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# Clonal dissemination of *Vibrio parahaemolyticus* displaying similar DNA fingerprint but belonging to two different serovars (O3:K6 and O4:K68) in Thailand and India

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## SUMMARY

Active surveillance of *Vibrio parahaemolyticus* infection among hospitalized patients in Calcutta, India, showed the appearance of the O4:K68 serovar for the first time in March 1998 alongside the continued predominant incidence of the O3:K6 serovar. Strains belonging to both these serovars have been reported to possess pandemic potential. The genomes of O3:K6 and O4:K68 strains and for comparison, non-O3:K6 and non-O4:K68 strains isolated from two different countries, India and Thailand, were examined by different molecular techniques to determine their relatedness. The O3:K6 and O4:K68 strains from Calcutta and Bangkok carried the *tdh* gene but not the *trh* gene. Characterization of representative strains of these two serovars by ribotyping and by arbitrarily primed-polymerase chain reaction (AP-PCR) showed that the isolates had identical ribotype and DNA fingerprint. Pulsed-field gel electrophoresis (PFGE) performed with the same set of strains yielded nearly similar restriction fragment length polymorphism (RFLP) patterns for the O3:K6 and O4:K68 isolates from Calcutta and Thailand. Phylogenetic analysis of the *Not*I RFLP showed that the O3:K6 and O4:K68 strains formed a cluster with 78–91% similarity thus indicating close genetic relationship between the two different serovars isolated during the same time-frame but from widely separated geographical regions. The non-O3:K6 and non-O4:K68, in contrast, showed different ribotype, AP-PCR and PFGE patterns.

## INTRODUCTION

*Vibrio parahaemolyticus*, a seafood-borne halophilic pathogen has emerged as a significant bacterium causing outbreaks of gastroenteritis across the globe [1–3]. Molecular epidemiological studies of this halophile have revealed that the virulence of the strains depend on the possession of either the *tdh* gene encoding the thermostable direct haemolysin (TDH) manifested as the Kanagawa phenomenon (KP) [4, 5], or the *trh* gene encoding the TDH-related haemolysin

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(TRH) or both [6, 7]. The presence of the *trh* gene has recently been shown to be correlated to urease production which is an unusual trait for *V. parahaemolyticus* [1, 8, 9].

For the most part of the history of *V. parahaemolyticus* infections, diverse serovars of the organism have been known to cause gastroenteritis. This is evident from the 11 O types and 65 K types currently recognized in the serotyping scheme of *V. parahaemolyticus*. However, the past few years have witnessed an unusual change in the pattern of occurrence of *V. parahaemolyticus* infections primarily due to the emergence and dominance of a unique serovar, the O3:K6 serovar, in causing diarrhoeal disease in India and in other parts of the world [2, 3, 10]. In Calcutta, the O3:K6 serovar was found to be the cause of 50–80% of the *V. parahaemolyticus* infections from February 1996 to August 1996 [3, 10] and was subsequently found responsible for gastroenteritis in other parts of Southeast Asia and also in the United States [2]. Like the O3:K6 serovar, a recent report has indicated the appearance of another serovar, O4:K68, which reportedly has the potential to spread and which by AP-PCR analysis was found to display a similar fingerprint as that of the O3:K6 clone [2].

Extensive molecular studies of the O3:K6 isolates from Calcutta by ribotyping with *Bgl*I enzyme yielded five ribotypes although a majority of the O3:K6 strains (78·6%) were shown to belong to the R4 ribotype [10]. Molecular genetic studies, based on AP-PCR and *toxRS* analysis by Matsumoto and colleagues [2] have shown that the O3:K6 strains isolated in eight countries including India and from international travellers are clonal. Investigations based on a similar approach have also hypothesized the possible evolution of, or diversification into the O4:K68 clone from the existing O3:K6 pandemic clone. Thus, the stabilization of a single clone in causing outbreaks of diarrhoeal illness and its capacity to cross international borders potentiates the theory of the first pandemic in the history of *V. parahaemolyticus* [2].

In this study, we present a comparative molecular analysis of the two dominant pandemic clones of *V. parahaemolyticus*, O3:K6 and O4:K68, isolated from two different geographical locations along with non-O3:K6 and non-O4:K68 strains used as controls in an effort to further understand the molecular basis of the evolution of such emerging serovars having enhanced propensity to spread.

