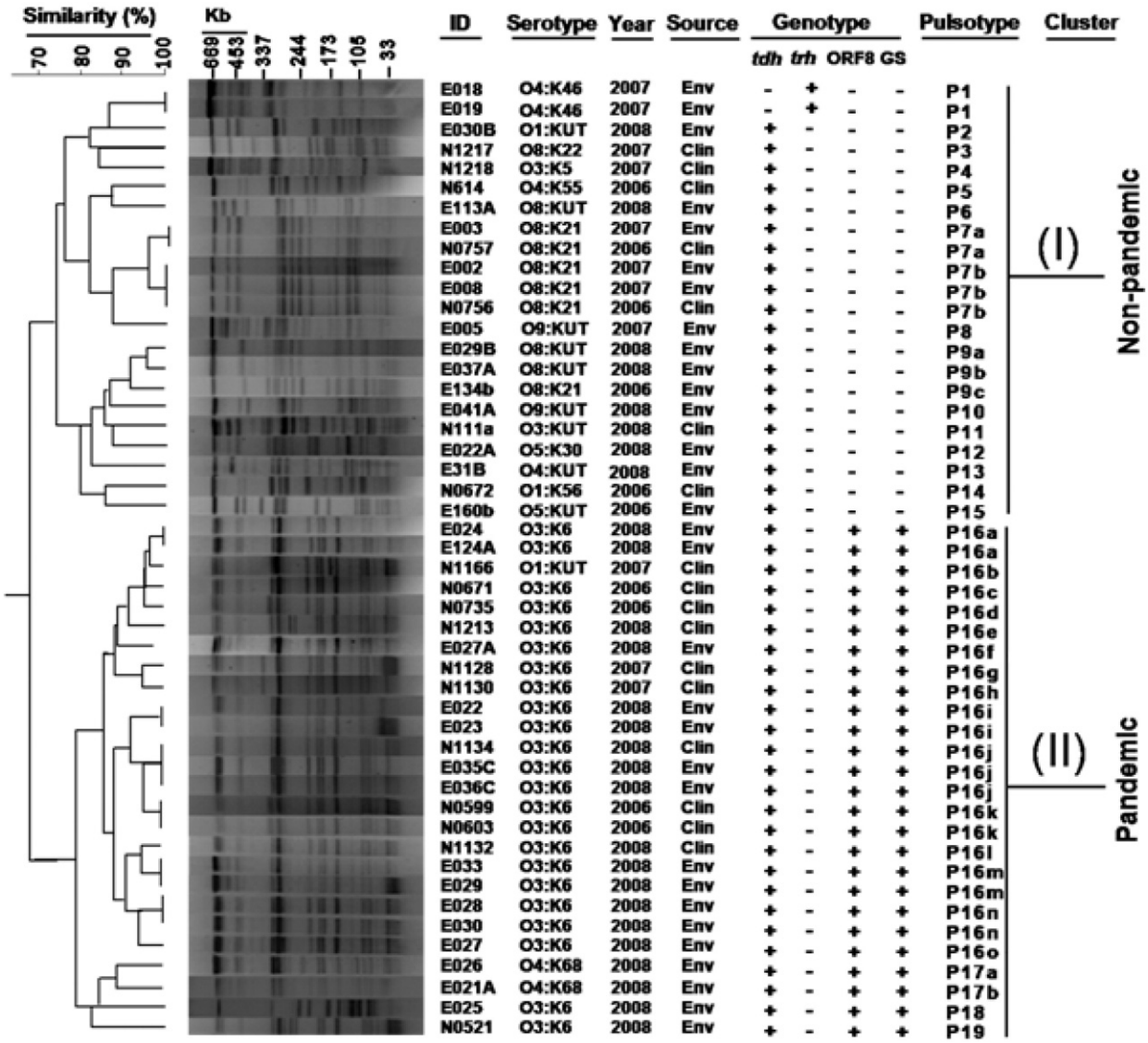


**Fig. 2.** PFGE patterns of *SfiI*-digested genomic DNA of potentially toxigenic *V. parahaemolyticus* strains isolated from the coastal ecosystem of Bangladesh. Strain identification number, serotype, year of isolation, environmental (Env) and clinical (Clin) sources and, genotypes of the *V. parahaemolyticus* strains are indicated. The dendrogram was constructed by the Bionumeric software (Applied Maths) using the Dice similarity coefficient and UPGMA of the PFGE profiles of the *V. parahaemolyticus* strains. The pandemic and non-pandemic groups were differentiated based on their genotypes and clustering patterns of the PFGE profiles of *V. parahaemolyticus* strains.

complexes' (Alam et al., 2009). Highly related PFGE patterns of the major *tdh*$^+$ *V. parahaemolyticus* serotypes (O3:K6 or O8:K21) isolated from natural surface waters, and strains belonging to the respective serotypes isolated from contemporary clinical sources indicate clonal link, suggesting surface water as the source of the *tdh*$^+$ *V. parahaemolyticus* responsible for diarrhea in Bangladesh. For example, pulsotype 16 is composed of both clinical and environmental O3:K6 strains and pulsotype 7 includes clinical and environmental O8:K21 strains (Fig. 2).

