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Rapid spread of the new clone of *Vibrio cholerae* O1 biotype El Tor in cholera endemic areas in India

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SUMMARY

Using molecular techniques, we investigated whether the clone of *Vibrio cholerae* O1 biotype El Tor which appeared in Calcutta, India, in 1994 has spread to other cholera endemic areas in the country. The ribotype of 31 of the 33 strains isolated from different parts of India during 1996 and 1997 was identical to the ribotype displayed by the new clone of *V. cholerae* O1 which emerged in Calcutta in 1994. Likewise, 12 of the 15 strains examined by pulsed-field gel electrophoresis (PFGE) showed identical profile to that exhibited by the new clone of O1. The restriction fragment length polymorphism (RFLP) of CTX genetic element of these strains also matched with the new clone of O1 which emerged after the outbreak of *V. cholerae* O139 in Calcutta. However, two strains (AH042 and AH046) isolated from an outbreak in Ahmedabad (western India) showed different CTX RFLP but had the same ribotype and PFGE profile as the new clone, whereas one strain from Goa (G2) showed distinct ribotype and PFGE profile and the CTX RFLP was identical to the O1 strains which prevailed before the genesis of O139 in Calcutta. The drug resistance pattern of most of the O1 strains examined in this study, except strain G2, was similar to that of the new clone of *V. cholerae* O1. None of the strains in this study carried plasmids. Molecular studies clearly show that the new expanded drug resistant clone of *V. cholerae* O1 has spread to all cholera endemic areas in India and also provide evidence for the evolution of new clones of the O1 serogroup.

INTRODUCTION

During the *Vibrio cholerae* O139 outbreak in India between 1992 and 1993, the new O139 serogroup associated with cholera replaced the existing toxigenic *V. cholerae* O1 biotype El Tor and became the dominant serogroup in Calcutta and in most parts of the Indian subcontinent [1]. However, in subsequent years, *V. cholerae* O1 biotype El Tor reappeared in the Indian subcontinent and became the dominant serogroup causing cholera and in the process replaced the O139 serogroup [2, 3]. Conventional typing methods, such as serotyping, biotyping and phage typing indicated that the O1 El Tor biotype apparently reappeared unaltered in Calcutta [4, 5]. However, the toxigenic *V. cholerae* O1 strains which appeared after the emergence of O139 serogroup were resistant to a variety of antibiotics when compared with *V. cholerae* O1 strains isolated before the emergence of the O139 serogroup [4].

Pulsed-field gel electrophoresis (PFGE) revealed

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that the strains of *V. cholerae* O1 which appeared after being temporarily displaced in Calcutta by the O139 serogroup exhibited different PFGE profiles as compared to strains isolated before the advent of the O139 serogroup and there was no carry-over of the O139 *rfb* genes in the new clone of *V. cholerae* O1 [6]. Comparison of the restriction fragment length polymorphism (RFLP) of the rRNA genes and CTX genetic element revealed that most of the strains isolated in Calcutta before the O139 epidemic belonged to a single known ribotype, whereas almost all El Tor O1 strains isolated after the epidemic belonged to a hitherto undescribed ribotype and the chromosomal location of CTX was different in before and after strains suggesting a new clone of O1 had appeared in Calcutta [5]. The objectives of this study was to investigate the extent of spread of the novel clone of *V. cholerae* O1 which emerged in Calcutta after the epidemic of O139 in the Indian subcontinent continent by examining their antibiogram and by using a variety of recently developed molecular techniques.

METHODS

Bacterial strains

A total of 33 strains of *V. cholerae* O1 biotype El tor were randomly selected out of 95 clinical strains received from different parts of India isolated between 1996 and 1997. Of the 33 strains, 6 were from the eastern region [1 from Bhillai (BHO2) and 5 from north Bengal (NB1 to NB4, NB6)], 14 from southern India [10 (MO563, MO565, MO568, MO569, MO571, MO574, MO575, MO576 and MO666), 3 (ALO11, ALO13 and ALO40) from Alleppey, 1 (VO66) from Vellore], 9 from western region [2 (AHO42 and AHO46) from Ahmedabad, 6 (G2, G7 to G12) from Goa], 2 from Delhi (DO28. and DO63) and 2 from northeast India (PDG6 and DG7, isolated from Dibrugarh, Assam). Of the 33 *V. cholerae* O1 strains selected, 32 belonged to the Ogawa serotype while one (DG6) belonged to the Inaba serotype. Strain 569B (classical *V. cholerae* O1), MAK757 (*V. cholerae* O1 El Tor) and CO840 [representing the new clone of *V. cholerae* O1 El Tor isolated in Calcutta (5)] were included as reference strains.

