

11. *Vibrio Parahaemolyticus*: The Threat of Another *Vibrio* Acquiring Pandemic Potential

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Introduction

Members of the genus *Vibrio* are autochthonous bacterial flora in the aquatic ecosystem and quite a few of them are associated with infections in humans and aquatic animals. Human infection follows either direct contact with aquatic environment or indirectly via contaminated food and water. Food-borne *Vibrio* infections tend to occur more frequently in developed countries while transmission of *Vibrio* infections in developing countries is, by and large, water-borne. Further, the magnitude of food-borne infections in developing countries is still incompletely understood for the want of robust scientific information.

Among *V. cholerae*, the toxigenic serogroups O1 and O139 are the etiologic serogroups that cause cholera, which are responsible for epidemics and pandemics mainly due to poor water supply and personal hygiene. The other important and most common seafood-borne halophilic *Vibrio* is *V. parahaemolyticus*. Since its discovery in 1953 (Fujino et al., 1953), many aspects on this pathogen were explored and established through extensive research. However, there is a burgeoning problem of food-borne illness throughout the globe. The relation between strains isolated from the environment and, those isolated from seafood, and human clinical isolates are poorly understood. Studies conducted in many countries have shown that *V. parahaemolyticus* is common flora in many coastal areas, fresh water bodies, plankton and in fishes.

Serotyping system of *V. parahaemolyticus* is based on somatic (O) and capsular (K) antigens that include 13 different O antigens and 71 different K antigens (Iguchi et al., 1995). Unlike *V. cholerae*, infection caused by *V. parahaemolyticus* has no relation with socioeconomic status, meteorological changes, quality of the water supply and general sanitation.

Early works used phenotypic assays to identify virulent strains of *V. parahaemolyticus*, but this has been replaced by more specific and less time consuming methods such as PCR (Tada et al., 1992; Bej et al., 1999) and DNA probe assays (McCarthy et al., 2000; Wong et al., 2000a). Recently, these and other genotypic assays have been employed to

environmental monitoring and seafood surveys (Elliot et al., 1998, Cook et al., 2002a). Through these molecular techniques, it is easy to identify the genetic variations and changes in the prevalence patterns of *V. parahaemolyticus*.

Until 1996, *V. parahaemolyticus* infections were associated with sporadic cases of gastroenteritis caused by multiple, diverse serovars. Since 1996, however, an increased incidence of gastroenteritis in many parts of Asia and United States has been associated with *V. parahaemolyticus* serovar O3:K6 (Okuda et al., 1997a; Bag et al., 1999; Chiou et al., 2000; Matsumoto et al., 2000). Furthermore, other serovars (O4:K68, O1:K25 and O1:K untypable [O1:KUT] etc.) which have been shown to be virtually identical to the O3:K6 serovar by a variety of molecular typing methods (Chowdhury et al., 2000a; Bhuiyan et al., 2002) and which have been postulated to be clonal derivatives of the O3:K6 strain.

Origin and spread of pandemic strains

In contrast to *V. cholerae*, infections of *V. parahaemolyticus* were usually not associated with exclusive serovars in the past. Historically, *V. parahaemolyticus* infections have been associated with sporadic cases caused by diverse serovars. In one report it was shown that the fatality rate of 44% for cases with septicemia due to *V. parahaemolyticus* (Hlady and Klontz, 1996). Beginning in February 1996, a new clone of *V. parahaemolyticus* belonging to the O3:K6 serovar was responsible for a dramatic increase in numbers of cases in Calcutta, India (Okuda et al., 1997a; Bag et al., 1999). During 1996, this serovar carrying *tdh* accounted for 50-80% of *V. parahaemolyticus* infections in Calcutta.

However, since 1996, the serovar O3:K6 has been associated with several large-scale outbreaks of illness, suggesting the emergence of a "new" clone with enhanced virulence and enhanced capacity to spread. The global spread of pandemic strain of *V. parahaemolyticus* O3:K6 is shown in Fig.1. An increase in the incidence of *V. parahaemolyticus* during 1997-98 was also ascribed to the increased

incidence of food poisoning caused by the O3:K6 clone in Japan (WHO, 1999). Many investigators demonstrated evidence supporting the hypothesis that the O3:K6 clone has recently emerged and has pandemic potential. Since 1998, *V. parahaemolyticus* strains belonging to other serovars such as O4:K68, O1:K25, O1:KUT (untypable), have also been isolated with increasing frequency from diarrhoeal patients in as many as 12 countries (WHO, 1999; Chowdhury et al., 2000a; Matsumoto et al., 2000; Bhuiyan et al., 2002). Pandemic strains belonging to serovars O1:K41, O1:K56, O3:K75, O4:K8, O4:K12, O4:KUT, and O5:KUT were also reported from a diarrhoea outbreak in Vietnam (Chowdhury et al., 2004b). Analysis of the strains at the molecular level indicated that they might have diverged from the pandemic O3:K6 strains by alterations of the O:K antigens (Chowdhury et al., 2000b, Matsumoto et al., 2000; Chowdhury et al., 2004b).

