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RESEARCH ARTICLE

VIBRIO PARAHAEMOLYTICUS IN WEST BENGAL, SEVERAL KILOMETRES AWAY FROM THE SEA

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ABSTRACT

We report a food-borne outbreak of diarrhoeal illness caused by Vibrio parahaemolyticus associated with consumption of Hindu Puja offerings, a completely vegetarian food. In the month of August 2014, stool samples from five hospitalised patients, was sent to us at Calcutta School Of Tropical Medicine, Kolkata in Cary-Blair transport media from CMOH office of District Hospital, Nandigram, Paschim Midnapore, West Bengal for Bacteriological examination. All the five cases were hospitalised with chief complaint of acute onset of pain abdomen, loose motion and vomiting, associated with fever, developing 24 hours after consumption of Puja offerings. Among 5 samples, 4 were positive for Vibrio parahemolyticus. All the 4 samples showed Kanagawa phenomenon in Wagatsuma agar. Of the 4 positive samples, 3 strains were 04:K8 serovar and 1 was O10:K60. An exhaustive study on serotyping Vibrio parahaemolyticus in the article, "A pulsed-field gel electrophoresis typing scheme for Vibrio parahaemolyticus isolates from fifteen countries" by Hin-chung Wong and his co-authors mention many serotypes but O10:K60 has not been found by them. Hence this is a new serotype causing diarrhoea. All strains were PCR positive for tdh but were negative for trh gene.

INTRODUCTION

Pathogenic strains of Vibrio parahaemolyticus, harbouring the thermostable hemolysin (TDH) encoded by tdh gene is known to cause diarrhoea outbreaks. Though consumption of raw or undercooked shellfish is the most common means of acquiring V. parahaemolyticus infection, ingestion of contaminated water is also a source of diarrhoeal outbreak (Patricia Tille 2014). The specimen arrived from Nandigram Midnapore of West Bengal which is surprisingly, situated several kilometres away from the sea. If pond water is to be blamed, epidemiologists must spread the awareness of such type of contamination. The 5 specimens were inoculated in Nutrient agar, 5% Sheep blood agar, Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar, CLED and Mac Conkey agar and incubated at 35^0C in ambient air for 24 hours. All the 4 samples have same cultural characteristics, biochemical pattern and antibiogram pattern. 1 sample out of the 5 was reported to have no growth of any organism.