## MATERIALS AND METHODS

### Hospital surveillance

Stool specimens or rectal swabs obtained from patients with diarrhoea admitted to the Infectious Diseases Hospital, Calcutta as a part of the diarrhoea surveillance study were bacteriologically examined within 1 h of collection. Stool samples were plated directly, while rectal swabs were plated after enrichment in alkaline peptone water, onto thiosulphate–citrate–bile salts–sucrose agar (Eiken Chemical Co. Ltd, Tokyo, Japan) as described previously [3]. Sucrose-nonfermenting colonies appearing on the agar medium were screened by using a multitest medium for presumptive identification [11]. Strains that yielded an alkaline slant and acid butt were examined by oxidase test, and the presumptive identification was subsequently confirmed by standard cultural and biochemical characteristics [12] and by the presence of the *V. parahaemolyticus toxR* gene [13].

Twenty-five clinical strains of *V. parahaemolyticus* (Table 1), isolated in 1998, were received from Bamrasnaradura Infectious Diseases Hospital, Nonthaburi Province, Thailand. The identity of these strains were confirmed by standard procedures as described above and also examined for the presence of the species specific regulatory *toxR* gene as described below.

### O:K serovar

The commercially available *V. parahaemolyticus* antisera kit manufactured by Denka Seiken Ltd, Tokyo, Japan was employed for serological typing. This kit contains 65 monovalent K types (K1–K71; K2, K14, K16, K27, K35, K62 are not included), 9 pooled polyvalent K groups (KI–KIX) and 11 O groups (O1–O11).

### Urease test

Urea test broth containing yeast extract 0·1 g; monopotassium phosphate 0·091 g; disodium phosphate 0·095 g; sodium chloride 20·0 g and phenol red 0·01 g in 1 l of distilled water was sterilized by autoclaving at 121 °C for 15 min. On cooling, filter (0·22 $\mu$)-sterilized urea (20 g/l) was added to the autoclaved medium, and dispensed into tubes. After inoculation, the organism was allowed to grow for 16–18 h at 37 °C

Table 1. *Characteristics of clinical* V. parahaemolyticus *strains isolated at the Bamrasnaradura Infectious Diseases Hospital, Thailand (1998)*

| O:K serovar* | No. of strains isolated | Presence of gene† | | Urease test |
|---|---|---|---|---|
| | | *tdh* | *trh* | |
| O4:K68 | 7 | + | − | − |
| O3:K6 | 5 | + | − | − |
| O1:KUT | 2 | + | − | − |
| O1:KUT | 1 | − | + | + |
| O1:K25 | 2 | + | − | − |
| O4:K9 | 2 | + | − | − |
| O4:K8 | 2 | + | − | − |
| O2:K28 | 1 | + | − | − |
| O12:K53 | 1 | + | − | − |
| ROUGH | 2 | + | − | − |

* UT, untypable.
† +, present; −, absent.

with shaking. Positive results were scored by a change from orange to pink colouration.

### Gene detection

The presence or absence of the *toxR*, *tdh*, *trh1* and *trh2* genes of *V. parahaemolyticus* strains isolated in Calcutta during the surveillance period was determined by the DNA colony hybridization test using polynucleotide probes under high stringency conditions as described previously [3, 14, 15].

PCR assay was performed to test for the presence of *toxR*, *tdh* and *trh* genes in the *V. parahaemolyticus* strains received from Thailand as described previously [13, 16]. Amplification was carried out using 25 $\mu$l volumes containing 2·5 $\mu$l of 10 × PCR amplification buffer (500 mM KCl; 100 mM Tris–HCl, pH 9·0; 0·1% Triton-X, 150 mM $MgCl_2$), 2 $\mu$l of dNTP (2·5 mM each); 2 $\mu$l (10 pmol/$\mu$l) of each of the primers, 0·2 $\mu$l (5 U/$\mu$l) of rTaq DNA polymerase (Takara Shuzo, Tokyo, Japan), 11 $\mu$l of sterile triple distilled water and 5·3 $\mu$l of template DNA. Template DNA was prepared by growing the bacteria in Luria broth (Difco, Detroit, USA) with 3% NaCl at 37 °C overnight, centrifuging the culture, resuspending the pellet in 500 $\mu$l of sterile distilled water, and boiling for 10 min. The cycling conditions included a preincubation at 94 °C for 5 min, followed by a middle step of 30 cycles at 94 °C for 1 min 30 s, 50 °C for 1 min 30 s, 72 °C for 1 min 30 s and a final extension of 72 °C for 10 min (for *tdh*); 30 cycles at 94 °C for 1 min, 55 °C for 1 min 30 s, 72 °C for 1 min 30 s (for *trh*); and 20 cycles at 94 °C for 1 min, 63 °C for 1 min 30 s, 72 °C for 1 min 30 s (for *toxR*) using an automated thermal cycler (Biometra, Gottingen, Germany). *V. parahaemolyticus* strain AMVP8 (*toxR*$^+$, *tdh*$^+$, *trh*$^+$) was used as a positive control for all of the virulence genes tested. Amplified products were electrophoresed in a 1% agarose gel (SRL, India), stained with ethidium bromide (Sigma, St. Louis, MO, USA), visualized, and documented using a video documentation system (Pharmacia Biotech, Uppsala, Sweden).

