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PATHOGENICITY

Studies on the genesis of *Vibrio cholerae* O139: identification of probable progenitor strains

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Summary. Four lines of evidence suggest that the recent outbreak strains of *Vibrio cholerae* O139 could have emerged from serogroup O1 strains typified by isolates M01 and M0477 described in this paper, which are neither truly classical nor truly El Tor in their biotype attributes. Firstly, like all O139 isolates, these O1 strains, isolated in Madras during and before the O139 outbreak, were resistant not only to polymyxin B but also to all biotype-specific choleraphages, i.e. classical phage Φ149 and El Tor phages e4 and e5. Secondly, the restriction fragment pattern (RFP) polymorphism displayed by these strains for the cholera toxin (*ctx*) gene, were identical with those produced by O139 isolates but were different from those of O1 type strains, namely *V. cholerae* 569B (classical) and *V. cholerae* MAK757 (El Tor). Thirdly, all the O139 isolates and the two O1 isolates carried an identical large number of copies of cholera toxin gene in their chromosomes. Finally, the outer-membrane protein profiles of strains M01 and M0477 were identical to those of O139 isolates but were different from those displayed by strains 569B and MAK757.

Introduction

Strains of *Vibrio cholerae* belonging to the O1 serogroup are responsible for the disease cholera and possess epidemic and pandemic potential. In contrast, the other serogroups of *V. cholerae*, collectively known as the non-O1 serogroups, are associated with sporadic cases of gastro-enteritis and extra-intestinal infections but do not manifest an epidemic potential. However, since October 1992, a typical cholera-like outbreak caused by strains of *V. cholerae* that do not agglutinate with the O1 antiserum started in Madras and soon spread to different parts of India[1,2] and Bangladesh.[3] In Calcutta alone, 13275 cases were reported between January and April 16, 1993.[4] These outbreak strains from India and Bangladesh were found to be serologically identical and were, therefore, assigned a new serogroup (O139) with the synonym Bengal.[5] All the O139 strains of *V. cholerae* isolated so far have been shown to produce cholera toxin (CT)[6] and to have a large number of tandem repeats of the toxin gene on their chromosomes.[7] In the early stage of the epidemic, infections due to *V. cholerae* O139 in an endemic area were mainly restricted to adults[8]—a subset of population which would otherwise be expected to be resistant to classical cholera because of their earlier exposure. The O139 strains could evade immune surveillance because of their altered surface antigen structure.

Even though the serological identity of O139 strains and their dissimilarity from *V. cholerae* O1 strains could explain the above phenomenon, it raised a very interesting question regarding the genesis of this new organism. This gave us the impetus to examine these strains in greater detail, to investigate their genetic configuration and to see how they relate to the other serogroups of *V. cholerae*. We thought that this might give some clue as to the genesis of the O139 serogroup. Analyses of outer-membrane protein (OMP) profiles and restriction fragment patterns (RFPs) have been used earlier for typing different strains of bacteria.[9,10] Moreover, RFP polymorphism of the *ctx* gene has been used to establish the identity of *V. cholerae* strains in the past.[11–14] Similar techniques were employed to search for the progenitor of the O139 serogroup by examining retrospectively strains of *V. cholerae* isolated in Southern India before and during

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the O139 outbreak in Madras. We report here identification of two strains, M01 and M0477, which although belonging to the serogroup O1, were identical with O139 strains with respect to all other characters examined. These strains may represent the strains from which O139 evolved.

Materials and methods

Bacterial strains, media and growth conditions

Fourteen strains of *V. cholerae* associated with the recent outbreaks of cholera-like illness in India and Bangladesh were examined. They included two from Amravati (P02, P07), two from Andhra Pradesh (AP1, AP2), one from Calcutta (SG24), three from Madras (M01, M0477, M028), two from Madurai (MD012, MD025), two from Vellore (V015, V020), India and two from Bangladesh (B02, B04). Of the 14 strains, 12 were confirmed as belonging to serogroup O139 by specific anti-O139 antisera;[5] the remaining two, M0477 and M01, isolated in Madras in July and early October 1992, respectively, were confirmed as belonging to serogroup O1. For purposes of comparison, three cholera toxin-producing *V. cholerae* non-O1 strains (VCE228, VCE232 and VCE353) isolated from the aquatic environs of Calcutta in 1986 were also included in the analysis.[15,16] Strains 569B (*V. cholerae* O1, Inaba) and MAK757 (*V. cholerae* O1, Ogawa) were used as the standard reference strains to represent the classical and El Tor biotypes, respectively. Cells were grown as described previously.[17]