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MATERIALS AND METHODS

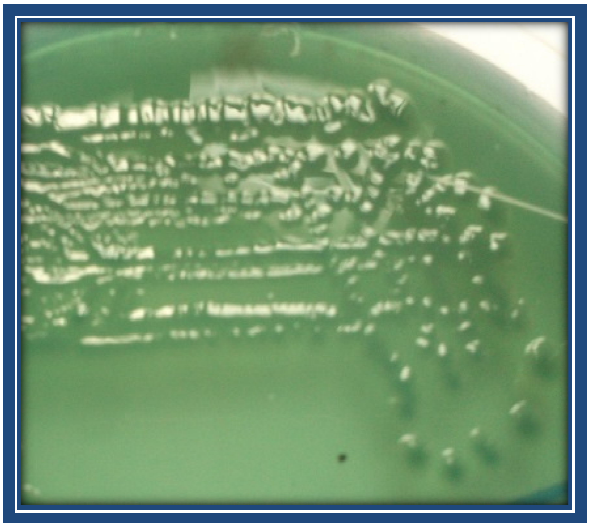


Fig 1. Colony in TCBS Agar

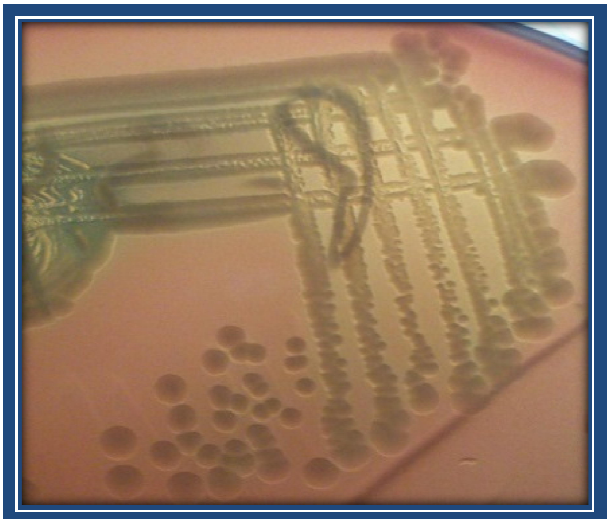


Fig 2. Hemolytic colony in Blood Agar

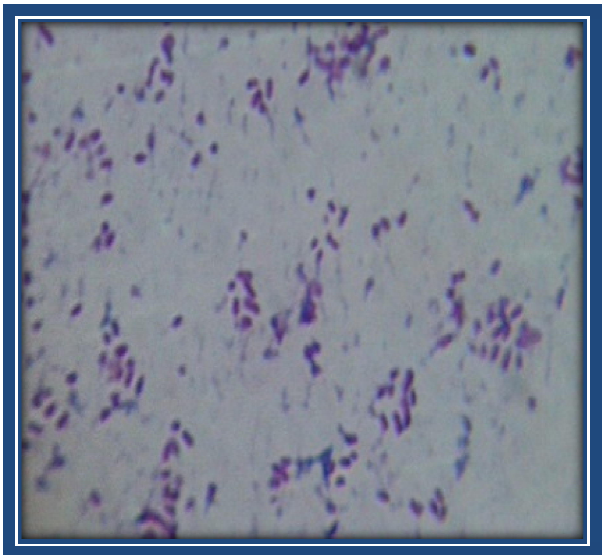


Fig 3. Gram Stain

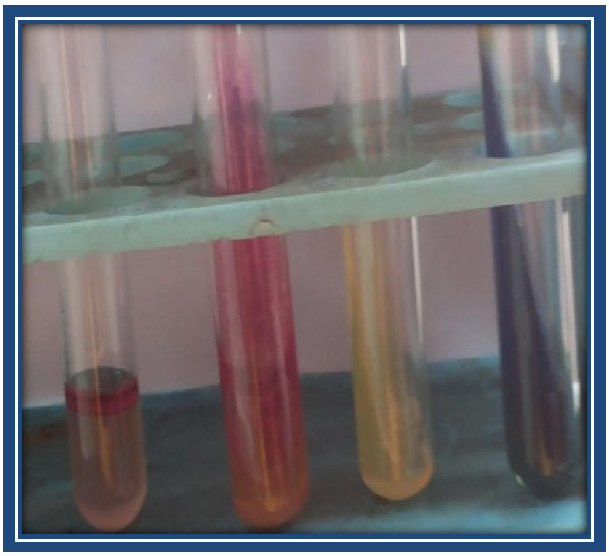
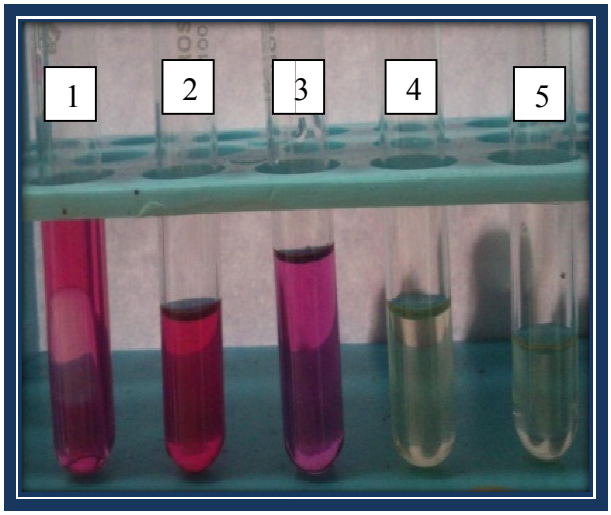
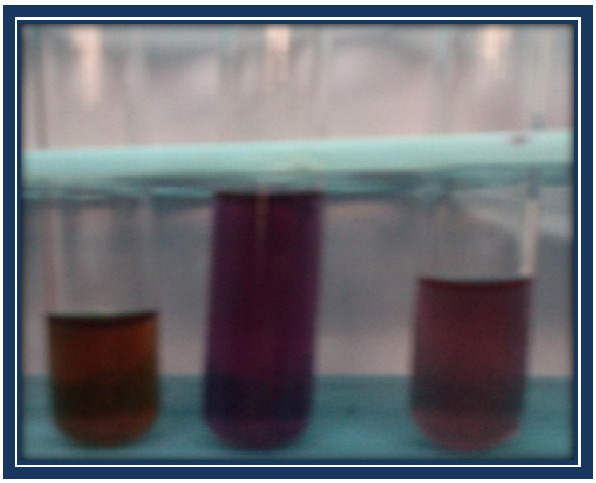


Fig 4. Indole: +ve TSI: K/A without gas
Urease: -ve Citrate: -ve



1. Glucose – F without gas 2. Arabinose – F 3. Mannitol – F
4. Lactose – NF 5. Sucrose – NF

Fig 5. Sugar Fermentation -> 'F' - sugar fermented; 'NF'– sugar not fermented



1. Arginine- negative 2. Lysine – positive 3. Ornithine – positive

Fig 6. Amino acid decarboxylation

Fig 7. Antibiogram pattern in Mueller Hinton Agar

Table 1.