### Bacterial strains

Ten pandemic isolates comprising 2 each of O3:K6 (VP155, VP165) and O4:K68 (VP232, VP234) isolates of *V. parahaemolyticus* from Calcutta and 3 each of O3:K6 (Tdp3, Tdp13, Tdp23) and O4:K68 (Tdp1, Tdp4, Tdp22) isolates from Thailand were chosen for the present study. Five non-pandemic strains belonging to different serovars, O2:K3 (VP191), O4:K13 (VP206), O3:K29 (VP263) from Calcutta and O1:K25 (Tdp10), O4:K9 (Tdp16) from Thailand were also included in this study. The 5 non-O3:K6, non-O4:K68 strains were isolated during the same period as the pandemic strains but the serovars were sporadically associated with diarrhoea and were used as external controls.

### DNA extraction

A modification of the method of Murray and Thompson [17] was applied for DNA extraction. In brief, cells from 18 h Luria broth culture were collected and resuspended in TE buffer (10 mM Tris–HCl; 1 mM EDTA; pH 8·0), treated with 10% SDS and freshly prepared proteinase K and incubated at 37 °C for 1 h. After incubation CTAB/NaCl (10% cetyl trimethyl ammonium bromide in 0·7 M NaCl) was added and incubated at 65 °C for 10 min. The aqueous phase was then treated with phenol–chloroform and DNA pellet was washed with 70% ethanol. The nucleic acid was suspended in TE and treated with RNAse at 37 °C for 30 min.

### Ribotyping

Restriction enzyme *Bgl*I (Boehringer–Mannheim Gmbh, Mannheim, Germany) was used for ribotyping of *V. parahaemolyticus* strains. *Bgl*I restriction enzyme was selected because this enzyme has successfully been

used to develop a ribotyping scheme for *V. cholerae* [18]. Enzyme-digested genomic fragments were electrophoresed, UV irradiated and transferred to nylon membrane (Hybond™ $N^+$, Amersham Life Science, Buckinghamshire, England) followed by hybridization with specific gene probes. A 7·5-kb *BamH*I fragment of the recombinant plasmid pKK3535 containing a rRNA operon of *E. coli* [19] was used as the *rrn* gene probe for ribotyping. Labelling of the probes, hybridization conditions, washing conditions of filters and detection of bands were performed using ECL detection system (Amersham Life Science, UK).

### Arbitrarily primed-PCR (AP-PCR)

AP-PCR fingerprinting [20] was carried out in 25 $\mu$l reaction mix containing 2·5 $\mu$l of 10 × PCR buffer [500 mM KCl, 100 mM Tris–HCl (pH 9·0); 0·1% Triton X-100, 150 mM $MgCl_2$], 20 ng of *V. parahaemolyticus* genomic DNA, 2·5 $\mu$l of 25 mM $MgCl_2$, 20 pmol of primers 1281 (5′-AACGCGCAAC) or 1283 (5′-GCGATCCCCA), 1 unit Amplitaq DNA polymerase and 2·5 $\mu$l of 2·5 mM dNTP under a drop of mineral oil for 45 cycles of: 94 °C for 1 min; 36 °C for 1 min; and 72 °C for 2 min in a automated thermal cycler (Biometra, Gottingen, Germany). After PCR, 6 $\mu$l of products were electrophoresed in 1% agarose gels containing 0·5 mg/ml ethidium bromide and photographed under UV light. The 1 kb DNA ladder (New England Biolabs Inc., USA) was used as size marker in all gels.

### Pulsed-field gel electrophoresis

To perform pulsed-field gel electrophoresis (PFGE), the genomic DNAs of the various *V. parahaemolyticus* strains were prepared in agarose plugs as described previously [21, 22]. Agarose blocks containing genomic DNA were equilibrated in restriction enzyme buffer for 1 h at room temperature and were cleaved in fresh buffer overnight at 37 °C. For complete digestion of the DNAs, 50 U of *Not*I enzyme (Takara, Shuzo Co. Ltd, Japan) was used. PFGE of the *Not*I digested inserts was performed by the contour-clamped homogeneous electric field method on a CHEF Mapper system (Bio-Rad, California, USA) with 1% PFGE grade agarose in 0·5 × TBE (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 $\lambda$ ladder; Bio-Rad, USA) was used as the molecular mass standard, and a minichiller (model 1000; Bio-Rad, USA) 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 20–300 kb. After electrophoresis, the gel was stained in ethidium bromide solution (1 $\mu$g/ml) for 30 min followed by destaining in water for 15 min twice and photographed under UV-light.

