Isolation and Characterization of Pandemic and Nonpandemic Strains of *Vibrio parahaemolyticus* from an Outbreak of Diarrhea in North 24 Parganas, West Bengal, India

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Abstract

The enteric pathogen *Vibrio parahaemolyticus* is known to cause epidemic and pandemic diarrhea. In industrialized countries, this pathogen causes sporadic or outbreaks of diarrheal illness associated with consumption of raw or improperly cooked seafood. This report describes a foodborne outbreak of gastroenteritis caused by *V. parahaemolyticus* in June 2011 following consumption of food served in a funeral ceremony at Habra, North 24 Parganas, West Bengal, India. About 650 people attended the function, of which 44 people had acute watery diarrhea with other clinical symptoms and 35 of them were admitted to the District Hospital for the rehydration treatment. All the three tested stool specimens collected from the hospitalized cases were culturally positive for *V. parahaemolyticus*, of which two strains belonged to the serovar O4:K8 and one was identified as O3:K6. In the group-specific PCR (GS-PCR) that identifies the pandemic strains of *V. parahaemolyticus*, only the O3:K6 serovar was positive. All the *V. parahaemolyticus* strains harbored the potential virulence gene (*tdh*) that encodes a thermostable-direct hemolysin and resistant to ampicillin but susceptible to other antimicrobials. In the pulsed-field gel electrophoresis (PFGE), the *Not*I restriction pattern of the O3:K6 strain was similar to that of a recent clinical strain from Kolkata but diverged from others isolated during the previous years. On the other hand, the non-pandemic O4:K8 strains were closely related but different from a recent strain from Kolkata. (227)

Introduction

Vibrio parahaemolyticus is a halophilic Gram-negative bacterium that causes acute gastroenteritis in human and has several combinations of somatic (O) and capsular (K) antigens, collectively known as serovars. Thermostable-direct hemolysin (TDH) and/or the TDH-related hemolysin (TRH) are the important virulence factors that have been identified in *V. parahaemolyticus*. These factors are reported in mainly clinical strains and hence are useful in the differentiation of clinical and environmental stains (Nair *et al.,* 2007). *V. parahaemolyticus* is one of the most important foodborne pathogens in US, Chile, Peru, France, Sapin, Indian, China, Japan, Thailand, Korea, Laos, and Mozambique, causing several diarrheal outbreaks at different periods of time (reviewed by Nair *et al.,* 2007).

Recently, *V. parahaemolyticus* has received global attention due to the emergence of a new O3:K6 clone, which is the first documented serovar related with its pandemic spread. The rapid spread of the O3:K6 strains to different countries after its first discovery in Kolkata, India further strengthened the pandemic concept of *V. parahaemolyticus* infection (Matsumoto *et al*., 2000). Remarkably, the pandemic strain O3:K6 has seven nucleotide changes in the *toxRS* operon compared to other serovars. Based on this strain-specific *toxRS* polymorphism, a group-specific PCR (GS-PCR) was developed for the identification of the pandemic O3:K6 strains (Matsumoto *et al*., 2000). In the following years, besides O3:K6, other serovars such as O4:K68, O1:K25, O1:K41, O1:K untypeable (UT), O1:K56, O3:K75, O4:K8, O4:K12, O4:KUT, and O5:KUT having the pandemic strain genetic properties emerged and spread to many countries (Chowdhury *et al*., 2004; Laohaprertthisan *et al*., 2003; Nair *et al*., 2007).

Epidemiological studies conducted in Kolkata showed the association of *V. parahaemolyticus* O3:K6 serovar in a foodborne diarrheal outbreak in 2003 (Sen *et al*.,

2007). In this article, we describe an unusual foodborne outbreak caused by *V. parahaemolyticus* in West Bengal India on June 22, 2011 after consumption of food served in a funeral ceremony. Strains of *V. parahaemolyticus* isolated from this outbreak were phenotypically characterized for serovars, antimicrobial susceptibility along with detection of virulence genes and clonality.