Ribotyping

Genomic DNA extractions and southern hybridization was performed following the method of Maniatis and colleagues [9]. Genomic DNA was digested with *Bgl*I restriction enzyme according to the manufacturers instructions (Boehringer Mannheim GmbH, Mannheim, Germany). Digested fragments were separated by agarose gel electrophoresis (0·8% gel) and southern hybridized on nylon membrane (Hybond™ N+, Amersham Life Science, Buckinghamshire, England). The rRNA gene probe was a 7·5 kb *Bam*HI fragment of pKK3535 which is a pBR322-derived plasmid containing an *Escherichia coli* rRNA operon consisting of one copy each of the genes coding for 5S rRNA, 16S rRNA. 23S rRNA and $tRNA^{Glu}$ [10, 11]. Labeling of the probes, hybridization and detection of bands were performed using the ECL detection system (Amersham Life Science).

Pulsed-field gel electrophoresis

Genomic DNAs of the various *V. cholerae* strains were prepared in agarose plugs as described previously [6]. Agarose blocks containing genomic DNA were equilibrated in restriction enzyme buffer for 1 h at room temperature and cleaved in fresh buffer at 37 °C. For complete digests of the DNAs, 40 U of *Not*I was used. PFGE of the inserts was performed using the contour-clamped homogenous electric field method on a CHEF Mapper™ system (Bio-Rad, Calif., USA) in 0·5 × TBE buffer (44·5 mM Tris-HCl, 44·5 mM boric acid, 1·0 mM EDTA [pH 8·0]) for 40·24 h. A DNA size standard (λ ladder, Bio-Rad) was used as the molecular mass standard and a model 1000 mini chiller (Bio-Rad) was used as the molecular mass standard and a model 1000 mini chiller (Bio-Rad) was used to maintain the temperature of the buffer at 14 °C. Run conditions were generated by the auto-algorithm mode of the CHEF Mapper™ pulsed-field electrophoresis system using a size range of 20–300 kb. The gels were stained in distilled water containing 1·0 μg ethidium bromide per ml for 1 h, destained in distilled water for 30 min and photographed under UV light.

RFLP of CTX genetic element

Genomic DNA extraction, southern hybridization, labelling of the probes, hybridization and detection of bands were performed as described for ribotyping. The restriction enzymes used for CTX genotyping were *Hin*dIII and *Bgl*I. The gene probe for cholera toxin (CT) was a 0·5 kb *Eco*RI fragment of pCVD27