The electrophoresis gel was then scanned in a Gel Doc 2000 gel documentation system (Bio-Rad, USA) for acquiring the image into the computer for analysis by a quantitation software (Quantity One) installed in the computer. The Quantity One software was used to compare the different *Not*I digested DNA patterns to elucidate a phylogenetic relationship between the O3:K6 and O4:K68 strains of *V. parahaemolyticus*. The unrooted dendogram was constructed on the basis of single-linkage method with percentage of matched bands ranging between 97 and 339·5 kb [23].

## RESULTS AND DISCUSSION

### Calcutta surveillance data

The results of the hospital surveillance for *V. parahaemolyticus* in Calcutta between January 1996 and December 1998 and the serologic and genotypic characteristics of the strains isolated during the above mentioned period are summarized in Table 2. As reported earlier [3], the strains belonging to the O3:K6 serovar and carrying the *tdh* gene but not the *trh* gene had a monthly isolation frequency of 50–80% until August 1996 and this trend was observed to continue till October 1996. In 1997, 42·7% of the *V. parahaemolyticus* strains belonged to the O3:K6 serovar and all these strains had identical genotypic traits ($tdh^+$, $trh^-$) as the ones isolated in the previous year (Table 2). The monthly detection rate of the O3:K6 strains ranged from 40 to 100% between January and September 1997 and 32–40% in 1998. The decrease in the isolation frequency of O3:K6 strains in 1998 was, however, complemented by the appearance, for the first time, of a unique O4:K68 serovar in March 1998, which, like the O3:K6 strains, carried the *tdh* gene but not the *trh* gene. Seven of 50 (14%) *V. parahaemolyticus* strains isolated in 1998 were identified as belonging to the O4:K68 serovar which thus dominated infections due to *V. parahaemolyticus* much in the same way as the O3:K6 type. Apart from O3:K6 and O4:K68 serovars, a third cluster of strains belonging to the O1:KUT serovar appeared to have a significant isolation profile both in 1997 (9 out of 75

Table 2. *Characteristics of* V. parahaemolyticus *strains isolated from patients with diarrhoea at the Infectious Diseases Hospital in Calcutta (1996–98)*

| Year and no. of strains | O:K serovar* | Presence of the gene† | | |
|---|---|---|---|---|
| | | *tdh* | *trh*1 | *trh*2 |
| 1996 | | | | |
| 68 | 3:6 | + | − | − |
| 6 | 4:8 | + | − | − |
| 6 | 4:55 | + | − | − |
| 7 | 5:UT | + | − | − |
| 3 | 1:1 | + | − | + |
| 3 | 8:22 | + | − | − |
| 2 | 1:38 | + | − | − |
| 2 | 8:41 | + | − | − |
| 2 | 12:61 | + | − | − |
| 1 | 1:56 | + | − | − |
| 2 | 1:UT | + | + | − |
| 1 | 2:3 | + | − | − |
| 1 | 3:UT | − | − | − |
| 1 | 4:4 | + | − | − |
| 1 | 8:21 | + | − | − |
| 1 | 8:39 | + | − | − |
| 1 | 3:29 | nd | nd | nd |
| 1 | 12:UT | nd | nd | nd |
| 10 | nd | nd | nd | nd |
| 1997 | | | | |
| 32 | 3:6 | + | − | − |
| 9 | 1:UT | + | − | − |
| 5 | 5:UT | + | − | − |
| 5 | nd | nd | nd | nd |
| 2 | 5:UT | − | − | − |
| 2 | R:UT | + | − | − |
| 2 | 4:13 | + | − | − |
| 2 | 2:3 | + | − | − |
| 2 | 1:38 | + | − | − |
| 1 | 3:7 | + | − | − |
| 1 | 4:8 | + | − | − |
| 2 | UT:UT | + | − | − |
| 1 | 12:UT | + | − | − |
| 1 | 4:4 | + | − | − |
| 1 | 1:56 | + | − | − |
| 1 | 4:55 | + | − | − |
| 1 | 1:26 | − | − | + |
| 5 | nd | nd | nd | nd |
| 1998 | | | | |
| 12 | 3:6 | + | − | − |
| 7 | 4:68 | + | − | − |
| 5 | 1:UT | + | − | − |
| 5 | 1:56 | + | − | − |
| 4 | 4:8 | + | − | − |
| 4 | 4:10 | + | − | − |
| 2 | 12:UT | + | − | − |
| 2 | UT:UT | + | − | − |
| 1 | 12:UT | − | − | − |
| 1 | 4:9 | + | − | − |
| 1 | 10:UT | − | − | − |
| 1 | 3:29 | + | − | − |
| 1 | 3:UT | − | − | + |
| 1 | 1:UT | − | − | + |
| 1 | 2:28 | − | − | + |

* UT, untypable. † +, present; −, absent.
nd, not done.