The O4 and K68 combination did not previously exist in the serotyping scheme of *V. parahaemolyticus*. The genesis of new serovars indicates that there is constant selection pressure on the organism to evolve since new antigenic determinants are likely to evade the host immune system better than those that already exist in the population.

Clinical manifestations

In infected individuals, *V. parahaemolyticus* can cause chills, fever, nausea, vomiting, watery to bloody diarrhoea, abdominal cramps, and in rare instances, death (Hughes et al., 1978; Blake et al., 1980). Survey conducted in Vietnam showed that 53% of the patients presented with watery stools and 6% reported blood in the stools (Tuyet et al., 2002). *V. parahaemolyticus* rarely causes septicaemia and fulminant necrotising fasciitis (Lim and Stebbings, 1999). Generally, soft tissue infections often progress to fatal septicemia. Most of the septicemia patients are immuno suppressed especially with leukemia and cirrhosis (Ng et al., 1999). For this reason, patients with immuno suppressed conditions or adrenal insufficiency should avoid raw seafood. *V. parahaemolyticus* elicits a strong immune response so that repeated episodes of gastroenteritis in the same individual are unlikely (Tuyet et al., 2002). A comparative study conducted in Vietnam showed that there is no difference in the expression of virulence between pandemic and non-pandemic strains of *V. parahaemolyticus* isolated from the diarrhoea patients as evidenced from the clinical symptoms (Chowdhury et al., 2004b).