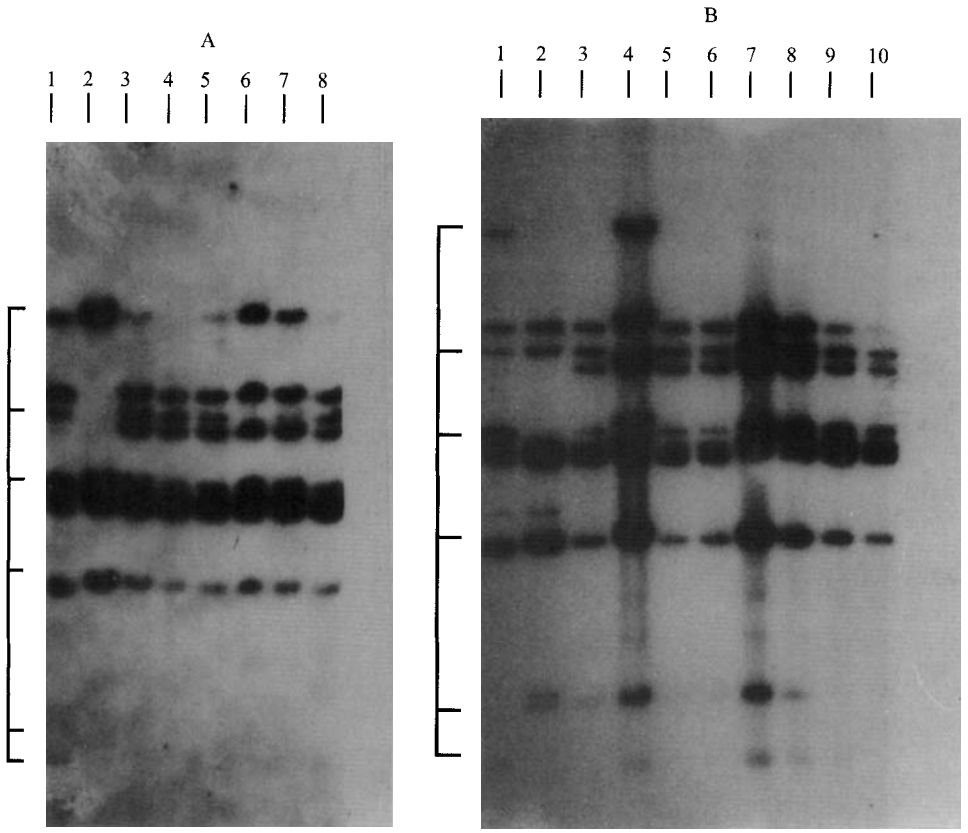

Fig. 1. Ribotypes of *V. cholerae* strains. A: Lanes 1–8: MAK757, 569B, CO840, AHO42, AHO46, BHO2, ALO11 and ALO40. B: Lanes 1–10: MAK757, G2, NB6, DG6, DG6, DG7, DO28, DO63, VO66, MO565 and MO571 (see the text). Positions of λ*Hin*dIII molecular size: markers run on same gel are indicated on the left side of the gel: top to bottom, 23·13, 9·41, 6·55, 4·36, 2·32 and 2·02.

which is a pBR325 derived plasmid containing an *Xba*I-*Cla*I fragment representing 94% of the gene encoding the A subunit of CT (*ctx*A) cloned with *Eco*RI linkers [11, 12]. A 0·38 kb *ctx*B probe spanning from base 1339 (*Nde*I site) to base 1727 [13] was a gift from Y. Singh (CBT, New Delhi, India).

Plasmid DNA isolation

For isolation of plasmid DNA, the modified method of Kado and Liu [14] was followed which involved incubation of cells at elevated pH (pH 12·5) at 56 °C for 30 min during the lysis steps [15].

Antibiotic susceptibility test

Susceptibility to antibacterial agents was examined by the disk diffusion method [7] using antibiotic impregnated disks (Hi-Media Laboratories, Bombay, India). The following antibiotic disks with concentration of the drug per disk as stated in the parentheses were used, ampicillin (10 μg), furazolidone (10 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), co-trimoxazole (25 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), streptomycin (10 μg), and tetracycline (30 μg). Characterization of strains as susceptible, intermediately resistant, or resistant was based on the diameter of the inhibition zones around each disk according to the manufacturers instructions, which matched the interpretive criteria for *V. cholerae* recommended by World Health Organization [8].

RESULTS

Altogether, three *Bgl*I ribotypes were observed among the 33 strains studied. The ribosomal banding pattern of representative 14 strains is presented in Figure 1*a*, *b*. The ribotype of 31 of the 33 strains examined was identical to the ribotype displayed by the new clone of *V. cholerae* O1 [5] which emerged in Calcutta (represented by CO840, Fig. 1*a*, lane 3, in this study). The isolate from Goa (G2) showed a ribotype (Fig 1*b*, lane 2) which resembled the pattern

