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Clonal Diversity among Recently Emerged Strains of *Vibrio parahaemolyticus* O3:K6 Associated With Pandemic Spread

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The genomes of the O3:K6 strains of *Vibrio parahaemolyticus* which abruptly emerged in Calcutta, India, in February 1996 and which demonstrated an unusual potential to spread and an enhanced propensity to cause infections were examined by different molecular techniques to determine clonality. No restriction fragment length polymorphism (RFLP) in the gene encoding the thermostable direct hemolysin was observed among the O3:K6 isolates of *V. parahaemolyticus*. Clonal diversity among the O3:K6 strains became evident by examining the RFLPs of the *rrn* operons and by the use of pulsed-field gel electrophoresis. Five ribotypes were distinguished among the O3:K6 strains examined, with ribotype R4 constituting the major type. Strains of O3:K6 isolated between June and August 1996 showed different pulsotypes compared to the pulsotypes of strains isolated before and after this period, indicating genetic reassortment among these strains, but those isolated between August 1996 and March 1998 showed identical or nearly similar pulsotypes. It is clear that there is a certain degree of genomic reassortment among the O3:K6 clones but that these strains are predominantly one clone.

Vibrio parahaemolyticus is a gram-negative marine bacterium that can cause seafood-borne gastroenteritis, but not all strains have the same pathogenic potential. Infections caused by *V. parahaemolyticus* are usually associated with diverse serovars. However, recent studies have shown the emergence of a unique serovar, serovar O3:K6, which appears to have the potential to spread and to be associated with infections more often than other serovars (19). Strains belonging to the O3:K6 serovar abruptly appeared in Calcutta, India, in February 1996 and accounted for 50 to 80% of the strains of *V. parahaemolyticus* (19). Furthermore, strains belonging to the same serovar have now been isolated from other Southeast Asian countries (19), from travelers at a quarantine station in Japan (6), and from a food-borne outbreak in the United States (3).

Although the pathogenic mechanism of *V. parahaemolyticus* is poorly understood, it has long been known that one of the hemolysins detected on a special blood agar medium, Wagatsuma agar, is a major virulence determinant (16). Most strains isolated from patients demonstrate this hemolytic activity, which is known as the positive Kanagawa phenomenon (KP) (13, 20). The hemolysin produced by KP-positive strains is thermostable in nature, and the hemolytic activity is not enhanced by the addition of lecithin, which indicates that it has direct action on erythrocytes (21) and which is thus called thermostable direct hemolysin (TDH). Additionally, a TDH-related hemolysin (TRH) produced by a KP-negative strain was reported in the last decade (4, 5). The *tdh* and *trh* genes, which encode TDH and TRH, respectively, share 70% nucleotide sequence identity (17). Molecular epidemiological studies revealed that not only strains that carry the *tdh* gene but also strains that carry the *trh* gene, or strains that carry both genes, are strongly associated with gastroenteritis (7, 23). Recently, a strong correlation between the presence of the *trh* gene and urease production, which is an unusual trait for *V. parahaemolyticus*, has been observed (11, 16, 25).

Intense investigations of the O3:K6 strains isolated in Calcutta in 1996 revealed that all 61 strains examined shared identical traits (*tdh* positive, *trh* negative, and urease negative), with only 2 strains having an antibiogram different from those of the other strains (19). In addition, the representative O3:K6 strains were shown to be genetically indistinguishable by arbitrarily primed PCR analysis and were therefore considered to be a single clone (19). The present report describes the further examination of the genomes of the O3:K6 strains of *V. parahaemolyticus* isolated in Calcutta by a variety of molecular typing methods to determine clonality among the strains. These additional molecular studies indicate that this collection of strains is composed of multiple clonal populations.

A total of 30 clinical *V. parahaemolyticus* O3:K6 strains isolated between February 1996 and June 1998 from patients admitted to the Infectious Diseases Hospital, Calcutta, were included in this study. The strains were selected to represent strains from different periods of isolation subsequent to the initial isolation of the O3:K6 strains in February 1996. The strains numbers of the selected strains (with the date of isolation in parentheses) are as follows: VP45 (5 February 1996), VP53 (28 February 1996), VP59 (21 March 1996), VP61 (22 March 1996), VP72 (12 April 1996), VP77 (17 April 1996), VP79 (23 April 1996), VP86 (10 May 1996), VP96 (30 May 1996), VP100 (4 June 1996), VP122 (9 July 1996), VP136 (2 August 1996), VP138 (6 August 1996), VP144 (17 August 1996), VP151 (20 September 1996), VP155 (8 October 1996), VP159 (4 November 1996), VP165 (12 March 1997), VP170

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(17 April 1997), VP176 (30 April 1996), VP178 (26 May 1997), VP186 (12 June 1997), VP187 (1 June 1997), VP199 (10 July 1997), VP208 (8 August 1997), VP210 (29 August 1997), VP218 (16 September 1997), VP233 (25 March 1998), VP238 (16 June 1998), and VP239 (18 June 1998). Strains were identified and characterized, and their O:K serovars were determined with specific antisera as described previously (18). *V. parahaemolyticus* strains were grown in a gyratory shaker at 37°C in Luria broth containing 3% NaCl and were maintained in nutrient agar with 3% NaCl at room temperature.