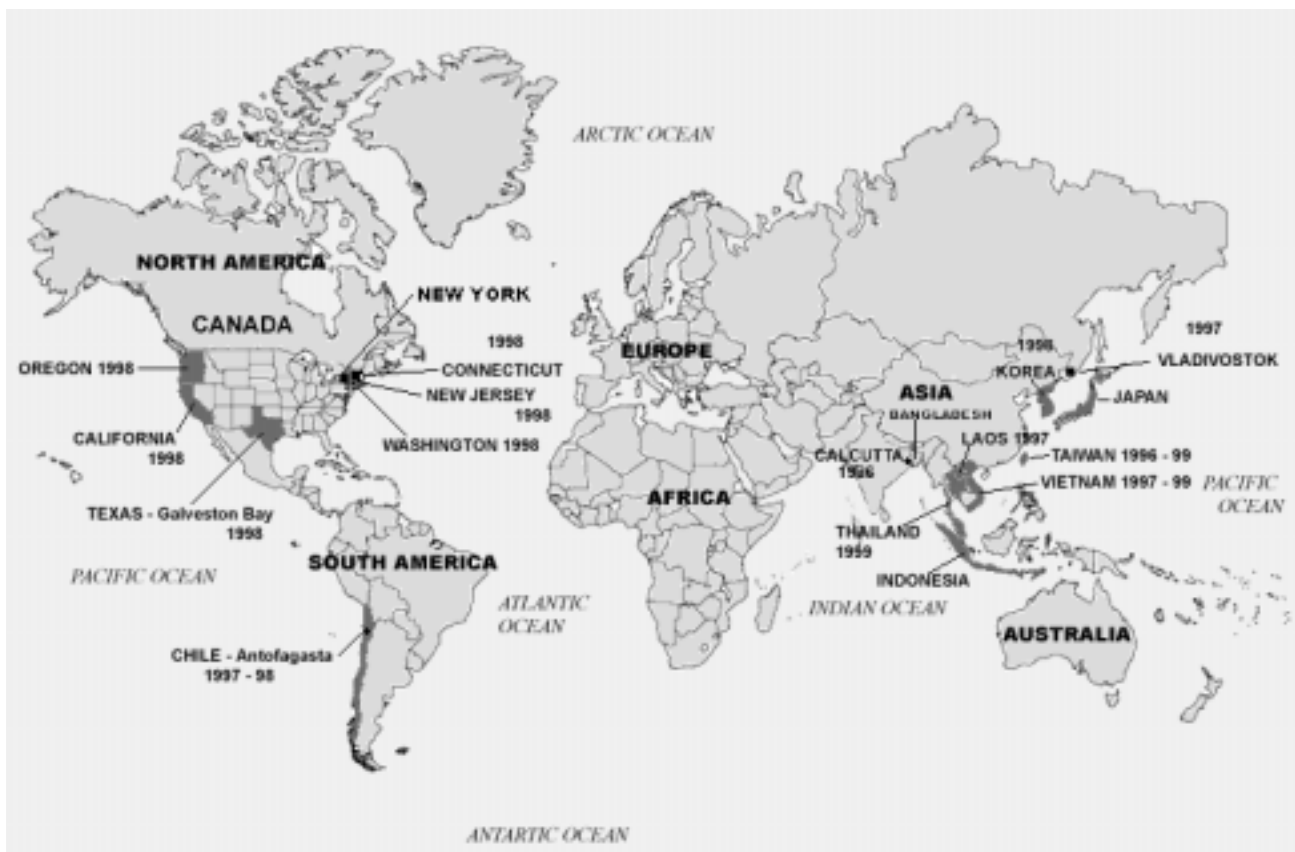


Fig1: The global spread of pandemic strain of *V. parahaemolyticus* O3:K6

Incidence in the environments

Studies conducted in Calcutta showed that *V. parahaemolyticus* could be detected in freshwater environments in association with fish and plankton (Sarkar et al., 1985). Generally, the virulence genes carrying strains of *V. parahaemolyticus* are less frequent in the environment. Incidence of virulent strains of *V. parahaemolyticus* was reported to be high (41.4 and 8.5% for *tdh* and *trh*, respectively) in Seto Inland Sea, Japan (Alam et al., 2003).

A food-borne pathogen

V. parahaemolyticus is a well-known seafood-borne pathogen or considered as zoonotic pathogen as molluscan and shrimps act as reservoirs of this pathogen (Cook et al., 2000b; DePaola et al., 2000; Daniels et al., 2001; Lozano-Leon et al., 2003). It was shown that *V. parahaemolyticus* could survive and proliferate in oysters in various environmental conditions (Gooch et al., 2002). A case control study conducted in Guam showed that the cases are more likely than controls to have eaten seafood (Haddock and Cabanero, 1994).

In Japan, a recent study showed that the incidence of *tdh* positive *V. parahaemolyticus* tend to be high in samples contaminated with relatively high levels of *V. parahaemolyticus*. Most of the *tdh*-positive strains belonged to the pandemic O3:K6 clone (Hara-Kudo, 2003). *V. parahaemolyticus* grows relatively fast in unrefrigerated oysters (Gooch et al., 2002). The pandemic strains of *V. parahaemolyticus* were proven to be more process resistant (50°C for about 3 min) than the non-pandemic environmental strains (Andrews et al., 2003).

Incidence/outbreaks

During 1973 to 1998, 40 outbreaks of *V. parahaemolyticus* infections were reported to the CDC and these outbreaks included more than 1000 illnesses (Daniels et al., 2000). Most of the outbreaks occurred during the warmer months and were attributed to seafood, particularly shellfishes. The median attack rate among the persons who consumed the implicated seafood was 56% (Daniels et al., 2000). In USA, the first outbreak due to the pandemic strain *V. parahaemolyticus* O3:K6 occurred between May 31 and July 10, 1998 and 416 persons in 13 states had gastroenteritis after the consumption of oysters harvested from Galveston Bay; all 28 stool specimens were culture positive for the pandemic strain (Daniels et al., 2001). In July-August 1998, large outbreaks of *V. parahaemolyticus* infection was reported from California, Oregon, Washington and

Texas (MMWR, 1999; DePaola et al., 2003). In subsequent months (July-September), the *V. parahaemolyticus* infection spread to Connecticut, New Jersey, and New York (MMWR, 1999). However, this serovar was not detected in any of the environmental and food isolates (DePaola et al., 2003). Like the spread of the epidemic strain of *V. cholerae* O1 in 1991 to coastal waters along the Gulf of Mexico, it is possible that the pandemic *V. parahaemolyticus* O3:K6 strain was introduced to U. S. coastal waters by ballast water discharged from ships, which had traveled to Asia (CDC, 1999; McCarthy et al., 2000).

Several outbreaks were also reported from Canada (Fyfe et al., 1997) and Spain (Lozano-Leon et al., 2003). In the latter, it was due to consumption of oysters contaminated with non-pandemic strains of *V. parahaemolyticus* (Martinez-Urtaza et al., 2004). The Canadian outbreak in British Columbia during 1997 might have been due to exceptionally warm-ocean surface water temperatures and heavy rainfall associated with El Nino conditions that appear to be conducive to the growth of *V. parahaemolyticus* (Fyfe et al., 1997). Between November 1997 and April 1998, several human gastroenteritis cases were reported in Antofagasta, Chile (Cordova et al., 2002). This outbreak was associated with the consumption of shellfish, and the etiologic agent *V. parahaemolyticus* was isolated both from the affected patients and the shellfish samples. However, in this investigation, tests were not used to determine whether the *V. parahaemolyticus* strains belonged to the pandemic type. In Russia, food poisoning outbreak due to this *Vibrio* was reported in the city of Vladivostok (Smolikova et al., 2001). In Taiwan, *V. parahaemolyticus* was responsible for 61 to 71% of the food-borne outbreaks during 1996 to 1999. Since 1996, the O3:K6 strains have caused numerous outbreaks in Taiwan, accounting for 51, 79, 61 and 65% of the outbreaks in 1996, 97, 98 and the first half of 1999, respectively (Wong et al., 2000b). The incidence of O3:K6 increased from 51% in 1996 to 83.8% in 1997 and prevailed during 1999 and accounted for 61.3% of all *V. parahaemolyticus* infections (Chiou et al., 2000). The emergence and dominance of pandemic strains of *V. parahaemolyticus* was also recorded in Korea, Laos, and Indonesia (Matsumoto et al., 2000).