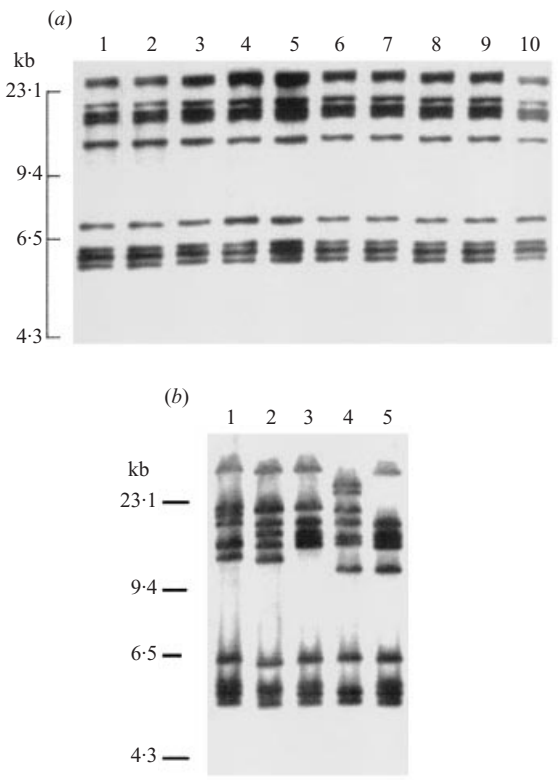


**Fig. 1.** Ribotypes of O3:K6, O4:K68 and non-O3:K6, non-O4:K68 strains of *V. parahaemolyticus* obtained by *Bgl*I restriction enzyme. (*a*) The O3:K6 and O4:K68 strains isolated from two different countries generated identical and reproducible pattern. Lanes 1, 2, O3:K6. Calcutta (VP155, VP165); lanes 3–5, O3:K6, Thailand (Tdp3, Tdp13, Tdp23); lanes 6, 7, O4:K68, Calcutta (VP232, VP234); lanes 8–10, O4:K68, Thailand (Tdp1, Tdp4, Tdp22). (*b*) Lanes 1–3, O2:K3 (VP191), O4:K13 (VP206), O3:K29 (VP263), respectively, from Calcutta; lanes 4, 5, O1:K25 (Tdp10), O4:K9 (Tdp16), respectively, from Thailand.

strains) and in 1998 (5 out of 50 strains). The rest of the strains belonged to diverse serovars. Only 1% of all the *V. parahaemolyticus* strains isolated during the entire period of surveillance carried the *trh* gene and 1% of the strains carried neither of the two virulence genes of *V. parahaemolyticus*. Most (98%) of the *V. parahaemolyticus* strains isolated during the surveillance period possessed the *tdh* gene.

## Thailand surveillance

The *V. parahaemolyticus* strains received from Thailand are listed in Table 1 along with the serologic, phenotypic and genotypic characteristics of the strains. Twenty-eight percent of the strains were serotyped as O4:K68 and 20% as O3:K6, thus,