### 3.7. Seroconversion and horizontal gene transfer (HGT) both play a role in shaping the population of toxigenic strains

Strains from two pandemic serovariants clustered with O3:K6, one clinical O1:KUT (pulsotype 16b) and two O4:K68 strains (pulsotype 17) (Fig. 2). Being genetically close to O3:K6, these are likely the result of seroconversion occurring in the local environment (Chen et al., 2011). In the estuarine environment of Bangladesh, the predominant *tdh*$^+$ serotype was O8:K21 during 2006 and 2007. A major shift in the *tdh*$^+$ serotype occurred in 2008 when the O3:K6 was predominant, and O8:KUT was found to be one of the *tdh*$^+$ non-pandemic serotypes, but not O8:K21. We presume, the *tdh*$^+$ O8:KUT might have been originated from O8:K21 via seroconversion (Bhoopong et al., 2007; Chen et al., 2011). Although the O8:KUT strains found in this study generally cluster closely with O8:K21 isolates, there are two clearly distinct clusters containing both of these serotypes (pulsotypes P6-P7 and pulsotype P9) (Fig. 2), suggesting high frequency of horizontal gene transfer (HGT) within and between the naturally occurring populations, as has been proposed recently for toxigenic *V. parahemolyticus* (Theethakaew et al., 2013; Kamruzzaman and Nishibuchi, 2008; Morita et al., 2013). The observed diversity of the *tdh*$^+$ serotypes, which included the pandemic serotype O3:K6 and the pandemic serovariants, and their temporal serotypic shift portray the estuarine aquatic environment to be an important natural habitat that allows genetic recombination among pathogenic *V. parahaemolyticus* that remains a potential threat for millions of people living in the coastal villages of Bangladesh.

## 4. Conclusions

Foodborne pathogen *V. parahaemolyticus* has been a significant concern for millions of seafood lovers worldwide. In the coastal villages of Bangladesh, diarrhea is endemic (Alam et al., 2006) and diarrheagenic *V. parahaemolyticus* serotypes occur naturally in the aquatic environment (Alam et al., 2009; Islam et al., 2004). The results presented in this study, coupled with the information available in the published literature clearly indicate the coastal and estuarine environment of the Bay of Bengal to be an important natural habitat supporting the survival and evolution of potentially toxigenic $tdh^+$ population, belonging to both the pandemic and non-pandemic serotypes of the bacterium. This study provides the first in situ evidence of the temporal serotypic shift of the major $tdh^+$ *V. parahaemolyticus* associated with diarrhea in this region. The emergence of the pandemic pathogen O3:K6 and its establishment as the major $tdh^+$ serotype in 2008 was remarkable because in the immediate past years, 2006–2007, and also in 2005, the O8:K21associated with diarrheal illness was the predominant $tdh^+$ serotype occurring naturally in the coastal and estuarine environment of Bangladesh. Also, remarkable was the fact that, in 2008 the pandemic serotype O3:K6 was accompanied by $tdh^+$ pandemic and non-pandemic serovariants, namely O4:K68 and O8:KUT, respectively; but not the O8:K21, predominantly found $tdh^+$ serotype occurring, in the preceding years, 2005–2007. While these results suggest possible seroconversion as the means of the observed serotypic shift, the high genetic diversity among the toxigenic population belonging to a respective serotype is attributable presumably to extensive HGT of virulence determinants (*tdh*, *trh*) and antigen coding genes (O and K) among the naturally occurring diverse population of *V. parahaemolyticus*. The serodiversity of $tdh^+$ strains, including the two novel $tdh^+$ serotypes, O5:K30 and O9:KUT occurring in the coastal and estuarine environment of Bangladesh might suggest broad host range for the major virulence factors in *V. parahaemolyticus* belonging to both the pandemic and non-pandemic clonal complexes, which makes the clinical management of the disease and its prevention potentially challenging. This study underscores the need for routine monitoring of the potentially pathogenic *V. parahaemolyticus* evolving in the estuarine surface water serving as the source of drinking water for millions in Bangladesh where many people dies each year due to recurrent diarrhea and related infectious diseases.

## Acknowledgments

This research was funded by the Japanese National Institute of Infectious Diseases (NIID) grant. icddr,b acknowledges with gratitude the commitment of the NIID, Japan to its research efforts. We gratefully appreciate the contribution of Mr. Nurul Amin Bhuiyan for his assistance to the research. icddr,b is also grateful to the Governments of Bangladesh, Canada, Sweden and the UK for providing core/unrestricted support.

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