Molecular Characterization of *Vibrio cholerae* O1 Biotype El Tor Strains Isolated between 1992 and 1995 in Calcutta, India: Evidence for the Emergence of a New Clone of the El Tor Biotype

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Sixty-one clinical strains of *Vibrio cholerae* O1 El Tor isolated in Calcutta before, during, and after the *V. cholerae* O139 Bengal outbreak were examined to see if the O1 strains of the post-O139 period were different from those in existence before. Comparison of the restriction fragment length polymorphism of the rRNA genes (ribotyping) and the CTX genetic element revealed that all "before" strains except 1 belonged to a single known ribotype, whereas all "after" strains except 2 belonged to a hitherto undescribed ribotype. Also, 23 of 25 "before" strains harbored two or more copies of CTX in tandem and also a "free" RS1 element away from CTX, whereas 19 of 21 "after" strains had a single copy of CTX and no free RS1 element. CTX occupied different chromosomal locations in "before" and "after" strains. These studies clearly showed that El Tor O1 strains, which displaced *V. cholerae* O139 in Calcutta, belonged to a new clone and suggested that there is a continuous genetic reassortment among El Tor strains of *V. cholerae* O1.

In 1992, an unprecedented event occurred in the history of the disease cholera, with the emergence of a novel causative serogroup classified as *Vibrio cholerae* O139 Bengal [1]. On the basis of its ability to rapidly spread through all areas in India and neighboring countries in which cholera is endemic and the propensity of the new serogroup to replace the existing O1 El Tor biotype, *V. cholerae* O139 was suspected as the new pandemic strain of cholera [2]. In Calcutta during the height of the O139 epidemic, the El Tor biotype of O1 was briefly displaced for a period of 6 months between January and June 1993 [2]. Surprisingly, however, in subsequent years, the El Tor biotype of *V. cholerae* O1 reappeared in Calcutta and in most parts of the Indian subcontinent and replaced the O139 serogroup, again becoming the dominant serogroup causing cholera [3–5]. The disappearance and reappearance of the O1 El Tor biotype and its subsequent predominance in areas in which O139 dominated in the preceding year is difficult to explain.

We embarked on a study to determine the clonality of *V. cholerae* O1 strains isolated during different time periods (1992–1995) from hospitalized patients with cholera, admitted to the Infectious Diseases Hospital in Calcutta. Our first approach was to examine the O1 strains using traditional typing systems, such as biotyping, phage typing, and identification of antimicrobial susceptibility patterns. Apart from some variations in the antibiotic susceptibility patterns, we were unable to differentiate strains of O1 isolated before, during, and after the genesis of *V. cholerae* O139; all of the O1 strains belonged to the El Tor biotype [6]. This prompted us to examine the *V. cholerae* O1 strains isolated in Calcutta during the different time periods by ribotyping—a technique that has been successfully used to type O1 strains isolated over 60 years from different parts of the world [7]. A further characterization of the strains was attempted through the examination of the structural organization and chromosomal location of the CTX genetic element in these strains.

The CTX genetic element comprises a 4.5-kb core region carrying *ctxAB, zot, ace, cep,* and *orf*U genes, flanked by two or more copies of a 2.7-kb direct repeat sequence termed RS1 [8, 9]. The number and arrangement of the CTX elements are known to vary in different strains of *V. cholerae.* While 70% of the El Tor strains examined thus far have been found to carry only a single CTX element, the rest have two or more copies arranged in tandem. In contrast, classical strains carry two copies of the CTX element located in the different regions of the chromosome [10, 11].

On the basis of these two kinds of analyses, we report our endeavors to characterize at the molecular level the *V. cholerae* O1 El Tor strains isolated in Calcutta before and after the emergence of *V. cholerae* O139 Bengal.

Materials and Methods

Bacterial strains. A total of 61 strains of *V. cholerae* O1 biotype El Tor were selected from 548 O1 strains available. These strains were categorized into groups on the basis of the time of isolation in relation to the genesis of the O139 serogroup as shown in table 1. Group I included 25 strains selected from a total of 92 strains isolated before the emergence of the O139 serogroup in Calcutta (from April to November 1992). Group II included 15

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Table 1. Characteristics of *V. cholerae* O1 El Tor strains isolated during different time periods in Calcutta.