Emergence of other serovars resembling the pandemic O3:K6 strain of *V. parahaemolyticus* has been recorded in many Asian countries. During 1999, the isolation rate of pandemic *V. parahaemolyticus* in southern Thailand increased to 76% and majority of the strains were O3:K6. The newer serovars such as O1:K25, O1:K41 and O4:K12 were also reported to share all the traits of the pandemic clone except for

their serovars (Laohaprerthisan et al., 2003). Studies conducted in Vietnam showed that the risk factor for *V. parahaemolyticus* related to high socioeconomic status of patients as this group could afford seafood (Tuyet et al., 2002). In contrast to *V. cholerae* infections, which tends to occur seasonally, no clear seasonality of the *V. parahaemolyticus* infections were observed in Vietnam. In India, the first outbreak of *V. parahaemolyticus* was reported from Vellore (Lalitha et al., 1983) due to non-pandemic strains. The pandemic strains first emerged in India and were associated with cases of diarrhoea admitted to the Infectious Diseases hospital in Calcutta from February 1996 (Okuda et al., 1997a)

Supportive factors

Recent studies have shown that elaboration of higher amounts of vibrio ferrin by clinical isolates of *V. parahaemolyticus* are associated with a selective advantage for survival and proliferation under conditions of iron-limitation such as in the intestine (Yamamoto et al., 1994). *V. parahaemolyticus* exhibits significant adaptive acid tolerance and heat-shock response, and the envelope proteins induced by stress are suggested to be associated with virulence (Wong and Chen, 2003). When the O3:K6 strains of *V. parahaemolyticus* were tested with different environmental stress such as temperature of 50°C, pH 4, exposure to 0.2% NaCl, the *D* values (time required for 90% reduction in the count of viable cells) showed no appreciable difference between strains isolated before and after 1996 (Wong et al., 2000b).

Under low temperature and nutrient content, *V. parahaemolyticus* enters viable but non-cultivable state. The true resuscitation of non-culturable cells of *V. parahaemolyticus* occurred after spreading them onto agar medium supplemented with H₂O₂-degrading compounds such as catalase or sodium pyruvate (Mizunoe et al., 2000).

Virulence

V. cholerae and *V. parahaemolyticus* have distinct mechanisms to establish infection. *V. cholerae* mediated infections are generally associated with non-inflammatory diarrhoea. The clinical features of *V. parahaemolyticus* infection include inflammatory diarrhoea at the advanced stage of infection. The major inflammatory response due to *V. parahaemolyticus* infection at the onset of illness includes edema in lamina propria, congestion in blood vessels and hemorrhage in the duodenum and rectum (Qadri et al., 2003). In vitro adherence and cytotoxicity studies with human epithelial cells showed that O3:K6

strains exhibited higher levels of adherence and cytotoxicity to host cells than non-O3:K6 strains suggesting the higher pathogenic potential of pandemic clones. The enhanced adherence and cytotoxicity may contribute to the unique pathogenic potential of *V. parahaemolyticus* O3:K6 strains (Yeung et al., 2002).

The thermostable-direct haemolysin (TDH) is the best-studied virulence factor of *V. parahaemolyticus*. TDH causes intestinal fluid secretion as well as cytotoxicity in a variety of cell lines (Raimondi et al., 2000). The *tdh* gene encodes the production of TDH (Nishibuchi and Kaper, 1995; McCarthy et al., 2000). The Tdh of *V. parahaemolyticus* is related (71.5%) to a newly described haemolysin (VC delta TH) of *V. cholerae* O1, which is encoded by the *dth* gene (Fallarino et al., 2002). The *tdh* gene of *V. parahaemolyticus* is flanked by nucleotide sequences identical to the terminal inverted repeat sequences of *IS102* in the chromosome (Nishibuchi and Kaper, 1990). Low content of the G+C (30%) of *tdh* compared to the average G+C content of the *V. parahaemolyticus* chromosome suggests that this locus might be transferred by a mobile genetic element. The amount of TDH produced did not significantly differ between pandemic O3:K6 and non-pandemic O3:K6 strains (Okuda et al., 1997a).