OM preparation and SDS-PAGE

Outer membranes were prepared exactly as described by Sengupta *et al.*[18] without any further modifications. Proteins were analysed on a polyacrylamide 12·5% w/v SDS gel with a 4·0% w/v stacking gel. Membrane preparations containing *c.* 50 μg of protein were suspended in 25 μl of sample buffer containing SDS 2% w/v, β-mercaptoethanol 5% v/v, 0·0625 M Tris-HCl, pH 6·8, glycerol 10% v/v and bromophenol blue 0·0025% w/v. Samples were then heated at 100°C for 10 min and electrophoresed at room temperature (24°C) at constant current (35 mA) for 4 h. After electrophoresis was completed, the gel was fixed and stained with Coomassie Brilliant Blue R 250 (Sigma). Mol. wt standards (Sigma) were run concurrently on the gels, for mol. wt determination.

Southern blotting and DNA hybridisation

Chromosomal DNA was prepared according to the protocol described by Mekalanos[19] except that a higher concentration of lysozyme (20 μg/ml instead of 5 μg/ml) was used. Samples of 2 μg of the DNA preparations were digested with a variety of restriction endonucleases (Promega Corporation, USA) according to the manufacturer's instruction. Restricted fragments were separated by electrophoresis through agarose 0·7% w/v gels and Southern hybridised as described before.[17,20] The DNA probe for the *ctx* gene was a 1·8-kb *Xba*I + *Bgl*II fragment from the plasmid pCT5A11.[21] The DNA probe was prepared by electroeluting the restricted DNA fragments, followed by labelling with [^{32}P] dATP by nick translation.[22]

Resistance to polymyxin B

This was determined according to the method described earlier.[23] Briefly, overnight cultures of the test organism in nutrient broth were smeared on the surface of nutrient agar plates with a sterile applicator. Bacteriological sensitivity disks (Hi-Media Laboratories, Bombay, India) containing 50 units of polymyxin B were placed on the medium aseptically. The plates were then incubated at 37°C overnight and the results were recorded. The presence of a zone of inhibition around the disk identified the organism as sensitive to polymyxin B.

Phage sensitivity

To determine the sensitivity of the *V. cholerae* cells to various phages (O149 specific for classical biotype and e4, e5 for El Tor biotype), logarithmic phase cells were overlaid with soft agar (nutrient broth containing agar 0·8%) on nutrient agar plates. Suitable dilutions of the phage were then spotted on the plates. Results (formation of zone of lysis) were read after incubation at 37°C for 16 h.

All the experiments were done at least three times.

Results

Analysis of the biotype of serogroup O1 strains

V. cholerae strains from cholera cases prevalent in India during the seventh pandemic before the recent epidemic were of El Tor biotype.[24] Against this background, the biotype of the serogroup O1 strains isolated at the same time or before O139 strains in Madras was examined. This was done by analysing their sensitivity to polymyxin B and to vibriophages. El Tor strains are resistant to polymyxin B, sensitive to phages e4 and e5 and resistant to phage Φ149. Classical strains are sensitive to polymyxin B, resistant to phage e4 and e5 but sensitive to phage Φ149. Two O1 strains in the collection, i.e. strains M01 and M0477, although resistant to polymyxin B, were resistant to all three phages e4, e5 and Φ149, indicating that they are neither truly El Tor nor truly classical in their biotype character. All O139 strains examined for these attributes had properties identical with M01 and M0477.

RFP analysis of the ctx-gene

RFPs of the 14 outbreak strains, three environmental CT-producing *V. cholerae* non-O1 strains (VCE228, VCE232 and VCE353) and the two ref-