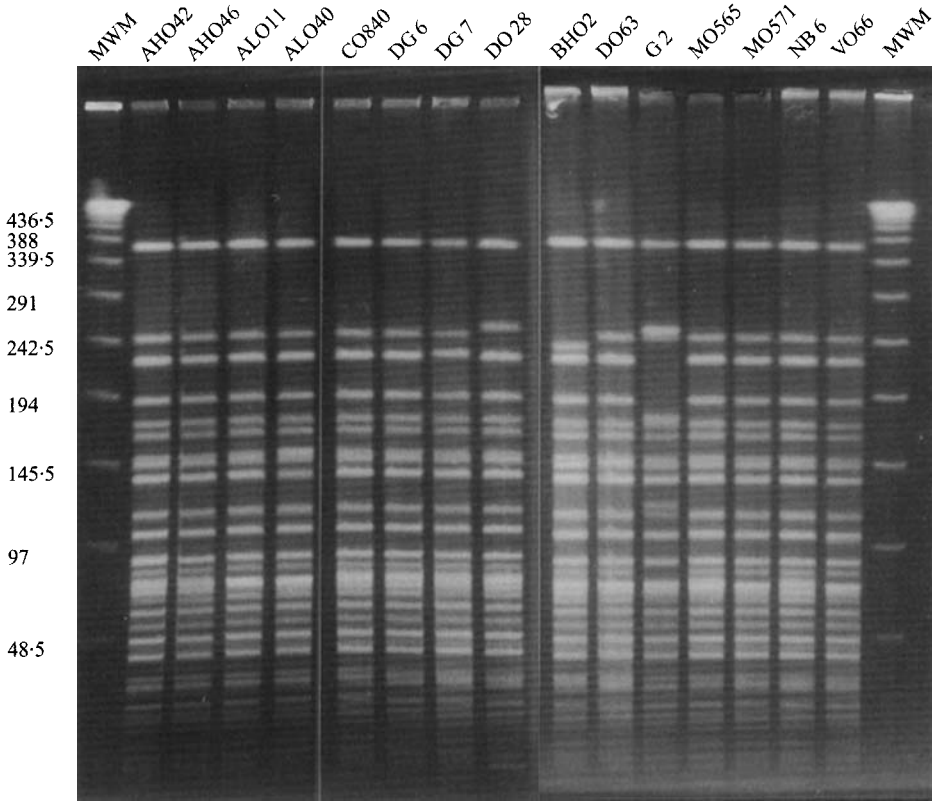

Fig. 2. *Not*I restriction patterns of 15 strains of *V. cholerae* O1 isolated from different parts of India during 1996 and 1997 when examined by pulsed-field gel electrophoresis. Size markers (λ ladder) was loaded in lanes marked MWM. The values are indicated in kb on the left side of the gel.

of MAK757 (*V. cholerae* O1 biotype El Tor, Fig. 1*b*, lane 1), except for the absence of 6·9 kb fragment. The strain from Dibrugarh (DG6) showed a ribotype banding pattern which resembled the ribotype of the new clone of *V. cholerae* O1 with the exception of a 9·45 kb band (Fig. 1*b*, lane 4). None of the two newly described ribotype patterns in this study matched with any of the ribotypes proposed in the standardized ribotyping scheme for *V. cholerae* by Popovic and colleagues [16].

The PFGE profiles of representative 15 *V. cholerae* O1 strains from different parts of India is shown in Figure 2. Of the 15 strains examined, 12 showed identical pattern to that exhibited by CO840 (Fig. 2), the new clone of O1 which appeared in Calcutta in 1994 [5]. The PFGE banding pattern of two strains DO28 and BHO2 isolated from widely distanced geographical locations resembled each other and differed slightly from the Calcutta clone by only one band in the 242·5 kb region. The PFGE profiles exhibited by strain G2 was, however, very different from that exhibited by the new O1 clone. The PFGE study thus correlated well with ribotyping in that strain G2 showed a distinctly different pattern by both the typing methods.

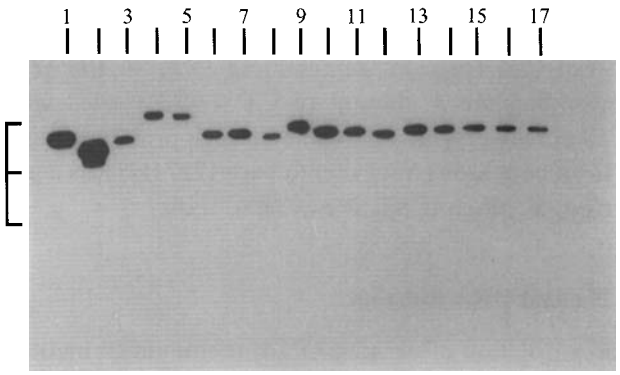

Fig. 3. Southern blot hybridization of *Hin*dIII digested *V. cholerae* chromosomal DNA using *ctx*A probe. Lanes: 1–17: MAK757, 569B, CO840, AHO42, AHO46, BHO2, ALO11, ALO40, G2, NB6, DG6, DG7, DO28, DO63, VO66, MO565, and MO571. Positions of λ*Hin*dIII molecular size markers run on same gel are indicated on the left side of the gel: top to bottom, 23·13, 9·41, 6·55 and 4·36.