Another virulence factor, the TDH-related haemolysin (TRH), encoded by the gene *trh*, has been discovered in clinical strains lacking *tdh* (Honda et al., 1987, 1988). The *trh* shares 69% identity with *tdh2* and the sequence difference were distributed throughout the gene (Nishibuchi et al., 1989). Most of the environmental strains possessing *trh* have been reported in Asia (Wong et al., 2000b). *V. parahaemolyticus* harbouring *trh* nearly always produces urease, which is atypical for other *V. parahaemolyticus* (Okuda et al., 1997b; Osawa et al., 1996; Suthienkul et al., 1995), and the genes may be part of a pathogenicity island (Park et al., 2002). Neither urease nor *trh* has been shown to enhance the virulence in *V. parahaemolyticus* strains possessing *tdh* gene. The association between urease-production and possession of the *trh* gene is due to a genetic linkage between the structural gene for urease (*ureC*) and *trh* on the chromosome of *V. parahaemolyticus* (Iida et al., 1997). The urease activity is known to increase gastric acid tolerance in *Yersinia enterocolitica*, (DeKoning-Ward and Robins-Browne, 1995) and *Helicobacter pylori* (Turbett et al., 1992).

The capsular polysaccharide (CPS) of *V. parahaemolyticus* is reported to play an important role in the adherence of the pathogen to epithelial cells as evidenced by comparing less CPS producing strains with opaque colonies of the same strain (Hsieh

et al., 2003). In addition, treatment of *V. parahaemolyticus* with anti CPS but not anti-LPS serum decreases the level of bacterial adherence (Hsieh et al., 2003). These results indicate that CPS is one of the factors in bacterial adherence to its target cells.

Antibody-secreting cell components are seen in patients infected with *V. parahaemolyticus*. At the onset of diarrhoea, the levels of C-reactive protein and nitric oxide metabolites increased in systemic circulation (Qadri et al., 2003). The major inflammatory response due to *V. parahaemolyticus* infection at the onset of illness includes edema in lamina propria, congestion in blood vessels and hemorrhage in the duodenum and rectum (Qadri et al., 2003). A monomeric serine protease of *V. parahaemolyticus* was found to exert toxic effect on different cell lines and induced tissue haemorrhage and death in experimental mice (Lee et al., 2002). Unlike *V. cholerae*, in *V. parahaemolyticus* genes responsible for type III secretion systems (TTSS) were identified (Makino et al., 2003). The TTSS is a central virulence factor for many enteric pathogens.

Susceptibility to antibiotics

The antibiogram analysis is a useful typing method for some pathogens (Rudolph et al., 1998). In Calcutta, strains of *V. cholerae* isolated after the epidemic of serogroup O139 revealed an expanding antibiotic resistance to a variety of drugs compared to the O1 serogroup isolated before the advent of the O139 serogroup (Mukhopadhyay et al., 1995). It is surprising to note that in the same geographical area, both the pandemic and non-pandemic strains of *V. parahaemolyticus* strains did not exhibited multidrug resistance. Similarly, in Taiwan, the sensitivity pattern of the recently isolated *V. parahaemolyticus* O3:K6 and the strains isolated before 1996 showed no difference (Wong et al., 2000a).

Molecular techniques for the detection of *V. parahaemolyticus*

PCR technique has become a routine method in clinical and food diagnostic laboratories due to its specificity, sensitivity and due to the ability to bring about a rapid detection. Comparison between microbiological testing using conventional culture and PCR shows that molecular techniques carry the potential for time saving and are amenable to automated handling of large number of samples. PCR has widely been used as a diagnostic tool in clinical laboratories for more than a decade but its application to the food industry is just beginning.

Early detection of pandemic strains of *V. parahaemolyticus* will help to increase seafood safety and decrease the risk of infectious outbreaks caused by this pathogen. The timely resolution of seafood-associated outbreaks requires prompt and accurate detection of pathogenic *V. parahaemolyticus*. Conventional detection of vibrios to species level is difficult and relies mainly on biochemical and NaCl tolerance tests. Many PCR assays have been recently formulated for the identification of *V. parahaemolyticus* with higher specificity and sensitivity (Venkateswaran et al., 1998; Kim et al., 1999; Robert-Pillot et al., 2002). As both pathogenic and non-pathogenic strains of *V. parahaemolyticus* exists in seafood, application of PCR specific for the virulence genes (*tdh* and *trh*) will help in detection of pathogenic strains of *V. parahaemolyticus* which will consequently reduce the risk of food-borne illness (Dileep et al., 2003). In seafood such as oysters, squid, mackerel and yellowtail, unknown substances inhibited PCR targeting the *tdh* gene. A DNA purification step with the use of silica membrane or glass fiber method increased the detection sensitivity (Hara-Kudo et al., 2003).