Genomic DNA extraction and Southern hybridization were carried out as described by Sambrook et al. (22). Restriction enzymes *Bgl*I and *Hin*dIII (Boehringer Mannheim GmbH, Mannheim, Germany) were used for ribotyping and *tdh* genotyping of the O3:K6 strains of *V. parahaemolyticus*, respectively. Enzyme-digested genomic DNA fragments were electrophoresed, UV irradiated, and transferred to a nylon membrane (Hybond-N^+; Amersham Life Science, Buckinghamshire, England), followed by hybridization with specific gene probes. A 7.5-kb *Bam*HI fragment of the recombinant plasmid pKK3535 containing an rRNA operon of *Escherichia coli* (2) was used as the *rrn* gene probe for ribotyping. The gene probe for *tdh* was a 415-bp *Pst*I fragment of pCVD518 representing 71% of the structural gene coding for TDH (15). Labeling of the probes, hybridization conditions, washing conditions for the filters, and detection of bands were performed with the ECL detection system (Amersham Life Science).

To perform pulsed-field gel electrophoresis (PFGE), the genomic DNAs of the various *V. parahaemolyticus* strains were prepared in agarose plugs as described previously (1, 27). Agarose blocks containing genomic DNA were equilibrated in restriction enzyme buffer for 1 h at room temperature and were cleaved in fresh buffer at the appropriate incubation temperature. For complete digestion of the DNAs, 50 U of *Not*I was used. PFGE of *Not*I-digested inserts was performed by the contour-clamped homogeneous electric field method on a CHEF Mapper system (Bio-Rad, Richmond, Calif.) in 0.5× TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1.0 mM EDTA [pH 8.0]) for 40 h 24 min. A DNA size standard (bacteriophage λ ladder; Bio-Rad) was used as the molecular mass standard, and a minichiller (model 1000; Bio-Rad) was used to maintain the temperature of the buffer at 14°C. Run conditions were generated by the autoalgorithm mode of the CHEF Mapper PFGE system by using a size range of 20 to 300 kb. *Ceu*I-digested DNAs were electrophoresed on 1% agarose (Bio-Rad) with the Pulsaphor Plus System (Pharmacia, Uppsala, Sweden), with the pulse time interpolated between 10 and 50 s for 24 h at 10 V/cm at 4°C. Phage λ multimeric DNA (Bio-Rad) and yeast chromosomal DNA (New England Biolabs, Boston, Mass.) were used as molecular mass markers. After electrophoresis, the gel was stained by placing it in ethidium bromide (1 μg/ml) for 30 min and was destained by placing it in water for 15 min twice. The DNA bands were visualized with UV transilluminator and were photographed with a Polaroid photo apparatus.

The *tdh* gene probe hybridized with two *Hin*dIII fragments of 2.5 and 1.3 kb for all strains examined (data not shown). No restriction fragment length polymorphism (RFLP) of the genes encoding TDH was observed among the O3:K6 strains examined in this study. Most of the KP-positive strains carry two copies of the *tdh* gene in their genomes, and since *Hin*dIII cleaves DNA sequences outside the *tdh* structural genes, the number of copies of the *tdh* gene can be determined (15). For clinical strains of *V. parahaemolyticus* isolated in Thailand, the *Hin*dIII restriction fragment patterns of *tdh* and *trh* genes could be classified into five and four types, respectively, and a strong association between the restriction fragment patterns of *tdh* and *trh* was observed (24).

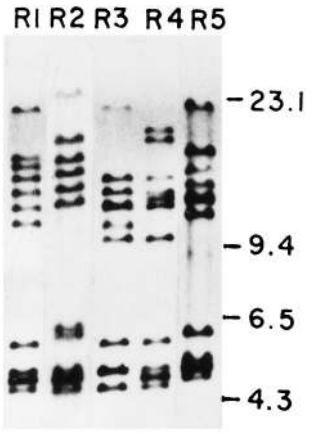

FIG. 1. Representative results for the five ribotypes of the O3:K6 *V. parahaemolyticus* strains encountered in this study. R1, strain VP86; R2, strain VP199; R3, strain VP208; R4, strain VP155; and R5, strain VP96. Numbers on the right are molecular sizes (in kilobases).