			Studied characteristics				
Group	Strain no.	Date of isolation	Ribotype	CTX RFLP pattern	No. of CTX elements	Chromosomal* location of CTX	Lysogenic phage
I	VC1	24/3/92	RI	A	Two	2	−
	VC2	1/4/92	RI	A	Two	2	−
	VC3	1/4/92	RII	A	Two	2	−
	VC5	2/4/92	RI	A	Two	2	−
	VC12	16/4/92	RI	A	Two	2	−
	VC14	24/4/92	RI	—	ND	ND	−
	VC20	7/5/92	RI	A	Two	2	−
	VC35	20/5/92	RI	A	Two	2	−
	VC41	26/5/92	RI	A	Two	2	−
	VC44	27/5/92	RI	A	Two	2	−
	VC48	2/6/92	RI	A	Two	2	−
	VC54	8/6/92	RI	A	Two	2	−
	VC55	11/6/92	RI	A	Two	2	−
	VC56	11/6/92	RI	A	Multiple	2	−
	VC59	17/6/92	RI	A	Two	2	−
	VC69	4/7/92	RI	A	Two	2	−
	VC70	23/7/92	RI	A	Multiple	2	−
	VC72	4/8/92	RI	A	Multiple	2	−
	VC73	7/8/92	RI	A	Two	2	−
	VC80	7/9/92	RI	A	Multiple	2	+
	VC91	20/10/92	RI	A	Two	2	+
	VC99	11/11/92	RI	A	Two	2	+
	VC100	12/11/92	RI	A	Two	2	+
	VC105	1/12/92	RI	Neither A nor B	One	2	+
	VC106	1/12/92	RI	A	Two	2	+
II	CO222	21/7/93	RI	A	Two	2	+
	CO327	28/9/93	RIII	A	One	1	−
	CO334	29/9/93	RI	A	Two	2	−
	CO366	1/10/93	RIII	B	One	1	+
	CO370	7/10/93	RIII	B	One	1	+
	CO371	7/10/93	RI	A	Multiple	2	−
	CO374	8/10/93	RIII	B	One	1	+
	CO387	13/10/93	RIII	B	One	1	−
	CO394	15/10/93	RIII	B	One	1	+
	CO407	19/10/93	RIII	B	One	1	+
	CO416	1/11/93	RIII	B	One	1	−
	CO417	1/11/93	RIII	B	One	1	−
	CO423	4/11/93	RIII	B	One	1	−
	CO424	4/11/93	RIII	B	One	1	−
	CO427	5/11/93	RIII	B	One	1	−
III	CO458	28/3/94	RI	A	Two	2	−
	CO459	29/3/94	RIII	B	One	1	−
	CO460	29/3/94	RIII	B	One	1	−
	CO461	29/3/94	RI	A	Two	2	−
	CO471	11/4/94	RIII	B	One	1	−
	CO473	12/4/94	RIII	B	One	1	−
	CO474	12/4/94	RIII	B	One	1	−
	CO475	13/4/94	RIII	B	One	1	−
	CO580	16/4/94	RIII	B	One	1	−
	CO650	8/7/94	RIII	B	One	1	−
	CO720	11/8/94	RIII	B	One	1	−
	CO770	12/9/94	RIII	B	One	1	−
	CO810	19/10/94	RIII	Neither A nor B	One	2	−
	CO825	9/11/94	RIII	B	One	1	−
	CO835	6/12/94	RIII	B	One	1	−
	CO839	9/1/95	RIII	B	One	1	−
	CO840	3/2/95	RIII	B	One	1	−
	CO860	25/4/95	RIII	B	One	1	−
	CO910	17/5/95	RIII	B	One	1	−
	CO950	7/6/95	RIII	B	One	1	−
	CO970	16/6/95	RIII	B	One	1	−

NOTE. Dates of isolation are given as day/month/year. ND, not determined.