Materials and Methods

Background of the Outbreak: In June 21, 2011 about 650 peoples attended a funeral ceremony at Iswarigacha, Habra, North 24 Parganas, West Bengal. A total of 44 people developed typical symptoms of food poisoning including watery diarrhea with or without abdominal pain and vomiting after consumption of some non-vegetarian dishes prepared by the caterer. Some were admitted in the District Hospital and rests were treated at home. An investigation coordinated by the West Bengal State Department of Health detected an index case on 22.06.11 at 4.00 a.m. Out of 44 cases, 35 were admitted in hospital rest were treated as outpatients. In the sex ratio analysis, females were most affected (68.2%) than the male patients. Three rectal swabs and water samples were collected and sent to the laboratory on June 23, 2011. Water samples collected from the tap of the venue but the leftover food materials and served water could not be collected. The outbreak was declared over on 26.06.11.

Stool culture: The non-bloody diarrheal samples were collected as rectal swabs and transported to the laboratory in the Cary-Blair medium. The rectal swabs were processed for common enteric pathogens such as pathogenic vibrios, salmonellae, shigellae, campylobacters and diarrheagenic *Escherichia coli* using enrichment and selective plating media (WHO, 1987). After enrichment of the rectal swabs in the in alkaline peptone water (pH 8.6) for 6 hrs at 37°C, the cultures were plated on the thiosulfate citrate bile salt sucrose (TCBS) agar (Eiken, Tokyo, Japan). Sucrose-non-

fermenting green colonies suspected as *V. parahaemolyticus* on TCBS agar were subsequently tested for fermentation reactions in triple-sugar-iron agar, hemolysin, urease and oxidase positively. Confirmation of the isolates as *V. parahaemolyticus* was done by standard methods (WHO, 1987).

Serotyping: Serotyping of *V. parahaemolyticus* was done using a commercially available antisera test kit (Denka Seiken, Tokyo, Japan) that has 11 O and 71 K antisera. Following manufacturer's instructions, an aliquot of the cell suspension in normal saline from the Luria agar culture was boiled for 2 hrs and used for O serotyping. The un-boiled cell suspension was used in the detection of capsular (K) antigen.

Antimicrobial susceptibility testing*:* Antimicrobial susceptibility testing was performed using disk diffusion method with commercially available discs such as ampicillin (10 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), furazolidone (100µg), norfloxacin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracycline (30 µg) and erythromycin (15 µg) (Becton Dickinson, Sparks Glencoe, MD, USA) in accordance with the criteria recommended by Clinical and Laboratory Standards Institute (CLSI, 2011). Sine there is no interpretive criterion for *V. parahaemolyticus* in the CLSI guidelines, breakpoints for *Enterobacteriaceae* was adopted. *Escherichia coli* ATCC 25922 was used as a quality control strain.

PCR assays: PCR assays were performed to detect the *toxR*, *tdh*, and *trh* genes by using the primers described previously (Chowdhury *et al*., 2004). GS-PCR was performed to detect the pandemic strain of *V. parahaemolyticus* (Chowdhury *et al*., 2004). Template DNA was prepared with the growth of the test strains in Luria broth (Difco) supplemented with 3% NaCl. The bacterial cells were washed and resuspended the cell

pellets in sterile distilled water. The cells were lysed and the DNA was denatured after boing the cell suspension for 10 min. Soups of this cell lysate were used as templates in the PCR assays. The PCR amplicons were electrophoresed in a 1% agarose gel stained in ethidium bromide and visualized under UV light using a gel documentation system (Geldoc 2000, BioRad, Hercules, CA).