For ribotyping of *V. parahaemolyticus* strains, we selected the *Bgl*I restriction enzyme because this enzyme has successfully been used to develop a ribotyping scheme for *Vibrio cholerae* (8). Five ribotypes were distinguished among the 28 *V. parahaemolyticus* strains tested, and the ribotypes were designated R1 through R5 (Fig. 1). The ribotypes obtained in this study exhibited stable and reproducible patterns consisting of 9 to 11 bands ranging from approximately 23 to 4 kb (Fig. 1). The most common ribotype was ribotype R4 (78.6%), while the other ribotypes were uncommon but were distinct from ribotype R4. Ribotype R4 was found throughout the study period, while ribotype R1 was found in May 1996 and June 1997, ribotype R5 was found in May 1996, ribotype R2 was found in July 1997, and ribotype R3 was found in August 1997.

To further examine the clonality of O3:K6 isolates, the genomic DNAs of representative O3:K6 strains isolated during different time periods were analyzed by PFGE following digestion with *Not*I. The enzyme *Not*I produced several well-separated fragments in all the *V. parahaemolyticus* strains examined (Fig. 2). By using phage λ multimeric DNA and yeast chromosomal DNA as molecular mass markers, the *Not*I fragment sizes were estimated. The results showed several distinct RFLPs among the O3:K6 isolates (Fig. 2). O3:K6 strain VP61, which was isolated on 22 March 1996, had a pulsotype which resembled the pulsotypes of the strains isolated after August 1996. However, the O3:K6 strains isolated between June and August 1996 showed different pulsotypes, indicating genomic reassortment among these strains during these few months. The *Not*I profiles of the genomes of O3:K6 strains isolated between August 1996 and June 1998 were identical or nearly similar. The PFGE results suggest that genetic differences did exist between O3:K6 isolates, but only for a brief period.

*Ceu*I is encoded by a class I mobile intron (12), and *Ceu*I digestion followed by PFGE is very useful for determination of intra- and interspecies genomic rearrangements and elucidation of the numbers of *rrn* operons in the genomes of prokaryotes. Since the enzyme cleaves genomic DNA only in a 19-bp sequence in the 23S rRNA gene of the *rrn* operon (10), it has also been successfully used to determine the number of *rrn* operons in the genomes of many organisms including *V. cholerae*, the type species of the genus *Vibrio*. When the genomes of representative *V. parahaemolyticus* O3:K6 strains

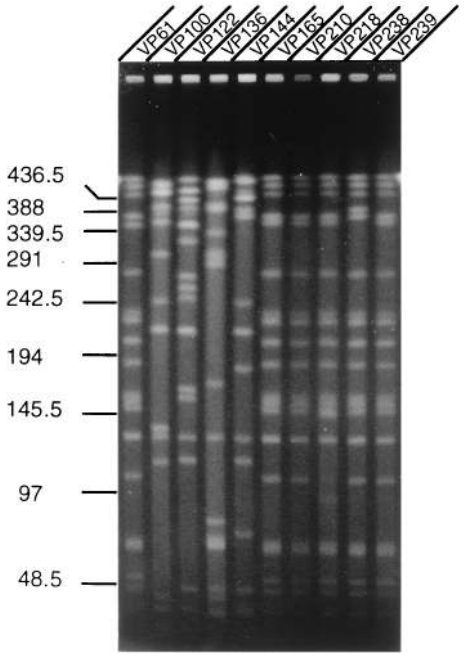

FIG. 2. PFGE of *Not*I-digested genomic DNAs of *V. parahaemolyticus* O3:K6 strains. The gel was stained with ethidium bromide. Numbers indicate the sizes of the molecular size markers (in kilobases). The RFLP patterns of strains isolated between February and August 1996 (strains VP61, VP100, VP122, and VP136) compared to the RFLP patterns of strains isolated between August 1996 and March 1998 (strains VP144, VP165, VP210, and VP218) are distinct. No RFLP was observed between the strains isolated in the later phase (June 1998) of the outbreak (strains VP238 and VP239).

were digested with *Ceu*I and pulsed-field gel electrophoresed, nine fragments were detected in the ethidium bromide-stained gel, indicating that there are at least nine *rrn* operons in the *V. parahaemolyticus* genome (operons C1 to C9; Fig. 3). It is noteworthy that, as in the *V. parahaemolyticus* genome, there are 9 to 10 *rrn* operons in the *V. cholerae* genome (14). Apart from detecting the *rrn* operon in the genomes of prokaryotes, *Ceu*I can serve as a tool for the rapid examination of the organizational variations in the genomes among various strains within a species (10). When we analyzed the *Ceu*I digestion profiles of O3:K6 strains, RFLPs between O3:K6 strains were observed (Fig. 3). The *Ceu*I-based RFLPs found between the O3:K6 strains matched those for the *Not*I-digested genomes of respective *V. parahaemolyticus* isolates (Fig. 2 and 3). These results confirm that there is clonal diversity among the O3:K6 strains, and genetic rearrangements were evident in strains isolated during a brief interim period after the genesis of this unique O3:K6 clone of *V. parahaemolyticus*.