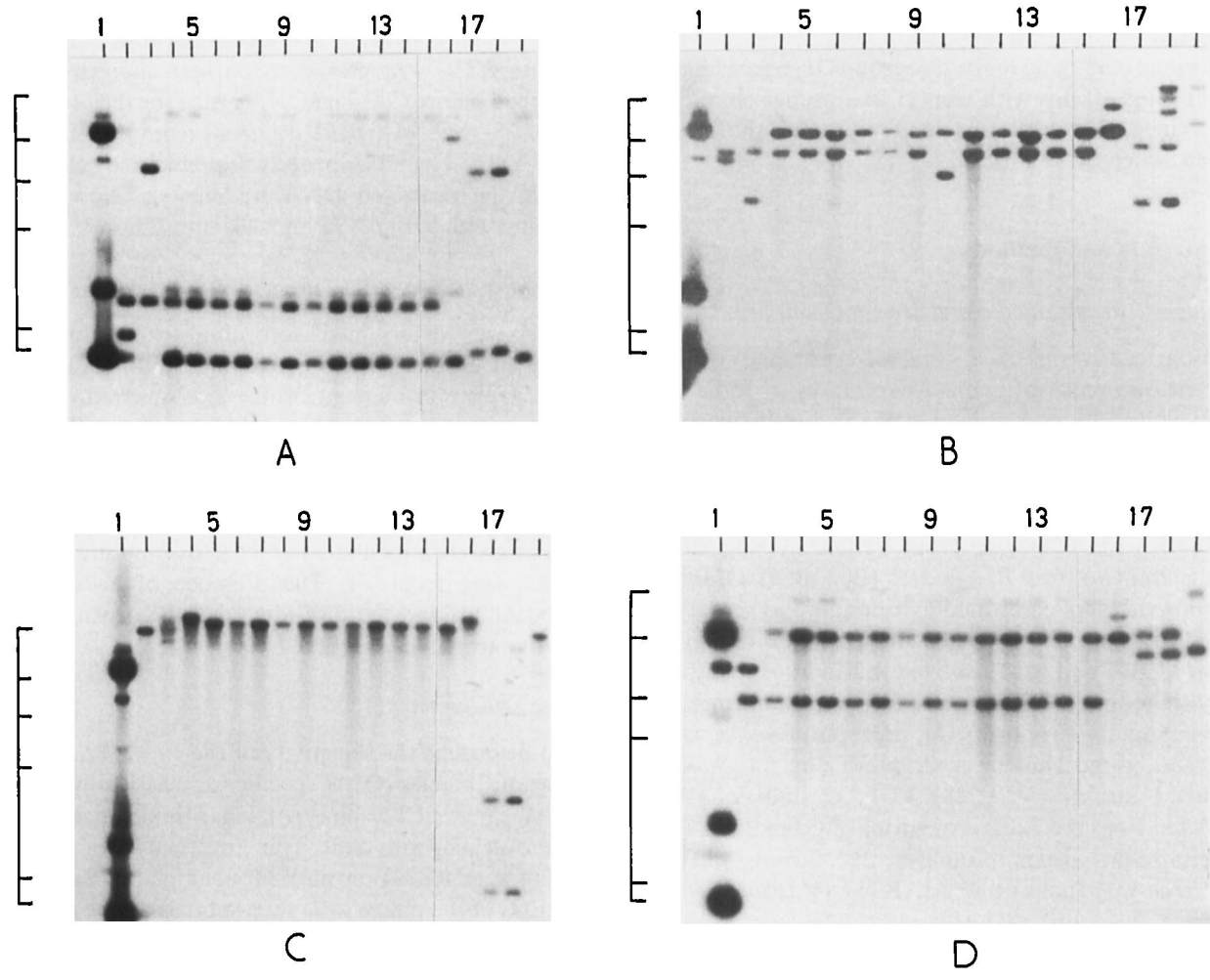

Fig. 1. Southern blot analysis of restriction fragment pattern of *ctx* gene among *V. cholerae* strains. Genomic DNA was digested with: panel **A**, *Xba*I + *Bgl*II; **B**, *Xba*I + *Bam*H1; **C**, *Hin*dIII; **D**, *Pst*1. Lane **1**, plasmid pCT5A11; **2–19**, MAK757, 569B, AP1, AP2, B02, B04, M01, M028, MD012, MD025, P02, P07, SG24, V015, V020, VCE228, VCE232, VCE353. Positions of λ-*Hin*dIII molecular size markers run on the same gel are indicated by bars from top downwards in kb: 23·13, 9·41, 6·55, 4·36, 2·32 and 2·0.