Pulsed-field gel electrophoresis: PFGE was performed according to the PulseNet protocol (Parsons *et al*., 2007) with slight modification (Kam *et al.,* 2008). DNA digestion with restriction endonuclease *Not*I (25 U/plug; Fermentas, Germany) was performed at 37°C overnight. The digested DNA of *Salmonella* Braenderup strain H9812 with *Xba*I was used as a molecular weight marker. The restriction fragments were resolved on a CHEF II Mapper system (Bio-Rad) in 0.5X TBE with 300 μM thiourea and the following running conditions: 6 V/cm for 18 h at 14°C, with 2-40 sec switch time and pump speed of 0.7 l/min. Gels were stained in ethidium bromide (25 min), destained in distilled water for 45 min and photographed under UV light. The PFGE patterns were analyzed using the BioNumerics version 4.0 software (Applied Maths, Sint Martens Latem, Belgium) after normalization of the TIFF images with *Salmonella enterica* serotype Braenderup size standard. Clustering was performed using the unweighted pair group method (UPGMA) and the Dice correlation coefficient with a position tolerance of 1.5%. The PFGE profiles of O3:K6, O4:K8 from the previous collection were made for clonal comparison.

Results

Of the 650 people attended the funeral ceremony 44 had severe diarrhea after consumption of food served after the function. Thirty five were admitted to the District

Hospital at North 24 Parganas. The index case was a 22-year-female with severe dehydration admitted on June 22, 2011 at 4:00 AM. The outbreak peaked during the morning of June 22, 2011. All the admitted cases were treated with IVF or ORF depends on the severity and oral norfloxacin was given in divided doses. There was no casualty in this outbreak.

Rectal swabs collected from three patients were sent to the laboratory for screening common enteric pathogens. Microbiological analysis of the 3 samples collected during the investigation revealed that all the samples were positive for *V. parahaemolyticus* and no other enteric pathogen was detected. Patients positive for *V. parahaemolyticus* were adult males with age between 20 and 40 years. The *V. parahaemolyticus* isolates exhibited hemolytic activity on sheep blood agar but none was urease positive.

The O and K antigens of the *V. parahaemolyticus* strains determined by slide agglutination showed that one was serovar O3:K6 and the other 2 belonged to O4:K8 (Table 1). PCR results for the identification of the pandemic strain and the virulence genes are shown in Table 1. The serovar O3:K6 was positive in the GS-PCR confirming its similarity with the pandemic strains but the serovar O4:K8 strains were negative in this PCR. All the three *V. parahaemolyticus* strains harbored *tdh* but not the *trh* gene (Table 1). In the antibiotic susceptibility assay, all the strains were resistant to ampicillin and streptomycin but susceptible for tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, norfloxacin, and nalidixic acid.

To confirm the clonal relationship, *V. parahaemolyticus* O3:K6 (NICED 459) and O4:K8 strains (NICED 458 and NICED 459) isolated in this outbreak, 4 strains of

O3:K6 from Kolkata outbreak cases in 2003 (SC 188, SC 189, SC 192, SC193) and two strains of O3:K6 and O4:K8 from diarrheal patients from the Infectious Diseases Hospital (IDH), Kolkata during in 2011 were included in the PFGE. In the dendrogram analysis, the recent outbreak O3:K6 *V. parahaemolyticus* showed identical profile with an IDH O3:K6 strain but differed with the 2003 outbreak strains (Fig. 1). The current O4:K8 strains on the other hand were closely related but distinct with IDH O4:K8 isolates (Fig. 2).

Discussion

The pandemic serovars of *V. parahaemolyticus* has emerged and became a persistent pathogen associated with diarrhea in many countries. In Kolkata, even though the isolation rate of *V. parahaemolyticus* is low (~3%) among the hospitalized diarrheal patients, the pandemic strains continued to persist with frequent changes in the serovars (unpublished data). The pandemic serovar O3:K6 has emerged in Kolkata in 1996 (Matsumoto *et al*., 2000) and has caused an outbreak in Kolkata during 2003 (Sen *et al.,* 2007). Systematic surveillance conducted in USA suggests that the burden of diarrhea is increasing due to infection caused by the vibrios, mainly by *V. parahaemolyticus* (Newton *et al.,* 2012). In addition, several other investigations carried out all over the world indicate the association of consumption contaminated seafood with the pandemic strains of *V. parahaemolyticus* (reviewed by Nair *et al*. 2007).