The *orf8* PCR was found to be highly sensitive and specific in detecting the pandemic *V. parahaemolyticus* O3:K6 strains (Myers et al., 2003). PCR assay targeting the thermolabile-haemolysin (*th*) was reported to be useful for detecting *V. parahaemolyticus* (Bej et al., 1999). The R72H DNA sequence, composed of a non-coding region and a phosphatidylserine synthetase gene is highly conserved in *V. parahaemolyticus* and can be used for species-specific detection by PCR (Lee et al., 1995; Robert-Pillot et al., 2002).

For the detection of pandemic strains of *V. parahaemolyticus*, two PCR systems are widely in use. The group-specific PCR targets unique variations in the *toxRS* sequence of pandemic strains (Matsumoto et al., 2000) while the *orf8* PCR is useful for the identification of integrated filamentous phage having a unique open reading frame ORF8 (Nasu et al., 2000; Myers et al., 2003). To simplify the assay system, a multiplex PCR was designed to distinguish pandemic serogroup strains from other *V. parahaemolyticus* strains (Okura et al., 2003). However, in many recent findings it was shown that the ORF8 was absent in considerable number of pandemic strains of *V. parahaemolyticus* (Bhuiyan et al., 2003; Laohaprerthisan et al., 2003; Chowdhury et al., 2004b).

Khan et al. (2002) have reported that all the O3:K6 pandemic strains of *V. parahaemolyticus* isolated from the Texas and New York outbreaks yielded an 850-bp DNA fragment along with other amplicons in the

enterobacterial repetitive intergenic consensus (ERIC) PCR. The primers designed from this 850-bp gene sequence specifically identified in O3:K6 strains with 327-bp amplicon, whose function and homology were not reported in the Genbank. However, this PCR was not validated with other pandemic and non-pandemic strains of *V. parahaemolyticus*. A recently developed real-time PCR for the detection of *tdh*, a stable marker of pathogenicity in *V. parahaemolyticus* was found to be rapid compared to more resource intensive conventional culture techniques (Blackstone et al., 2003). Based on the 23S rRNA gene sequence, a species-specific probe was used for the identification of *V. parahaemolyticus* (Sparagano et al., 2002).

Molecular typing

Interspecies characterization of pathogenic bacteria for epidemiological purpose can now be effectively achieved by molecular typing methods ERIC-PCR, restriction fragment length polymorphism (RFLP) in rRNA genes (ribotyping), pulsed-field gel electrophoresis (PFGE) and RFLP analysis of the genetic locus encoding the polar flagellum (Fla locus) or the virulence genes (*tdh/trh*) have been used to type *V. parahaemolyticus* strains.

RFLP of the *tdh* gene was shown to be a less discriminating method in the molecular typing of pathogenic *V. parahaemolyticus* strains (Bag et al., 1999), since, all the tested strains have two copies of *tdh* as reported earlier (Nishibuchi and Kaper, 1995). Ribotyping determines the number of *rrn* operons in the bacterial genome. For ribotyping, *V. parahaemolyticus* genomic DNA is fragmented either with *Hind*III or *Bgl*I digestion and hybridization with cDNA probe of *E. coli* 16S and 23S RNA genes (Wong et al., 1999; Bag et al., 1999). *rrn*-mediated recombination is one of the mechanisms by which pathogenic bacteria maintain their genomic plasticity and the possibility of genome rearrangements is greater when there are more *rrn* operons in the genome of any organism (Krawiec and Riley, 1990).

In *V. cholerae*, *Bgl*I restriction enzyme was successfully used to develop a ribotyping scheme (Koblavi et al., 1990). Studies conducted in Calcutta showed that the pandemic O3:K6 strains belonged to 5 different ribotypes with R4 being the most common with strains isolated during 1996-97 (Bag et al., 1999). This study also indicated that the strains isolated during brief interim period of time showed genetic instability, but over a period of time the clone stabilized as evidenced by the constant prevalence of R4 ribotype. The non-pandemic strains, including 'old O3:K6' strains used in this study exhibited heterogeneous ribotype profiles that were distinct from each other, as well as from the R4 pattern

exhibited by the pandemic strains. Thus, the new O3:K6 did not seem to have originated from the 'old O3:K6' group of strains. Ribotyping results of northeastern US *V. parahaemolyticus* outbreak strains showed similarity with the Asian clone, but different from Texas outbreak (Gendel et al., 2001). Combination of serotyping and ribotyping showed that the Pacific Coast *V. parahaemolyticus* strains appeared to be distinct from that of either the Atlantic coast or Gulf coast (DePaola et al., 2003).