* Site 1 is chromosomal location common to both 569B and MAK757; site 2 is location of other CTX element in 569B.

strains selected from a total of 23 strains of *V. cholerae* O1 isolated during the O139 epidemic in Calcutta (July to December 1993). Group III included 21 strains selected from a total of 433 strains of *V. cholerae* O1 isolated after the O139 epidemic in Calcutta (February 1994 to June 1995). The process of randomization in selection of strains was ensured by blinding the date of isolation to the person who selected the strains from a monthly batch of strains. Strains *V. cholerae* 569B (O1, Inaba) and *V. cholerae* MAK757 (O1, Ogawa) were used as the standard reference strains to represent the classical and El Tor biotypes, respectively. After selection, the strains of *V. cholerae* O1 representing different time frames were maintained in brain-heart infusion broth (Difco, Detroit) supplemented with 15% glycerol at −70°C. The strains of *V. cholerae* O1 were routinely grown as described before [12].

Southern blotting and DNA hybridization. Chromosomal DNA was prepared as reported earlier [13]. Samples of 2 μg of the DNA preparations were digested with a variety of restriction endonucleases (Promega, Madison, WI) according to the manufacturer's instructions. Restricted fragments were separated by electrophoresis through 0.7% (wt/vol) gels and Southern-hybridized as described [13].

The DNA probe for the *ctx+RS1* and *RS1* was a 1.8-kb *Xba*I+*Bgl*II and 2.7-kb *Not*I fragment, respectively, from the plasmid pCT5A11 [14]. A 0.38-kb *ctxB* probe spanning from base 1339 (*Nde*I site) to base 1727 [15] was a gift from Y. Singh (Centre for Biochemical Technology, New Delhi). The *zot* probe used was a 0.7-kb *Eco*RI+*Pst*I fragment from pMZP11 [16]. A 0.8-kb *Sma*I fragment and 1.5-kb *Sst*I+*Pvu*II fragment of plasmid pKK3535 containing the 16S and 23S rRNA *Escherichia coli* genes, respectively [17], were used as *rRNA* probes. These were labeled separately and mixed together in equimolar amounts for hybridization. DNA from phage ϕO139 was prepared as described elsewhere [18].

Probes were prepared as described before [13], followed by labeling with [^{32}P]dATP (specific activity, 3000 Ci/mmol^{-1}; Bhabha Atomic Research Centre, Mumbai, India) by nick translation [19].

Results

From a total of 548 consecutive *V. cholerae* O1 strains isolated from hospitalized cholera patients, 61 were randomly selected to represent three distinct periods of time in relation to the genesis of the O139 serogroup. The relative prevalence of *V. cholerae* O1 and O139 serogroups in Calcutta during the period of the O139 outbreak (July 1993 to December 1993) was 10.5% and 89.5%, respectively, while the relative prevalence of the O1 and O139 serogroups after the O139 outbreak (February 1994 to June 1995) was 84.9% and 15.1%, respectively. The selected strains of *V. cholerae* O1 were analyzed for the restriction fragment length polymorphism (RFLP) of their rRNA genes (ribotyping) and CTX genetic element with respect to a number of enzymes.

Ribotyping. For ribotyping, the restriction endonuclease *Bgl*I was chosen because this enzyme had been shown to produce good discriminatory patterns for *V. cholerae* [7]. Three different ribotypes were observed among the strains examined in this study. These were designated as RI, RII, and RIII, as exemplified by lanes 1, 5, and 7, respectively, in figure 1. All group I strains with the sole exception of VC3 (table 1) belonged to ribotype RI. The strain VC3 was the only strain found to belong to ribotype RII, which differed from ribotype RI by the absence of a single band of 6.0 kb (figure 1, lane 5). In contrast, all *V. cholerae* O1 strains isolated after July 1993 (group II and III strains) with the exception of 5 (table 1) belonged to ribotype RIII, which differed from ribotype RI by the presence of a single extra band of 8.6 kb. The 5 exceptions, which were isolated after June 1993, were found to belong to ribotype RI.