Culture	Nutrient agar – small circular transluscent colonies. 5% sheep blood agar – 2-3 mm size haemolytic colony Mac Conkey agar- pale, Non-lactose fermenting colonies TCBS agar - 2-5 mm in diameter, green colour colonies CLED medium – no growth
Gram stain	Short, curved Gram negative bacilli, with rounded ends .Some were typically comma shaped.
Motility (hanging drop preparation)	Darting motility present
Biochemical reactions (from nutrient agar)	Oxidase – positive Catalase – positive Nitrate reduction test – positive Indole – positive Triple sugar iron media – acid /alkali without gas Urease – negative Citrate – negative Arginine- negative Lysine – positive Ornithine – positive Growth in 3%, 6% & 8% NaCl salt concentrations – growth present 0% & 10% NaCl salt concentrations – no growth Glucose, Arabinose, Mannitol – Fermentation positive Lactose , Sucrose – no fermentation
Kanagawa phenomenon in wagatsuma agar	Present
Antibiotic sensitivity by kirby-bauer disk diffusion method	Sensitive to Amikacin(30), Tetracycline(5), Ciprofloxacin(5), Chloramphenicol(30), Ceftriaxone(30), Cotrimoxazole(25), Azithromycin(15) Resistant to Amoxycillin-clavulinic acid and Ampicillin
Serotyping	3 Strains are O4:K8 1 Strain is O10:K60

Table 2.

Biochemical test	Result	Conclusion
Catalase	Positive	Excludes Enterococcus spp
Oxidase	Positive	Excludes Proteus spp
Ornithine decarboxylase	Positive	Excludes P.damselae and Grimontia hollisae
Growth in 8% Nacl	Positive	Excludes Aeromonas & Vibrio mimicus
Lactose fermentation & Onpg test	Negative	Excludes Vibrio vulnificus
Polymixin b	Sensitive	Further excludes Vibrio vulnificus
Kanagawa phenomenon	Positive	Confirms Vibrio parahemolyticus

Green colony in TCBS are produced by

- Vibrio parahaemolyticus
- Vibrio mimicus
- Vibrio vulnificus
- Proteus spp
- Aeromonas spp
- Photobacterium damselae
- Grimontia hollisae and
- Enterococci spp

DISCUSSION

Vibrio parahaemolyticus is a gram-negative halophilic bacterium that is a leading cause of seafood-borne gastroenteritis. It is one of the most important causes of acute gastroenteritis in Asia. Since 1996, one serotype in particular, V. parahaemolyticus O3:K6 (and its clonal derivatives O4:K68, O1:K25, and O1: KUT), has received increasing notoriety, as it is the first documented V. parahaemolyticus serotype to cause pandemic disease. However at present, serotype-based detection and surveillance efforts have been complicated by serotype transition and variation within the pandemic lineage, as additional serotypes (O1:K26, O6:K18 O1:K41, O4:K12, O1:K56, O3:K75, O4:K8, O4:KUT, and O5:KUT) from specific locales have now been identified as having been derived from the original pathogenic O3:K6 clone (Carolyn E. Meador *et al.,* 2007). It was originally isolated in 1951 in Japan as the causative agent of an outbreak of food poisoning caused by sea fish. Since then it has been identified in several countries as an important cause of acute gastroenteritis. It inhabits the coastal areas, where it is found in fish, arthropods such as shrimps and crabs and concentrated by filter feeders such as oysters. In Kolkata, it has been found in small pond fish (Ananthanarayan and Paniker 2013**)**. Vibrios can exist in the environment in several forms, from planktonic form to spherical ultramicrocell form and Viable But Non-Culturable form (VBNC). It is still not clear whether ultramicrocell and VBNC form are related. Planktonic form is most easily studied and they occur in water column as free-living individual cells. This form is seen during adequate nutrition and favourable environmental conditions. When nutrients become limited, the Vibrios lose their flagella and