The *Bgl*I-generated ribotypes obtained from this study also indicate that there are at least nine or more *rrn* operons in the genome of *V. parahaemolyticus*, because all strains tested had 9 to 11 bands in the autoradiogram. It seems that most of the *rrn* operons of *V. parahaemolyticus* do not have a *Bgl*I site in the coding sequence. However, this needs further investigation to establish clearly the exact copy number of the *rrn* operon in this bacterium. *rrn*-mediated recombination may be one of the mechanisms by which pathogenic bacteria may maintain their genomic plasticity or diversity, and the possibility of genome rearrangements is greater when there are more *rrn* operons in the genome of any organism (9).

Intraspecies characterization of pathogenic bacteria for epidemiological purposes can now be achieved by several approaches. The development of molecular techniques has allowed the comparison of strains by examination of their genomes. We applied three such techniques to determine the clonality of the O3:K6 *V. parahaemolyticus* strains which abruptly appeared in Calcutta in February 1996 and which we previously defined as clonal (19). We used *Not*I and *Ceu*I for PFGE analysis of the genomes of O3:K6 strains, and to our knowledge, this is the first report to describe an analysis of the genome of *V. parahaemolyticus* with these two enzymes. Previously, Wong et al. (26) used *Sfi*I to distinguish a large number of *V. parahaemolyticus* strains isolated from different outbreaks in Taiwan, and they grouped all the strains into 14 PFGE types. From the present study it appears that the *Not*I enzyme can also be used to differentiate various *V. parahaemolyticus* strains because it produces a small number of large fragments with a uniform distribution of sizes separable by PFGE.

Although Southern analysis with the *tdh* gene as a probe failed to differentiate the strains, it confirmed that there are two chromosomal gene copies in strains showing a typical hemolysin-positive phenotype, as reported earlier (16). However, clonal diversity among O3:K6 isolates of *V. parahaemolyticus* became evident when we used the PFGE technique and the ribotyping method. The infection caused by the O3:K6 clone of *V. parahaemolyticus* can be categorized as an emerging infectious disease, considering the extent of its geographical spread. At this point we are not certain where this clone originated, but chronologically, it would appear that the clone abruptly established itself as an entity in Calcutta in February 1996 (19). Subsequently, the clone appears to have spread to other areas in Southeast Asia and now seems to have crossed over to the North American continent. This study indicates that the strains isolated during a brief interim period showed genetic instability, but over a period of time the clone stabilized. The global spread of this clone of *V. parahaemolyticus* needs to be carefully monitored.

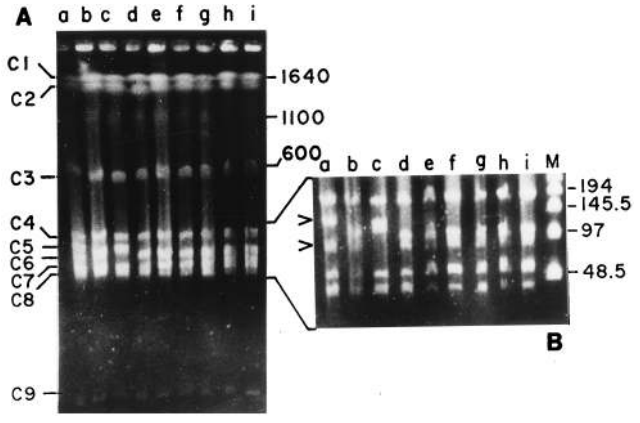

FIG. 3. PFGE of *Ceu*I-digested genomic DNAs of *V. parahaemolyticus* O3:K6 isolates and identification of number of *rrn* operons. The enzyme-digested DNAs were electrophoresed, with pulse times interpolated between 20 and 200 s for 24 h at 10 V/cm at 4°C to separate *Ceu*I fragments (operons C1 to C9) (A), and for better resolution of *Ceu*I operons C4 to C8, the pulse time was interpolated between 5 and 100 s for 24 h at 10 V/cm at 4°C (the region for operon C4 to C8 is expanded in panel B. Since *Ceu*I has sites only in *rrn* operons in the bacterial genomes examined so far, it appears that there are nine *Ceu*I sites in the genome of *V. parahaemolyticus* and hence probably nine *rrn* operons. Lanes: a, VP100; b, VP122; c, VP136; d, VP144; e, VP165; f, VP165; g, VP210; h, VP218; i, VP238; and M, bacteriophage λ multimeric DNA as marker. Numbers indicate the sizes of the molecular size markers (in kilobases). The arrowhead indicates RFLP between initial isolates only in operons C5 and C6 (lanes a to d). Strains isolated from the later phase of the outbreak had identical *Ceu*I pulsotypes (lanes e to i).

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