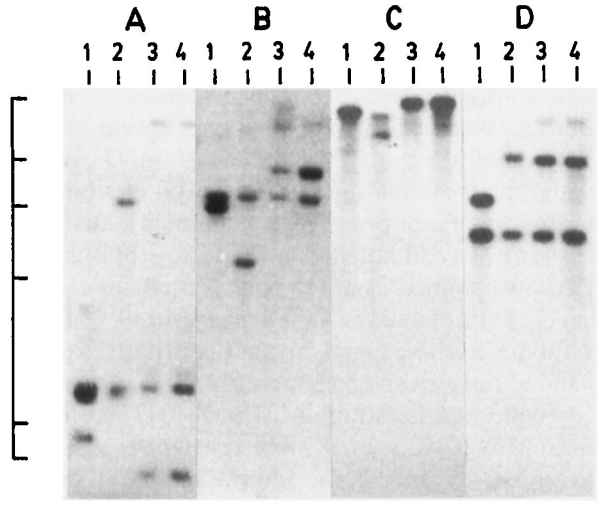

Fig. 2. Southern blot analysis of restriction pattern of *ctx* gene in *V. cholerae* isolates. All details are exactly as in fig. 1. Lanes **1–4**, MAK757, 569B, M0477, M028.

erence strains of *V. cholerae* (MAK757, 569B) were compared as shown in figs. 1 and 2. RFPs of the toxin gene exhibited by the outbreak strains of *V. cholerae* were very different from those of MAK757 and 569B (figs. 1 and 2). Ten of the 14 outbreak isolates produced identical patterns with respect to all the enzymes or enzyme combinations (*Xba*I, *Hin*dIII and *Xba*I + *Bgl*II). Of the remaining four, the pattern displayed by AP1 differed from those displayed by the other outbreak strains only with respect to *Hin*dIII (fig. 1, panel C), the MD012 pattern was different with respect to *Xba*I + *Bam*HI (fig. 1, panel B), and the pattern generated by V020 (fig. 1, lane 16 in all panels) was distinct with respect to all enzymes. Such variation in *ctx* RFP among the O139 isolates may be due to an instability of the *ctx* gene in the chromosomes of O139 isolates.[25]

It was also of interest that RFPs of strains M01 and M0477 (fig. 1, lane 8 and fig. 2, lane 3), which belong to serogroup O1, appeared to be identical to those of the O139 outbreak isolates and dissimilar from those of El Tor and classical strains, MAK757 and 569B.

Presence of multiple copies of ctx gene in outbreak isolates

We have shown elsewhere that four of the O139 isolates carry an identical large number of copies of

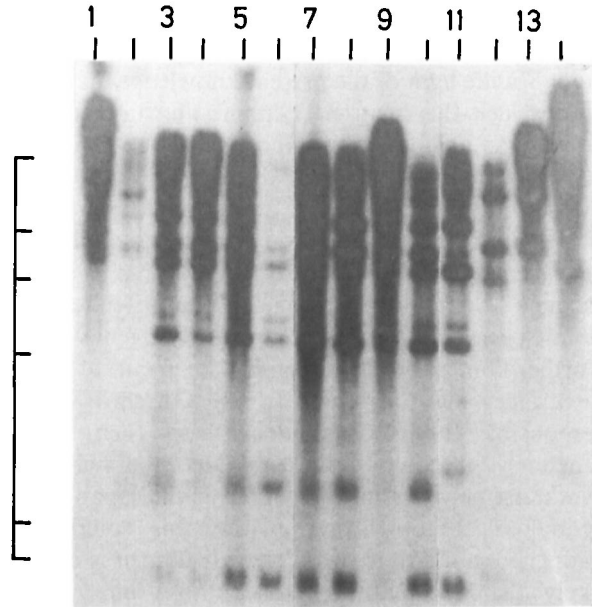

Fig. 3. Southern blot analysis of partial *Xba*I-*Bgl*II digests. Lanes **1–14**, MAK757, 569B, AP2, B04, M01, M0477, MD025, P02, P07, V015, V020, VCE228, VCE232, VCE353. Positions of λ-*Hin*dIII molecular size markers run on the same gel are indicated by bars, from top downwards in kb: 23·13, 9·41, 6·55, 4·36, 2·32 and 2·0.

the *ctx* gene on their chromosomes.[7] To determine if this was true generally for the outbreak isolates, a similar experiment was performed in which chromosomal DNA samples were digested partially with a combination of *Xba*I + *Bgl*II, electrophoresed on a gel, transferred to a membrane and then probed with the *ctx* gene probe. The profile obtained is shown in fig. 3. As has been described elsewhere,[7] such a pattern is possible only if the *ctx* gene is duplicated several times in the chromosome. Fig. 3 also reveals that the *ctx* gene is duplicated an identical number of times in all outbreak strains. Again of interest was the fact that strains M01 and M0477 produced profiles (fig. 3, lanes 5 and 6) which were identical with those produced by the O139 isolates and not with the ones produced by El Tor or classical type strains of *V. cholerae* O1.