Southern blot analysis of *Hin*dIII digested genomic DNA of the strains studied with *ctx*A probe revealed a single band at 18 kb for all strains (Fig. 3, lane 3,

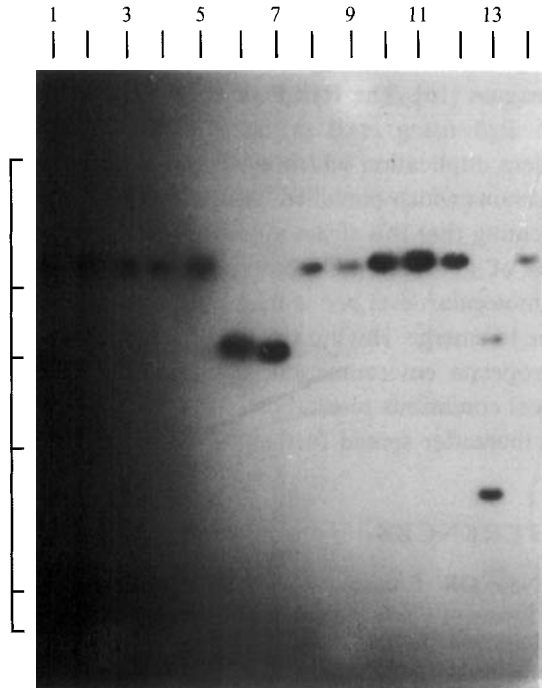

Fig. 4. Southern blot hybridization of *Bgl*I digested chromosomal DNA using *ctx*B probe. Lanes 1–14: MO565, MO571, DO28, DG6, DG7, AHO42, AHO46, ALO11, NB6, BHO2, DO63, ALO40, G2, and VO66. Positions of λ*Hin*dIII molecular size markers run on same gel are indicated at the left: top to bottom, 23·13, 9·41, 6·55, 4·36, 2·32 and 2·02.

lanes 6–17) except two strains (AHO42 and AHO46) which showed a single band at 23 kb (Fig. 3, lanes 4 and 5). However, with *Bgl*I digested genomic DNA, examined with *ctx*B DNA probe revealed a single band at 12 kb for all the strains examined (Fig. 4, lanes 1 to 5, 8–12 and 14) with the exception of the strains AHO42 and AHO46 which showed a single band at 7·2 kb (Fig. 4, lanes 6 and 7) and strain G2 which showed two bands at 7·2 and 3·5 kb suggesting tandem duplication of the CTX genetic element (Fig. 4, lane 13).

None of the 14 strains studied harboured plasmids when examined by the modified method of plasmid isolation recommended for *V. cholerae* [15]. The 14 strains of *V. cholerae* O1 isolated from different parts of India were resistant to ampicillin, co-trimoxazole, furazolidone, nalidixic acid and streptomycin with the exception of strain G2 which was susceptible to nalidixic acid and co-trimoxazole. All the strains examined were sensitive to tetracycline, norfloxacin, ciprofloxacin, gentamicin and intermediately resistant to neomycin. The drug resistance pattern of these strains was similar to the drug resistance pattern of the strains of *V. cholerae* O1 El Tor isolated in Calcutta after the O139 outbreak [4].