In the present investigation, we have identified two types of *V. parahaemolyticus,* one is the pandemic O3:K6 serovar that is positive in the GS-PCR assay and the other is the non-pandemic O4:K8 serovar. During 2009, these two serovars were associated with diarrheal outbreaks in Guangdong, China (Ke *et al.,* 2011). We found that both the

serovars harbored the *tdh* virulence gene but not the *trh* gene. Though *V. parahaemolyticus* is an environmental organism, presence of *tdh* gene makes them virulent and hence detection of this marker gene is important (Meador *et al.,* 2007). Based on the GS-PCR results and DNA fingerprinting, the serovar O4:K8 was designated as a pandemic strain along with O3:K6 in 1999 (Chowdhury *et al.,* 2000). Interestingly, in this study, the two O4:K8 strains were negative in the GS-PCR indicating the recent changes at the molecular level.

Based on the surveillance of enteric pathogens conducted at the IDH, the serovars O3:K6 and O4:K8 harboring *tdh* is identified in the present outbreak is not new in this region. The PFGE profile of an O3:K6 strain isolated in from a diarrheal patient highly matched with a similar phenotypic strain isolated from a diarrheal case from the IDH during 2011. This strain similarity indicates that a circulating pandemic O3:K6 strains has involved in the recent outbreak. However, the pandemic *V. parahaemolyticus* O3:K6 serovar that has caused an outbreak in 2003 were different with the 2011 strains. On the other hand, O4:K8 strains isolated in the current outbreak and the IDH strain isolated in 2011 were clonally different.

V. parahaemolyticus strains are generally susceptible to commonly used antimicrobials in the treatment of diarrhea. We observed that except for ampicillin all the tested strains were susceptible to all the antimicrobials. The patients administrated with the fluoroquinolone responded to the treatment confirming the laboratory susceptibility results. Since the food samples were not microbiologically screened, we are not sure about the source of the *V. parahaemolyticus* encountered among diarrheal patients. There is a high possibility of secondary contamination as local culinary practice may

not allow the pathogens to survive. Possibility in the involvement of food-handlers cannot be ruled out since one of the early investigations conducted in this region has shown that the carrier status of *V. parahaemolyticus* is 21.4% (Sircar et al. 1979).

In summary, this finding has detected an association of *V. parahaemolyticus* O3:K6 and O4:K8 serovars with an acute diarrheal outbreak. Since leftover foods were not available, the suspected pathogen could not be screened for tangible evidence. However, circumstantial evidence suggests a link with consumption of contaminated food with this pathogen. Considering the recurrent foodborne outbreak in this region, the public health authorities ought to give due importance to the pandemic strains of *V. parahaemolyticus.*

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Disclosure Statement

No competing financial interest exist

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Table 1. Serotypes and genetic traits of *V. parahaemolyticus* strains isolated from diarrheal outbreak in Habra, North 24 Parganas

Strain	O: K Serotype	Genetic marker				Antibiogram
		Gene			Pandemic marker	
		toxR	*tdh*	*trh*	GS-PCR	
NICED 459	O3:K6	+	+	-	+	AM, S
NICED 458	O4:K8	+	+	-	-	AM, S
NICED 460	O4:K8	+	+	-	-	AM, S

Abbreviation: AM, ampicillin; S, streptomycin

Figure legends

Fig.1. *Not*I digested chromosomal DNA banding patterns of pandemic *V. parahaemolyticus* (O3:K6) strains. Strain NICED 459 was isolated during the outbreak 2011 while IDH 03525 was the recent *V. parahaemolyticus* isolated from Kolkata in 2011. Strains SC188, 189, 192, and 193 were isolated during the outbreak in Kolkata, 2003.