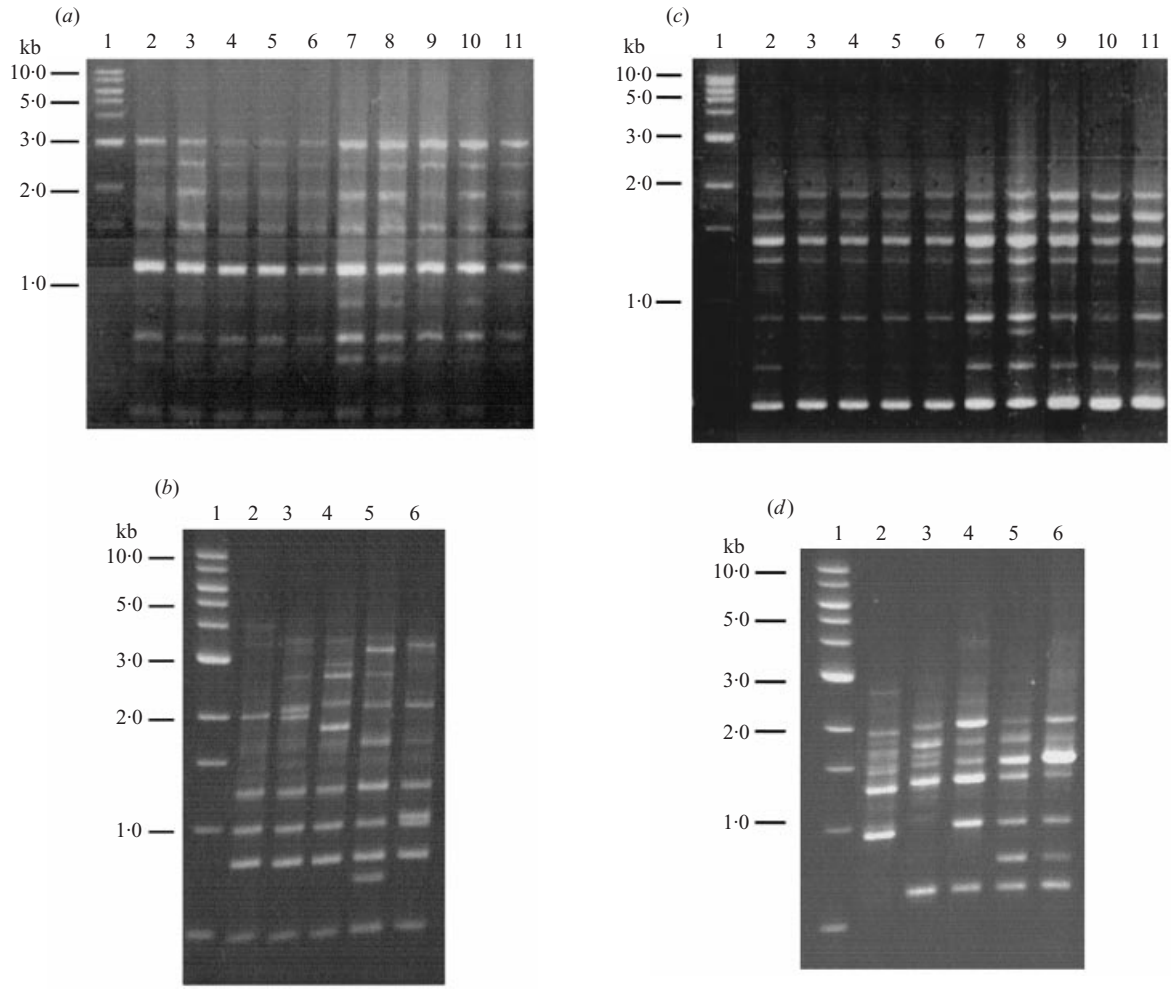


**Fig. 2.** Comparison of AP-PCR profiles obtained with DNA templates of O3:K6, O4:K68 and non-O3:K6, non-O4:K68 strains isolated from India and Thailand with primer 1281. (*a*) Lanes 2, 3, O3:K6, Calcutta (VP155, VP165); lanes 4–6, O3:K6, Thailand (Tdp3, Tdp13, Tdp23); lanes 7, 8, O4:K68, Calcutta (VP232, VP234); lanes 9–11, O4:K68, Thailand (Tdp1, Tdp4, Tdp22). Lane 1, 1 kb marker (New England Biolabs). (*b*) Lanes 2–4, O2:K3 (VP191), O4:K13 (VP206), O3:K29 (VP263), respectively, from Calcutta; lanes 5, 6, O1:K25 (Tdp10), O4:K9 (Tdp16), respectively, from Thailand. Lane 1, 1 kb marker (New England Biolabs). (*c*) Lanes 2, 3, O3:K6, Calcutta (VP155, VP165); lanes 4–6, O3:K6, Thailand (Tdp3, Tdp13, Tdp23); lanes 7, 8, O4:K68. Calcutta (VP232, VP234); lanes 9–11, O4:K68, Thailand (Tdp1, Tdp4, Tdp22). Lane 1, 1 kb marker (New England Biolabs). (*d*) Lanes 2–4, O2:K3 (VP191), O4:K13 (VP206), O3:K29 (VP263), respectively, from Calcutta; lanes 5, 6, O1:K25 (Tdp10), O4:K9 (Tdp16), respectively, from Thailand. Lane 1, 1 kb marker (New England Biolabs).

showing a dominance of these serovars in causing gastroenteritis in Thailand. Like in Calcutta, the strains belonging to both the serovars had the *tdh* gene but not the *trh* gene and did not hydrolyse urea. One strain, O1:KUT, was found to carry the *trh* gene and was also found to be positive for the urease test.

### Molecular analysis of the O3:K6 and O4:K68 strains isolated in India and Thailand

The present study was undertaken with the objective of analysing the relatedness of the two proposed pandemic serovars of *V. parahaemolyticus*, isolated from India and Thailand, to potentiate the hypothesis of a common clonal origin for the two serovars [2]. In this study, the O3:K6 strains isolated in Thailand and the O4:K68 strains isolated from the two countries were characterized and compared with the O3:K6 strains of India (VP155 and VP165) which have been characterized and reported earlier [3]. The molecular profiles of these two serovars were also compared with *V. parahaemolyticus* strains belonging to serovars other than O3:K6 and O4:K68 isolated from India and Thailand.