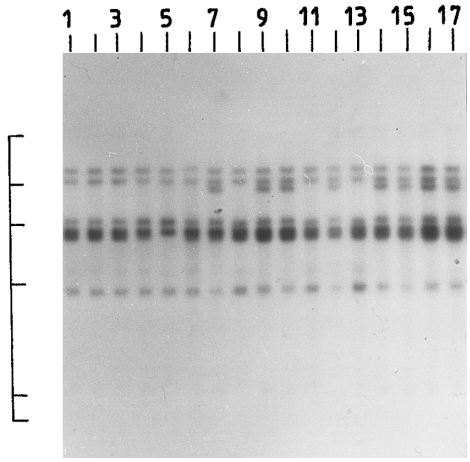


Figure 1. Ribotypes of *V. cholerae* O1 strains isolated before (group I), during (group II), and after (group III) *V. cholerae* O139 outbreak. Lanes 1–6: VC1, VC5, VC44, VC70, VC3, and VC106 (group I strains); lanes 7–10: CO327, CO371, CO387, and CO427 (group II strains); lanes 11–17: CO458, CO460, CO461, CO471, CO650, CO810, and CO970 (group III strains). Patterns for only few representative strains shown. Positions of λ-*Hin*dIII molecular size markers run on same gel are indicated at left: top to bottom, 23.13, 9.41, 6.55, 4.36, 2.32, and 2.02 kb.

RFLP of the CTX genetic element. RFLP of the CTX genetic element obtained with the enzyme combination *Xba*I+*Bgl*II, using the *ctxB* probe, exhibited four different patterns, with two patterns, designated as A and B (for example, figure 2A, lanes 3–9, and 2B, lanes 4 and 7–10), predominating. All group I strains except 2 displayed profile A (table 1), while all group II and group III strains, except 5 isolated after June 1993, displayed pattern B (table 1). The group I strain VC105 belonging to ribotype RI displayed a profile that was different from both A and B (figure 2A, lane 10). In case of VC14, no signal was obtained (table 1). The group II and III strains that belonged to RI displayed pattern A (table 1). Strain CO810, like VC105, was unique in the sense that even though it was a group III strain and like all other group III strains belonged to RIII, it had a CTX RFLP profile that did not match with that of any other strain (figure 2B, lane 5).

Organization and the chromosomal location of the CTX genetic elements. To examine how different these strains were from one another, the number, arrangement, and chromosomal

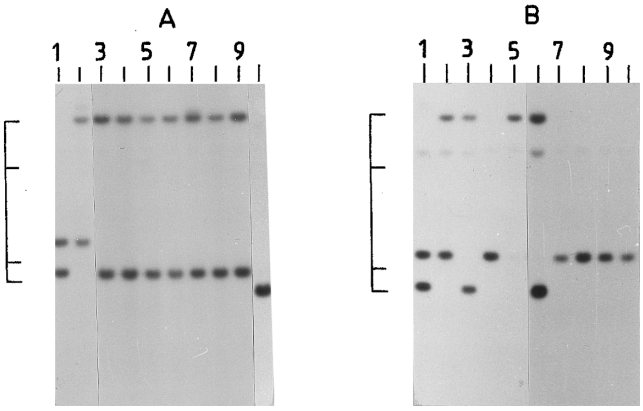


Figure 2. Southern blot hybridization of *Xba*I+*Bgl*II–digested *V. cholerae* O1 chromosomal DNA, with *ctxB* probe. **A**: Lane 1, MAK757; 2, 569B; 3–10 (strains isolated before O139 outbreak), VC1, VC5, VC3, VC44, VC70, VC100, VC106, and VC105. **B**: Lane 1, MAK757; 2, 569B; 3 and 4 (strains isolated during O139 outbreak), CO334 and CO387; 5–10 (strains isolated after O139 outbreak), CO810, CO461, CO471, CO460, CO650, and CO970. Patterns for only few representative strains are shown. Positions of λ-*Hin*dIII molecular size markers run on same gel are indicated at left: top to bottom, 6.55, 4.36, 2.32, and 2.02 kb.

location of the CTX genetic element in these strains were determined. Southern hybridizations were carried out with *ctxB, zot, ctx+RS1,* and *RS1* probes after digesting the chromosomal DNAs from these organisms with *Ava*I, *Bgl*II, *Pst*I, and *Xba*I, either separately or in combination. For comparison, 2 reference strains, *V. cholerae* 569B, which has two well-separated CTX elements on its chromosome, and *V. cholerae* MAK757, in which the tandemly duplicated CTX element occupies the same chromosomal location as one of the two in 569B, were also included in this analysis.

In case of single digestions, with the *ctxB* probe, all group I strains (except VC105) and 5 strains, CO222, CO334, CO371, CO458, and CO461, belonging to groups II and III exhibited two bands in the autoradiograph, as shown for the representative strains VC1 and CO371 (figure 3A–D, lanes 3 and 4). In contrast, all group II and III strains, except the 5 mentioned above, displayed only one band (figure 3A–D, lane 5).