Analysis of OMPs

The OMP of the 14 outbreak strains displayed strikingly similar profiles characterised by the presence of a major band of 37 kDa (fig. 4: panel A, lanes 4–15 and panel B, lanes 4 and 5). However, the pattern was very different from the profiles exhibited by all other strains studied. The predominant band in the strain 569B profile was of 40 kDa (fig. 4: lane 3, panel A; lane 6, panel B), whereas the profile of the strain MAK757 was characterised by the presence of two equally intense bands of 41 and 39 kDa (fig. 4: lane 2, panel A and lane 7, panel B). The patterns displayed by the environmental isolates VCE228 and VCE232 (fig. 4, lanes 2 and 3, panel B) were identical with that of the classical O1 strain 569B and that displayed by strain VCE353 (fig. 4, lane 1, panel B) was similar to the pattern displayed by strain MAK757. Interestingly, the OMP profiles of strains M01 and M0477 (fig. 4, panel A, lanes 8 and 9), which belong to serogroup O1, were identical with the OMP profiles of the other outbreak strains belonging to the O139 serogroup. Among the outbreak strains, minor differences were apparent among strains isolated from the same geographical location. For example, Amravati isolates P02 and P07 (fig. 4, lanes 12 and 13, panel B)—both of which were isolated from the same location and within the same time frame—displayed similar but distinct patterns.

Discussion

The genesis of the novel strain of *V. cholerae*, which appears to have epidemic and pandemic potential, has evoked global interest. As never before in the recorded

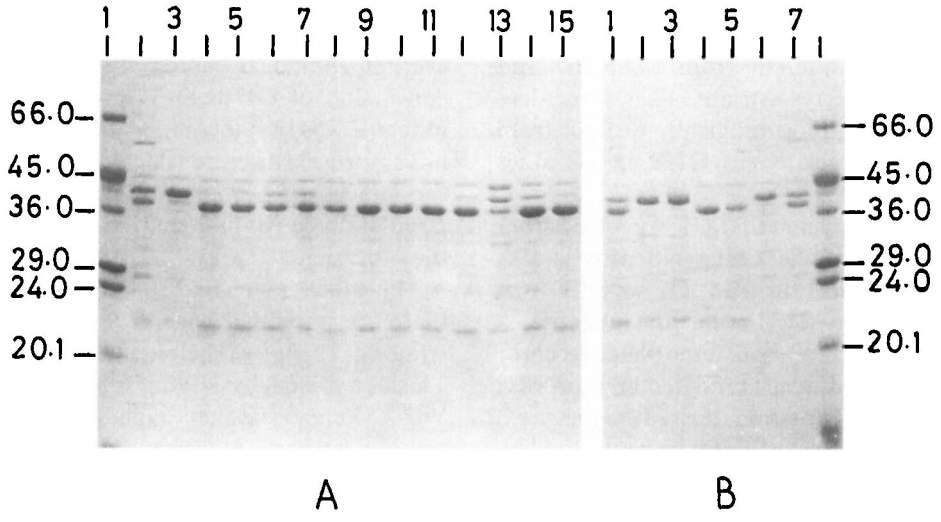

Fig. 4. SDS-PAGE of outer membrane of strains of *V. cholerae*. Panel **A**: Lane **1**, SDS mol. wt markers (Sigma MW-SDS-70L) from top downwards in kDa: bovine albumin 66·0; egg albumin 45·0; glyceraldehyde-3-dehydrogenase 36·0; carbonic anhydrase 29·0; trypsinogen 24·0; trypsin inhibitor 20·1; α-lactalbumin 14·2. Lanes **2–15**: MAK757, 569B, AP1, AP2, B02, B04, M01, M0477, MD012, MD025, P02, P07, SG24, M028. Panel **B**: lanes **1–7**, VCE353, VCE232, VCE228, V015, V020, 569B, MAK757; **8**, mol. wt standards as in lane 1, panel A.

history of the disease, a cholera epidemic has been caused by a non-O1 strain. The way the new strain evaded the pre-existing immunity against serogroup O1 prevalent in a cholera-endemic population has aroused great concern and is reminiscent of the antigenic variation observed in certain parasitic diseases.[26] There is already a great deal of speculation as to whether the new strain, recently assigned to the serogroup O139, is a mutant of the O1 serogroup. This conjecture is strengthened by the observation that recent cholera epidemics in India before the present outbreak were always due to serogroup O1, El Tor strain.[24] Co-isolation of serogroup O1 strains with O139 strains during the O139 epidemic and the similarity of behaviour of two O1 strains, M01 and M0477 with O139 isolates in respect of their phage and polymyxin B sensitivity, indicated a possible transition from serogroup O1 to O139 and suggested that strains M01 and M0477 could be the representatives of O1 strains from which the strain of serotype O139 could have evolved. To compare these two serogroup O1 strains with O139 at the molecular and genetic level, their OMPs and the RFP polymorphism of their *ctx* genes were analysed.