DISCUSSION

For the past few years, we have been attempting to understand the molecular variation in successive isolates of *V. cholerae* O1 and O139 and to discern if these genetic variations dictate the emergence of new clones of *V. cholerae*. The emergence of *V. cholerae* O139 in 1992 in Calcutta and the concurrent disappearance of *V. cholerae* O1 biotype El Tor for a brief period of 6 months provided a unique opportunity in that it punctuated the otherwise continuous incidence of *V. cholerae* O1. This enabled us to identify two groups of *V. cholerae* O1, one that prevailed before the genesis of O139 and the other that reappeared after the epidemic of O139 in Calcutta. Phenotypic analysis of these 2 groups of strains of *V. cholerae* O1 using conventional biochemical, physiological and serological traits did not reveal differences [4] and therefore by the conventional traits, one would conclude that both the groups of *V. cholerae* O1 were identical. However, changes in antibiotic susceptibility were observed among strains of *V. cholerae* O1 isolated after the genesis of O139 and higher percentage of O1 strains were resistant to co-trimoxazole, furazolidone and nalidixic acid when compared with strains of O1 isolated before or during the outbreak of O139 [4]. This heterogeneity was confirmed by molecular analysis which showed distinct variations between the two groups of O1 strains in their ribotype, CTX RFLP [5] and PFGE profiles [6] leading us to conclude that those O1 strains which reappeared after the O139 epidemic in Calcutta, actually, belonged to a hitherto undescribed new clone.

The present study is a continuation of our earlier efforts to determine, using the previously established molecular markers [5], if the appearance of the new clone of O1 was an event restricted to Calcutta or whether the new clone had spread to other cholera endemic areas in the country. The strains examined in the study were isolated from different cholera endemic areas in India during 1996 and 1997. The drug resistance pattern of most of the strains of *V. cholerae* O1 examined was similar to the new clone of *V. cholerae* O1 which emerged in Calcutta in 1994 [4]. Interestingly, the antibiotic sensitivity pattern of the new clones of O1 was nearly identical to the O139 serogroup except that the new clone of O1 was

additionally resistant to nalidixic acid. At this point, both O1 and O139 are resistant to co-trimoxazole and furazolidone. Although the transmission of drug resistant plasmid from *Shigella flexneri* to *V. cholerae* has been established, this was not stable under drug-free condition [17, 18]. None of the strains in this study carried plasmids and therefore plasmids did not appear to encode drug resistance [14]. It has recently been documented that a new type of conjugative transposon encodes resistance to sulphamethoxazole, trimethoprim and streptomycin in *V. cholerae* O139 and this could be conjugally transferred from *V. cholerae* O139 to *V. cholerae* O1 and *Escherichia coli* strains, where it integrated into the recipient chromosomes in a site-specific manner independent of *rec*A [19].

Most of the strains examined in this study had identical *Bgl*I ribotype, *Not*I PFGE profiles and the RFLP of CTX genetic element matched with the new clone of O1 which emerged after the O139 outbreak in Calcutta [5]. However, two strains (AHO42 and AHO46) isolated from an outbreak in Ahmedabad (western India) had different CTX RFLP pattern but had the same ribotype as well as PFGE profile as the new clone, whereas one strain (G2) isolated from Goa showed distinct ribotype and PFGE profile and the CTX RFLP was identical to the O1 strains which prevailed before the genesis of O139 in Calcutta [5]. The strain DG6 from Dibrugarh showed a unique ribotype but the PFGE pattern was identical to that exhibited by the new clone. The evidence of the spread of the new expanded drug resistant clone of *V. cholerae* O1 throughout the Indian subcontinent as shown by our study further substantiates the spread of a single clone of *V. cholerae* in cholerae endemic areas in the country. Using molecular typing techniques including PFGE and ribotyping, we have recently documented that this new clone of *V. cholerae* O1 has also spread into the African continent [20].

Our studies are beginning to show that clones of *V. cholerae* O1 or O139 emerge, purge the existing clone and spread spatially in waves. Further, we have observed that emergence of a new clone is preceded by a shift in the antibiogram of the existing clone. In this study, strain G2 isolated in Goa represents this phenomenon in that this strain has a distinct ribotype and PFGE profile, showed polymorphism in the CTX genetic element and has an altered antibiogram as compared to the clone which emerged in Calcutta. Interestingly the ribotype of strain G2 did not match with either the ribo type of the new clone or with any of the ribotype proposed in the standardized ribotyping scheme for *V. cholerae* by Popovic and colleagues [16]. The RFLP of CTX genetic element with *Bgl*I using *ctx*B probe of strain G2 showed tandem duplication which was similar to that of the O1 strains which prevailed before the O139 outbreak indicating that this strain might be another emerging clone of *V. cholerae* O1 biotype El Tor. Changes at the molecular level *per se* may not be enough for the clone to emerge. Having emerged the clone must have appropriate environmental, climatic and epidemiological conditions to establish itself in a specific area and thereafter spread further.

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