take on a small round morphology called spherical ultramicrocell. Cytoplasmic granules disappear and the nuclear region of the cell becomes condensed. When environmental temperature falls, they are primarily found in sediments or attached to surfaces such as shellfish or copepods and may require the production of specific enzymes or lectins for such attachment. Cells may enter VBNC during starvation and/or cold when cells are physiologically active but will not grow under routine laboratory conditions (Judith A Johnson ?**)**. Acanthamoeba castellanii has been noted to support the survival of Vibrio parahemolyticus but this protozoan does not internalize the pathogen and apparently secretes a factor that supports a dormant phase (Laskowski-Arce and Orth 2008). Some strains of Vibrio parahaemolyticus, Vibrio cholerae, and non-01 V cholera produce a bacterial-cell-associated, heat-stable material that is cytotoxic for hela cells. Cytotoxicity is completely neutralised by antibody to purified Shigella dysenteriae 1 (Shiga) toxin but not by antibody to purified cholera toxin (Alison D. O'Brien *et al.,* 1984). Most Vibrios produce a single polar flagellum with a sheath that allow the bacteria to move through liquids and respond to food sources through chemotaxis and are involved in biofilm formation. Vibrio parahaemolyticus produces monotrichous flagella and 60 genes that are mostly contained within two regions, are required for the expression of the flagella. Flagella are required for full virulence of both V. cholera and V.parahaemolyticus. On solid or highly viscous media, some Vibrios produce elongated swarmer cells that produce both polar and peritrichous flagella. The large numbers of peritrichous flagella are not sheathed, have a different wavelength than the polar flagella, and allow the organism to swarm across the surface of the agar. V.parahaemolyticus shows this swarming behaviour and the lateral flagella are encoded by a separate region of the chromosome from the polar flagella and controlled by the scr ABC genes (Judith A Johnson ?).

Though further work is required to determine the role of pilli in virulence, it has been found that non-epidemic strains of V. cholerae, V. parahaemolyticus, and V. vulnificus also have pili that may be involved in colonization. In V. parahaemolyticus, the LPS O-side chain is very short and is termed as lipooligosaccharide (LOS). LOS is the major determinant of serogroup and an important virulence factor in gram negative bacteria. V. parahaemolyticus has 13 O antigens and more than 60 K antigens V. parahaemolyticus produces a capsule but its role in virulence is not known. V.parahaemolyticus has two circular chromosomes; it contains 4832 genes, one of which may have started as a megaplasmid, but both now contain essential genes. Comparison of the V parahaemolyticus genome with that of V. cholerae showed many rearrangements within and between the two chromosomes. Genes for the type III secretion system (TTSS) were identified in the genome of V parahaemolyticus; V cholerae does not have these genes (Kozo Makino *et al.,* 2003). It is not known whether all Vibrios share this unique genetic organization. Plasmids are common in Vibrios, but their functions are largely unknown. Recently, a number of transmissible integrons encoding antibiotic-resistance genes have been found on plasmids or on the chromosome of Vibrios. There are over 180 tailed and at least ten filamentous phage, both lytic and lysogenic, that infect Vibrios. These can be used for phage typing schemes and may be responsible for horizontal gene transfer. General transducing phages have also been described for V. parahaemolyticus. The role of this phage in the ecology and virulence of Vibrios remains to be determined. However, all of these recent developments in the genetics of Vibrios suggest that horizontal gene transfer and recombination are relatively frequent events and the genome of Vibrios is plastic and continually evolving. It is likely that we will see the emergence of new pathogenic clades of V.cholerae and other Vibrios in the future.

The TTSS is a central virulence factor of diarrhoea-causing bacteria such as shigella, salmonella, and enteropathogenic Escherichia coli, which cause gastroenteritis by invading or intimately interacting with intestinal epithelial cells. It is suggested that V parahaemolyticus and V.cholerae use distinct mechanisms to establish infection. This finding explains clinical features of V parahaemolyticus infections, which commonly include inflammatory diarrhoea and in some cases systemic manifestations such as septicaemia, distinct from those of V. cholerae infections, which are generally associated with non-inflammatory diarrhoea. V.parahaemolyticus-mediated disease has traditionally been thought to be associated with two virulence factors, the thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH). Strains producing TDH are responsible for the β-type hemolysis on Wagatsuma agar that is known as the Kanagawa phenomenon (KP), most clinical strains are KP+, while environmental strains tend to be KP−. However, strains carrying either the tdh or the trh gene (or both genes) are considered virulent strains (Shirai *et al.,* 1990). In addition to these two virulence factors, recent analysis of the genome sequence of V.parahaemolyticus strain RIMD2210633 (KP+, serotype O3:K6, pandemic group member) suggests that another virulence factor, the type III secretion system (T3SS), may also play a role in the disease manifestation of V. parahaemolyticus infections (Makino *et al.,*2003). Unlike the genomes of other Vibrio species, which appear to contain one set of T3SS genes, that of the sequenced V. parahaemolyticus O3:K6 pandemic group strain contains two sets of T3SS genes. The first set of T3SS genes is found on chromosome 1 (T3SS1), is similar in genetic structure and organization to the Yersinia sp. T3SS, and appears to be present in all V. parahaemolyticus isolates tested, regardless of origin. The second set of T3SS genes is found on chromosome 2 (T3SS2) embedded within a ~80-kb pathogenicity island that harbors two copies of the tdh gene. T3SS2 does not appear to be similar to any bacterial T3SS other than the V. cholerae non-O1/non-O139 T3SS (Dziejman *et al.,* 2005) and is reported to be found only in KP+ isolates (Park *et al.,* 2004). Importantly, both T3SS1 and T3SS2 in V. parahaemolyticus appear to be functional.