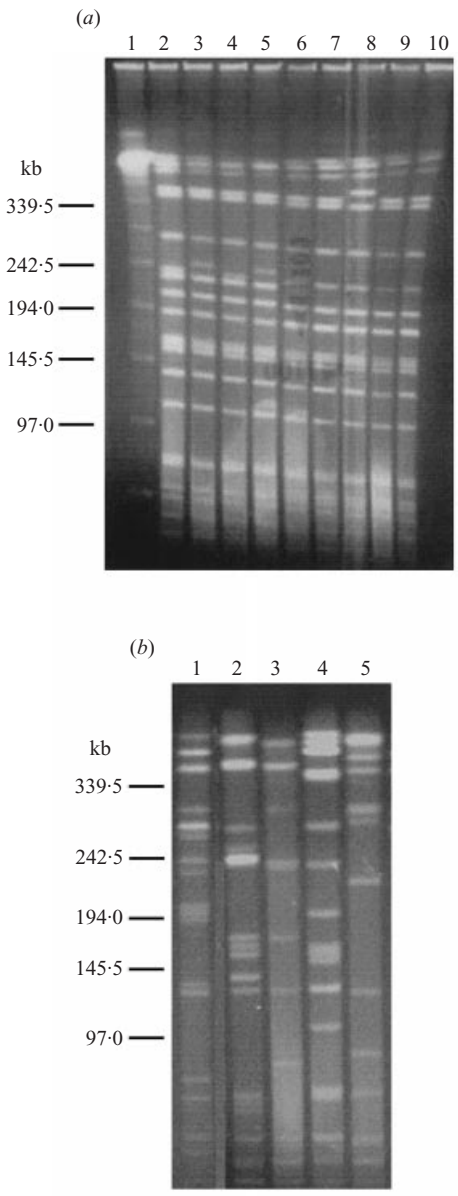


**Fig. 3.** PFGE of *Not*I digested genomic DNAs of O3:K6, O4:K68 and non-O3:K6, non-O4:K68 strains of *V. parahaemolyticus*. (*a*) Lane 2, Calcutta, O3:K6, Calcutta (VP165); lanes 3–5, O3:K6, Thailand (Tdp3, Tdp13, Tdp23); lanes 6, 7, O3:K6, Calcutta (VP232, VP234); lanes 8–10, O4:K68, Thailand (Tdp1, Tdp4, Tdp22). Lane 1, 48·5 kb marker (BioRad, USA). (*b*) Lanes 1–3, O2:K3 (VP191), O4:K13 (VP206), O3:K29 (VP263), respectively, from Calcutta; lanes 4, 5, O1:K25 (Tdp10), O4:K9 (Tdp16), respectively, from Thailand.

*Bgl*I restriction endonuclease was used for ribotyping the pandemic O3:K6 and O4:K68 strains and the non-pandemic strains belonging to various serovars of *V. parahaemolyticus*. Previous *Bgl*I ribotyping studies of the O3:K6 strains isolated in Calcutta (VP155 and VP165) have shown them to belong to the R4 pattern which constitutes the major ribotype (78·6%) among the five ribotypes currently circulating in Calcutta, India. Like the O3:K6 strains of India, the O3:K6 strains of Thailand (Tdp3, Tdp13 and Tdp23), O4:K68 strains of India (VP232 and VP234) and O4:K68 strains of Thailand (Tdp1, Tdp4, and Tdp22) were shown to belong to the R4 ribotype pattern. The ribotypes obtained in this study exhibited stable and reproducible pattern consisting of 11 bands ranging from approximately 23–4 kb (Fig. 1*a*). The identical ribotype pattern obtained for all the 10 isolates of *V. parahaemolyticus* belonging to two different serovars could explain for a common clonal origin of the O3:K6 and O4:K68 strains isolated in India and in Thailand. As shown in Figure 1*b*, the R4 pattern is distinct from the ribotype pattern obtained for the other serovars. Each of the non-pandemic strains exhibited 10 bands in different arrangements.

To determine and compare the extent of clonal relationship between O3:K6 and O4:K68 isolates and between these two pandemic clones and other non-O3:K6 and non-O4:K68 isolates from India and Thailand, the *V. parahaemolyticus* strains were analysed by AP-PCR using two sets of primers. AP-PCR fingerprinting carried out on the five representative isolates from each of the two pandemic serovars generated identical arrays of anonymous DNA fragments with one primer (1281) (Fig. 2*a*). Interestingly, the other primer (1283) exhibited a slightly varied AP-PCR profile for the O4:K68 strains of India from those of Thailand as observed by the appearance of two additional polymorphic AP-PCR bands at the regions of 1·2 and 0·9 kb (Fig. 2*c*), although, the O4:K68 strains of Thailand had an indistinguishable AP-PCR profile from those of O3:K6 serovar from the two countries (Fig. 2*c*). This variation in the AP-PCR profile exhibited by the O4:K68 *V. parahaemolyticus* strains of India, however, requires further investigation by characterization of the additional polymorphic bands towards a better understanding of the parameters guiding microbial evolution and diversification. The non-O3:K6, non-O4:K68 strains of *V. parahaemolyticus* displayed AP-PCR patterns very different from the pandemic clones with both sets of primers (Figs. 2*b*, *d*).