In the case of strains typified by VC1 in addition to the 6.5-kb *Ava*I (figure 3A, lane 3, band d) and 11-kb *Xba*I, *Pst*I, and *Bgl*II (figure 3B–D, lane 3, bands e, h, and k), which were in common with the corresponding bands obtained for 569B with these enzymes (figure 3B–D, lane 2, bands e, h, and k), a 7.2-kb fragment (figure 3A–D, lane 3, bands c, g, i, and m) hybridized with the *ctx*B probe. As all of these enzymes bear a single site within the CTX+RS1 region [8, 9] and since the size of this region is 7.2 kb (4.5 kb + 2.7 kb) [8], it could be deduced that group I strains typified by VC1 had two copies of the CTX elements in tandem with one of the two elements flanked by RS1 on either side.

Bands on the autoradiograph other than the 7.2-kb band could originate only from fragments generated by one restriction endonuclease site present within CTX+RS1 and the other one in the adjacent chromosomal segment downstream of CTX. Since all of these bands were common to both 569B and VC1, it could be deduced that the tandemly duplicated CTX element in VC1 occupies a chromosomal site identical to that occupied by one of the two elements in 569B. It could also be deduced, from the presence of two bands of 2.2 and 6.5 kb in the Southern blot profile of *Xba*I+*Bgl*II–digested DNA (figure 2A, lanes 3–9), that it is the first CTX element that is flanked by RS1 on both sides.

Hybridization of the same blot with *ctx+RS1* probe, after stripping, gave rise to five bands of 11.5, 6.5, 5.2, 2.7, and 2.2 kb (figure 3E, lanes 1–3, bands n, o, p, q, and r, respectively), two of which, the 6.5- and 2.2-kb bands, were identical to those obtained with the *ctxB* probe (figure 2A, lanes 3–9). Since the data obtained with *ctxB* probe already established that there was only one RS1 element between the two ''core'' regions and that the second ''core'' element had no RS1 element downstream, the 2.7-kb band could come only if the first core element in the strains typified by VC1 had two upstream RS1 elements in tandem (figure 3, bottom). Of the two remaining bands of 11.5 kb and 5.2 kb (figure 3E, lanes 1–3, bands n and p), while one could arise from the region upstream of the CTX element, the origin of the other band could not be explained on the basis of the deduced structure of the CTX element of VC1 strains. Subsequently, it could be demonstrated that the other band originated from a ''free'' RS1 element present elsewhere in the chromosome of VC1 type strains (see below).

It was established that 3 strains, VC70, VC72, and VC80, in this collection had only one RS1 element upstream of the first ''core'' element. Correspondingly, the 2.7-kb band (figure 3E, lanes 1–3, band q) was missing in the hybridization pattern of the *Xba*I+*Bgl*II–digested DNA with the *ctx+RS1* probe (figure 3E, lane 4). The CTX genetic element in VC70, VC72, and VC80 and also in VC56 and CO371 was found to be tandemly duplicated several times, but its chromosomal location was the same as that found in other VC1 type strains. Also, they all carried a ''free'' RS1 element. The strain VC105 was like VC1, but its CTX element was not duplicated and it did not carry a ''free'' RS1 element (table 1, data not shown).

In an essentially similar manner, all group II and III strains (except the 5 mentioned earlier) were shown to have a single copy of the CTX genetic element (figure 3F, lanes 2 and 3) with the structure as shown for CO471 (figure 3, bottom). Chromosomal location of the CTX element in all of these strains except CO810 was identical to that found in MAK757. The CTX element in CO810 occupied the same site as that in VC1 (table 1, data not shown).