The outer membrane of *V. cholerae* strains contained three predominant proteins of 37–45 kDa. Even though the outbreak isolates were from diverse geographical locations, their OMP profiles displayed a striking homogeneity, and a pattern distinct not only from the ones exhibited by strains MAK757 and 569B but also from those produced by CT-producing non-O1 environmental strains. Furthermore, RFP analysis of the *ctx* gene clearly placed the outbreak strains as an entity distinct from the non-O1 environmental strains and from the reference strains representing the classical and El Tor biotypes of *V. cholerae* O1. All these factors taken together support the conjecture that O139 strains could be clonal in origin.[27]

Analysis of the above parameters for strains M01 and M0477 revealed very interesting features. Both these strains, although belonging to serogroup O1, had OMP and RFP patterns identical with those of the O139 serogroup but different from MAK757 and 569B, the representative type strains. Therefore, these are two strains which are serologically distinct from O139 but are closely related to O139 in all other attributes tested.

From these data it appears that O139 evolved from serogroup O1 strains of the kind typified by strains M01 and M0477. Transition from O1 to O139 was perhaps a multi-step event. In the first instance, a mutation leading to an alteration in the phage receptor site could have occurred which rendered an otherwise typical ancestral O1 El Tor strain resistant to phage e5. Consequently these strains could still be neutralised by the pre-existing immunity against O1 strains prevalent in the population in a cholera endemic area. In the next stage of evolution, perhaps a transition in the serotype from O1 to O139 occurred. This probably came about through a change in the *rfb* gene cluster[28] (responsible for the O-antigen biosynthesis) in the transitory O1 strains of *V. cholerae*, i.e. M01 and M0477, in this study. In the light of the present knowledge that O139 strains lack the sugar perosamine and instead have colitose as an integral component of the O-antigen LPS,[29] it is tempting to speculate that this was probably not the result of a single mutational event. One possibility is that an ancestral O1 strain acquired the genes encoding the pathway for colitose synthesis through horizontal gene transfer from a related bacterial species *via* a transducing and lysogenic phage. Furthermore, if the "acquired" segment of DNA integrated into the region of the O1 chromosome responsible for perosamine synthesis then, simultaneously, it could have eliminated the ability to synthesise perosamine and conferred the capability to synthesise colitose. Alternatively, this could have happened in multiple steps. Nevertheless, it is equally likely that the pathway for colitose biosynthesis already existed in the O1 strains in a cryptic form and at some stage of evolution this pathway was activated through some unknown mechanism. Many examples of the activation of cryptic genes by various mechanisms have been described. While it is not possible to know exactly what happened, nucleic acid sequence comparison of the *rfb* genes of O1 and O139 strains may throw some light on the probable mechanisms.

Finally, it is tempting to pose the question "why has this kind of conversion not taken place more frequently?" While no firm evidence is available, one could speculate that if this conversion involved gene transfer in the environment, then it had to be a very rare event. For such an event to take place it required the concurrence of not only the proximal presence of two genetically distinct organisms taking part in the gene transfer event but also of conditions conducive to the process. The natural aquatic environment is known to be highly variable, with changes in temperature, pH, nutrient availability etc., occurring on both daily and seasonal basis.[30] It may be significant in this aspect, that many of the O139 isolates are known to harbour prophage (unpublished observations). However, it should be noted that no matter how the conversion of O1 to O139 took place, the ultimate outcome was that these new strains (O139) acquired a novel surface structure which permitted them to elude the pre-existing immunity in the cholera-endemic population of Madras and then to spread rapidly to the other areas.

The other interesting finding in this study is that there are micro-heterogeneities among the outbreak strains of *V. cholerae* belonging to the O139 serogroup. This is typified by strains like P07 (Amravati) and V020 (Vellore) which agglutinate with the O139-specific antisera but show distinct OMP or RFP profiles in comparison with the other O139 strains. These strains again might represent important transitional strains which may bridge the gap in our understanding of how the new epidemic serogroup O139 came into being.

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