A third molecular typing method involving pulsed-field gel electrophoresis of *Not*I digested restriction fragments of the *V. parahaemolyticus* strains was performed to validate the extent of clonal relationship between the O3:K6 and O4:K68 strains established by the other molecular methods of ribotyping and AP-

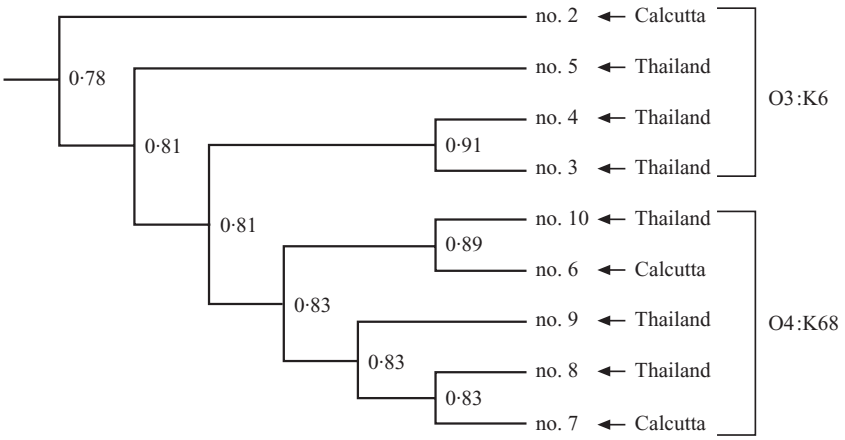


**Fig. 4.** Dendogram generated by single-linkage method with percentage of matched bands enumerating the degree of similarity of *Not*I restriction pattern of genomic DNA of O3:K6 and O4:K68 *V. parahaemolyticus* strains.

PCR, VP 165, O3:K6 isolate of Calcutta, for which a *Not*I PFGE profile has been reported [10], was used as the reference strain for a comparative analysis of the isolates belonging to two different serovars and to two different countries. *Not*I digestion of all the 14 strains of *V. parahaemolyticus* produced well separated fragments. A near similar RFLP pattern was observed for each of the pandemic strains thus indicating a high degree of relatedness between the two pandemic serovars (Fig. 3*a*). But the PFGE pattern of the *Not*I fragments of the non-O3:K6, non-O4:K68 strains showed considerable polymorphism among themselves and also when compared to the pandemic O3:K6 and O4:K68 strains (Fig. 3*b*). The relatedness between the genomic DNA restriction patterns of the pandemic strains were estimated and represented as a dendogram (Fig. 4). In the cluster analysis we found that the O3:K6 and O4:K68 *V. parahaemolyticus* strains from India and Thailand were related to each other at levels between 78 and 91 % (Fig. 4). The slight differences between the O3:K6 and O4:K68 isolates observed by AP-PCR and PFGE were in striking contrast to the differences observed between the pandemic and the non-pandemic strains. The molecular tools applied in this study thus illustrates a close genetic relatedness between the two pandemic serovars.

From the intensive characterization of the *V. parahaemolyticus* strains by various molecular methods and by chronology of appearance of the O3:K6 and O4:K68 serovars of *V. parahaemolyticus* in the two countries, we assume that (i) the O3:K6 strains of *V. parahaemolyticus* could be the precursor of the O4:K68 strains in which the *rfb* genes, coding for the somatic antigens, were altered or (ii) it is also possible that the two lineages have diverged from a common ancestor.

The successive appearance of the O3:K6 strains in 1996 and the O4:K68 strains in 1998 in Calcutta and their predominant association with gastroenteritis with respect to other serovars distinguishes them as a cluster of strains possessing potential to cause a pandemic spread unidentified in any previous *V. parahaemolyticus* strains. In this study, these two epidemiologically related strains have been shown to be genetically related by the various molecular typing methods. However, the occurrence of genetically similar strains expressing entirely different somatic and capsular antigens and the acquisition of pandemic traits by the two serovars of *V. parahaemolyticus*, unusual for this organism, is an enigma that requires to be explored to curb the spread of *V. parahaemolyticus*-mediated disease.

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