The *Ceu*I digestion of chromosomal DNA followed by PFGE is useful for the determination of intra- and interspecies genomic rearrangements and elucidation of the numbers of *rrn* operons in the genomes of prokaryotes. Since the *Ceu*I cleaves the DNA only in a 19-bp sequence in the 23 rRNA gene of the *rrn* operon (Liu and Sanderson, 1995), it has also been successfully used to determine the number of *rrn* operons in the genomes of many organisms including *V. cholerae*.

The method of PFGE for *V. parahaemolyticus* was first published in 1996 (Kubota, 1999). The *Sfi*I digested chromosomal DNA banding patterns were similar for serovars O3:K6 and O4:K68 isolated from seawater and patients in Japan (Kubota, 1999). Large number of *V. parahaemolyticus* strains isolated from different outbreaks in Taiwan showed at least 14 PFGE types after *Sfi*I digestion (Wong et al., 1996). After *Nci*I digestion, strains of O3:K6 isolated between June and August 1996 in Calcutta showed different pulsotypes compared to the pulsotypes of strains isolated before and after this period (Bag et al., 1999). The same study showed that the strains isolated during the later part exhibited identical or nearly similar pulsotypes, indicating the genome stability of the tested strains. In the PFGE, *V. parahaemolyticus* strains might show DNA degradation (Marshall et al., 1999). Wong et al. (2000b) observed that the strains of O3:K6 which appeared after 1996 formed eight closely related PFGE patterns, I1 being the predominant one (81%) consisting of strains from Taiwan, Korea, Japan, and India.

Multilocus sequence typing (MLST) has been used for differentiation of many pathogens (Kotestishvili et al., 2002, 2003) and to detect the role of recombination during bacterial evolution (Feil et al., 2000). Sequence analysis of amplified *gyrB*, *recA*, *dnaE* and *gnd* of different serovars of *V. parahaemolyticus* showed that pre-pandemic strains were highly variable and the pandemic O3:K6 isolates shared two alleles (Chowdhury et al., 2004a). The pandemic clones, defined by a positive GS-PCR for *toxRS* and the presence of ORF8 from the f237 phage, were genetically nearly identical at the sequence loci, in contrast to the four distinct serovars, O3:K6, O4:K68, O1:KUT, and O1:K25 (Chowdhury

et al., 2000b). These results reconfirm the results of molecular typing methods that showed multiple serovars occur in a single genetic lineage (Chowdhury et al., 2000b; Matsumoto et al., 2000; Nasu et al., 2000). Serovars of the pandemic strains may be selected in response to host immunological pressure.

Faecal pollution indicators and *V. Parahaemolyticus*

Bacteriological monitoring at oyster harvest sites in USA did not prevent O3:K6 outbreaks even though the formulated standards met current bacteriologic standards during harvest and fecal coliform counts in water samples were within acceptable limits. This suggests that current policy and regulations regarding the safety of raw oysters require reevaluation (Daniels et al., 2001).

Phylogenetic approach

Since early 1970s, there has been an avalanche of changes, and new insights into the phylogeny of prokaryotic species. The corner stone of natural classification above species level was the recognition that all the traits of an excellent phylogenetic marker are present in ribosomal (r)RNA and the genes encoding for rRNA. The most powerful and most extensively used phylogenetic marker molecule is the 16S rRNA and the genes coding for this molecule (rDNA). Amplified rRNA genes are sequenced directly by chain termination method or by automated DNA sequencer. Comparison of the 16S rDNA sequence with a database such as ribosomal database project (RDP) and ARBOR (ARB) (Stackebrandt, 2000), containing more than 12,000 sequences from more than 90% of all described genera provides an advantage unmatched by any other rapid screening method. In the 16S rRNA sequence analysis, *V. parahaemolyticus* is phylogenetically close to *V. cholerae*. However, *V. parahaemolyticus* genome showed many rearrangements within and between the two chromosomes (Makino et al., 2003).

Genomics and proteomics

Genetic analysis of *V. parahaemolyticus* showed that there is high genetic diversity in the species (Wong et al., 2000 a, b). Most of the marine vibrios are now shown to have two chromosomes (Yamaichi et al., 1999; Tagomori et al., 2002). Although the sizes of the large chromosomes of *V. parahaemolyticus* and *V. cholerae* are similar, the sizes of the small chromosomes are different. Phylogenetically, *V. parahaemolyticus*, is close to *V. cholerae*. Most of the open-reading frames present on the large and

small chromosomes of *V. cholerae* and *V. parahaemolyticus* are homologous, but the order and arrangements are different (Tagomori et al., 2002; Makino et al., 2003). Such differences imply frequent intrachromosomal genetic exchanges. A strong correlation between the presence of *trh* gene and urease production, which is an unusual trait for *V. parahaemolyticus*, has been observed (Magalhaes et al., 1992; Suthienkul et al., 1995). However, the urease gene cluster was not involved in the regulation of *tdh* and *trh* expression (Nakaguchi et al., 2003). The genetic uniformity of the pandemic strains of *V. parahaemolyticus* is quite opposite to the genetic diversity seen among the recently emerged *V. cholerae* O139 (Garg et al., 2003; Ramamurthy et al., 2003). The genetic diversity in toxigenic *V. cholerae* might contribute to their ability to cause recurrent epidemics, in particular, in cholera endemic areas. In the pandemic groups of *V. parahaemolyticus*, the gene diversity is less but the genes responsible for somatic (O) and capsular (K) antigenic biosynthesis appear to be dynamic.