Fig. 2. *Not*I digested chromosomal DNA banding patterns of pathogeniv *V. parahaemolyticus* (O4:K8) strains. Strain NICED 458 & NICED 460 was isolated during the outbreak 2011 while IDH 03062 was the recent *V. parahaemolyticus* isolated from Kolkata in 2011.

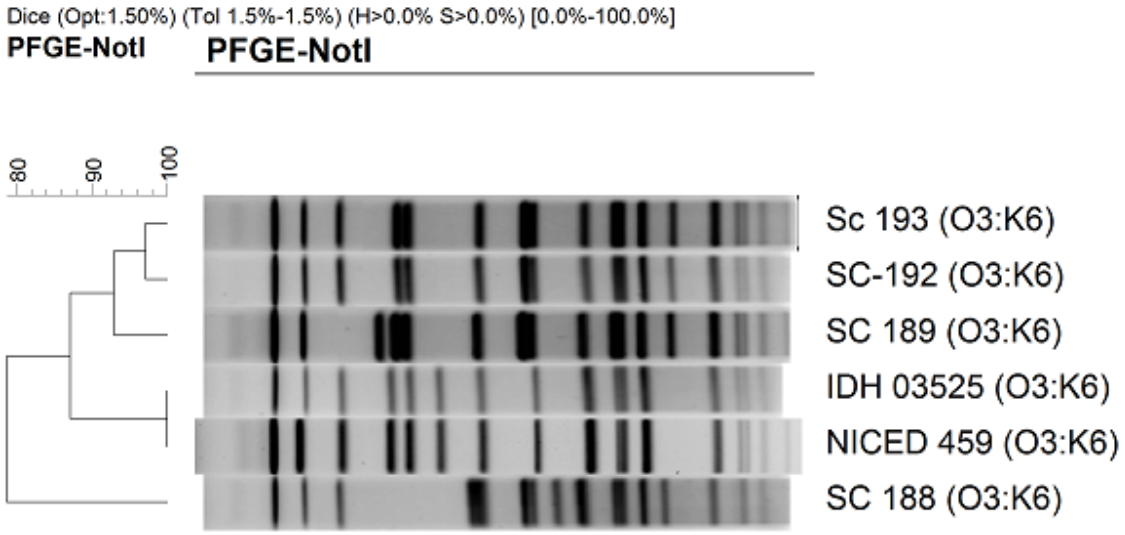
Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE-NotI
PFGE-NotI
80
90
100
Sc 193 (O3:K6)
SC-192 (O3:K6)
SC 189 (O3:K6)
IDH 03525 (O3:K6)
NICED 459 (O3:K6)
SC 188 (O3:K6)

Fig. 1

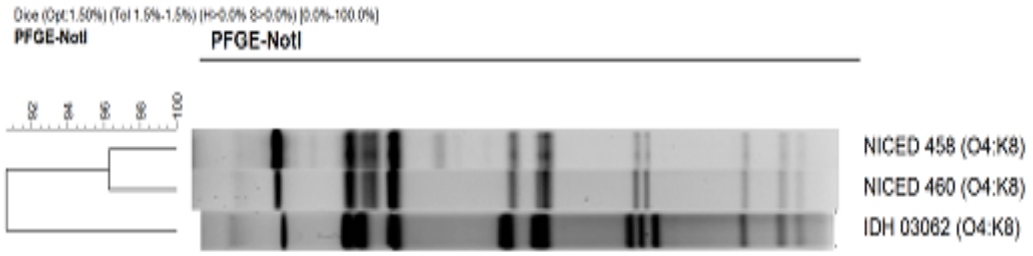
Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%)
PFGE-NotI
PFGE-NotI
92
94
96
98
100
NICED 458 (O4:K8)
NICED 460 (O4:K8)
IDH 03062 (O4:K8)

Fig. 2