As virulence factors for infection, this bacterium produces pore-forming toxins known as thermostable direct hemolysins (TDHs) and has two sets of T3SS: namely T3SS1 and T3SS-2 (Makino *et al.,* 2003). Histopathological study of the acute stage of infection with V. parahaemolyticus in humans has shown inflammatory responses with PMN infiltration, oedema of the lamina propria and hemorrhage. Also, the secretions of TNF-α and IL-1β, two pro-inflammatory cytokines were markedly induced (Qadri *et al.,* 2003). These results suggested

that infection with V. parahaemolyticus induces inflammatory responses in the intestinal mucosa. However, how host cells recognize infection with V. parahaemolyticus and regulate the inflammatory responses remain largely unknown. Since pore-forming toxins or virulence-associated secretion systems in bacterial infection mediate caspase-1 activation (Toma *et al.,* 2010; McCoy *et al.,* 2010, a, b), the possibility that V. parahaemolyticus might induce caspase-1 activation is considered. It was demonstrated that TDHs trigger NLRP3 inflammasome activation and that both the NLRP3 and NLRC4 inflammasomes are triggered in response to the T3SS1 in infected macrophages. It was further mentioned that two T3SS1-secreted effector proteins, VopQ and VopS, induce autophagy and Cdc42 inactivation, respectively; these processes consequently interfere mainly with NLRC4 inflammasome activation. Recognition of the activities of pore-forming toxins and the T3SS1 contribute to the host proinflammatory responses, and define the effector proteins that dampen the host responses by interfering with inflammasome activation. Preventing inflammasome activation by the T3SS1 effectors appears to be one strategy for bacterial evasion of the host proinflammatory responses. Consumption of raw or undercooked shellfish is the most common means of acquiring V. parahaemolyticus infection. Also consumption of contaminated water is a source of infection. Person-to-person transmission has not been documented, suggesting that the infective dose for normal persons is relatively high. Rarely, it has been cultured from asymptomatic people, and no carrier state has been identified. There is no known mammalian reservoir of infection. Gastroenteritis is the most common clinical illness; wound infections and septicaemia may be seen, but much less frequently (Hlady and Klontz 1996; Thomas *et al.,* 2007).

Enteric illness caused by V. parahaemolyticus comprises a broad spectrum of clinical manifestations ranging from mild watery diarrhoea to a frank, dysentery-like syndrome. Enteric illness commonly begins with the acute onset of explosive watery diarrhoea generally within 24 to 72 hours of ingestion of the contaminated seafood. Often, the diarrhoea is accompanied by mild to moderately severe cramping and abdominal pain associated with a low-grade fever, mild chills, and headache in less than half of cases. The fluid loss is rarely severe enough to cause decreased skin turgor or postural hypotension. Deaths caused by V.parahaemolyticus are rare, usually occurring in very young children, older adults, due to loss of sufficient quantities of fluid or persons with underlying disease. No treatment is required by most patients because the gastroenteritis is usually self-limited. However, antimicrobial therapy could be considered for those patients with diarrhoea lasting longer than 5 days (Morris 2003). Therapy with a tetracycline or quinolone would be expected to shorten the clinical course and duration of pathogen excretion. Antiperistaltic agents are of no clear-cut benefit. Oral rehydration or intravenous electrolyte therapy may be required for patients in extremes of age.

Conclusion

Vibrio parahaemolyticus infection is not uncommon at all. Even in Kolkata, it is found in pond fish, but how the contamination occurred in a Hindu puja offering is otherwise difficult to explain if we do not take into consideration the use of contaminated water. Second important fact which needs to be mentioned is the finding of serotype O10:K60. This particular serotype has not been implicated as a virulent serotype as yet. But according to Topley and Wilson, the recent development of genetics of Vibrios suggests that their genome is extremely plastic and continuously evolving. It is likely that we will see emergence of new pathogenic clades of different Vibrios in the future and probably the future is now.

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