Proteomics-based grouping method on the basis of their protein profiles and identification of unique protein has recently established to discriminate pandemic and non-pandemic strains of *V. parahaemolyticus* (Williams et al., 2004). In the pandemic strains, a histone-like DNA binding protein, HU- α has a C-terminal amino acid sequence different from those of non-pandemic strains of *V. parahaemolyticus*. It was reported that the gene encoding HU- α has a 16-kb insert in the 3' terminus of the ORF for this protein (Williams et al., 2004). This insertion sequence and its corresponding modification of the HU- α protein sequence are unique to the pandemic strains belonging to O3:K6, O4:K68, O1:K25, and O1:KUT serovars. The effect of this insertion sequence on the HU- α protein activity and its role in the pathogenesis are still unknown.

Filamentous phages

Bacteriophages are one of the mobile genetic elements that horizontally transfer genetic information to various bacterial species. These phages can convert a non-pathogenic strain to a pathogenic form (Bishai and Murphy, 1988). A filamentous phage, f237, is specifically and exclusively associated with pandemic strains of serovar O3:K6 (Nasu et al., 2000). Subsequently, in other serovars also ORF8 was identified (Iida et al., 2001). Phage f237 possesses 10 open reading frames (ORFs), including a unique ORF8 (Nasu et al., 2000). This phage, f237, has several genes in common with and a similar genomic structure as that of CTX phage (Waldor and Mekalanos, 1996), which is known to carry the genes for cholera

enterotoxin (*ctxAB*), the most important virulence factor of toxigenic strains of *V. cholerae*. Instead of *ctxAB*, f237 has *orf8*, but its role of virulence in *V. parahaemolyticus* is not established. In addition, ORF8 encodes a protein with similar combination of motifs to a *Drosophila* adhesive protein encoded by *plx* that has an adhesive function (Zhang et al., 1996). Probably, this mechanism might help the pandemic strains adhering to host intestine cells or to the surface of marine organisms and account for the high potency of infection. Prevalence of f237 phage in pandemic strains suggests that f237 might confer a so-far-unknown phenotype to the bacterium and perhaps protect against selective pressures before it infects humans (Iida et al., 2001).

A common RFLP homologous to the genome of Vf33 in many pandemic serovars of *V. parahaemolyticus* has also been reported (Chang et al., 2000). The phage Vf04K68 was isolated from the other pandemic *V. parahaemolyticus* strain belonging to O4:K68 serovar. The phage lacked a 1.8 kb DNA fragment present in the distinctive region of f237. Interestingly, the Vf04K68 infected the recipient O3:K6 strains and might act as genetic transmitter and may play some role in the pandemicity of *V. parahaemolyticus* infections (Chang et al., 2002).

Though the utility of PCR for the identification of ORF8 is useful in detecting the pandemic strains of *V. parahaemolyticus*, one report from Bangladesh has shown that some of clonally indistinguishable pandemic strains that were positive in GS-PCR did not amplify the *orf8* (Bhuiyan et al., 2002). This may not be due to elimination of the f237 phages as the ORF10 was detected in these variant strains (Laohapresthisan et al., 2003).

Future prospects

Repeated recovery of strains with similar genotypes from the patients far apart suggests that these changes are associated with increased fitness or, more specifically, transmissibility (Smith et al., 2000). Retrospective and prospective analysis of incidence and spread of *V. parahaemolyticus* O3:K6 strains clearly indicates that this serovar along with newly emerged serovars such as O4:K68, O1:K25, O1:KUT has similar genomic background with its gene pool recently enriched in order to maintain the virulence of these strains. Many molecular investigations carried out in the past clearly showed that the pandemic strains were under constant selection pressure to evolve as a more successful pathogen, a trend indicating positive selection and adaptive evolution. It may be hypothesized that these strains had an evolutionary advantage that also conferred on it the capability of rapidly acquiring serotype-

encoding genes. Studies on somatic and capsular synthesis genes might provide a great deal of information on the "hot spots" responsible for serotype changes. The adhesiveness hypothesis, other possible alternative virulence factors, competitive capability with other vibrios, and its population distribution in the natural environment, are needed to be investigated to understand the pathogenesis of pandemic strains of *V. parahaemolyticus*.

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