Structure of the sequences upstream of the common CTX elements were determined from the Southern hybridizations with the RS1 probe. From a detailed analysis of the profiles obtained (figure 4, top), the structure of the regions upstream of the CTX elements in VC1, CO471, 569B, and MAK757 was deduced (figure 4, bottom). In the case of VC1 type strains,

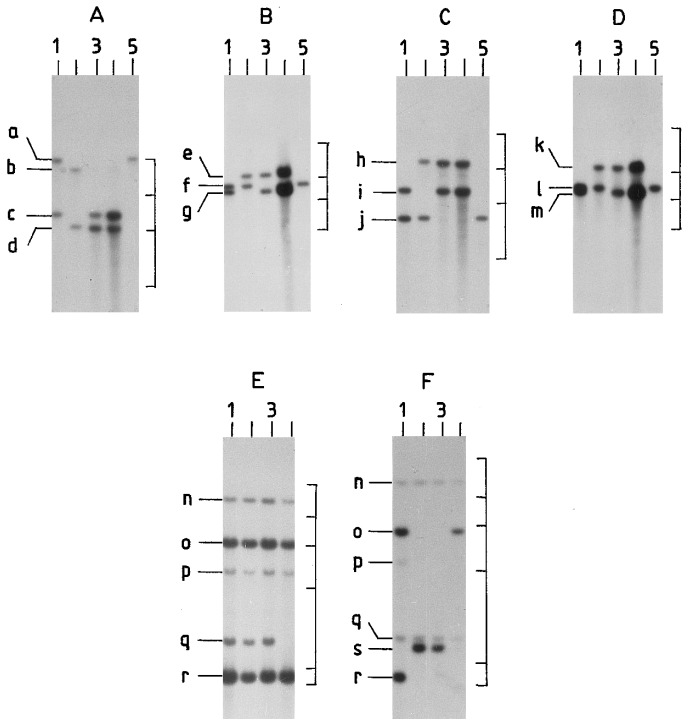
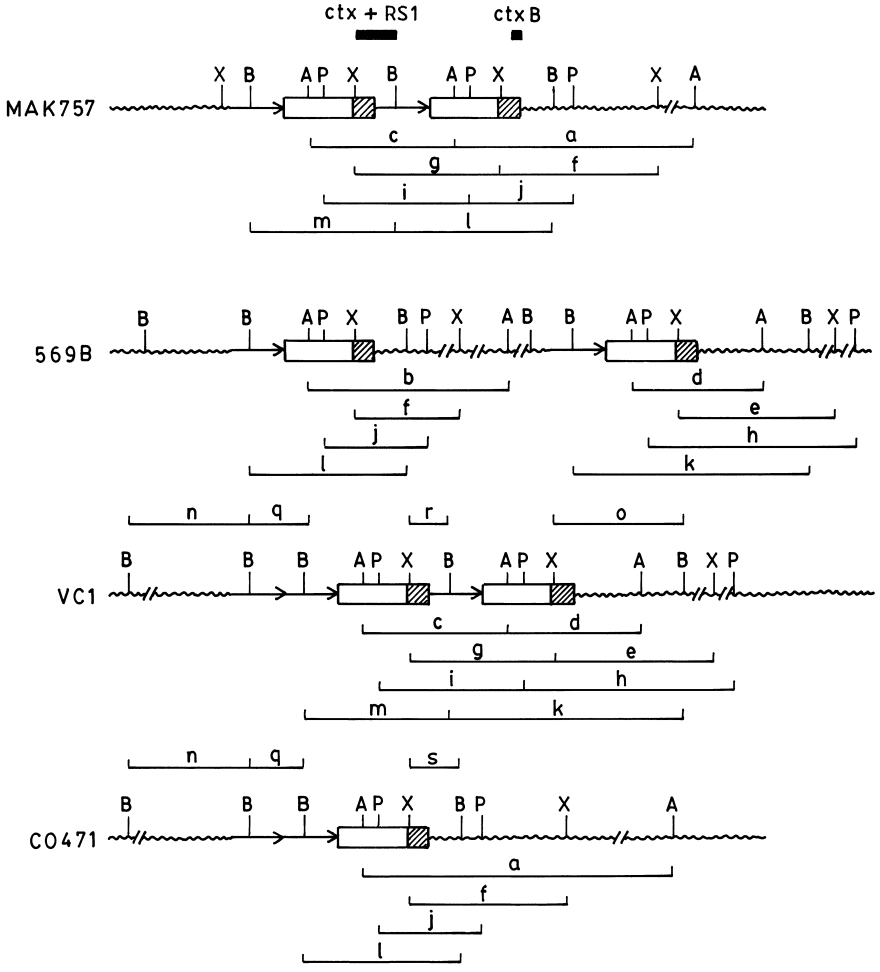
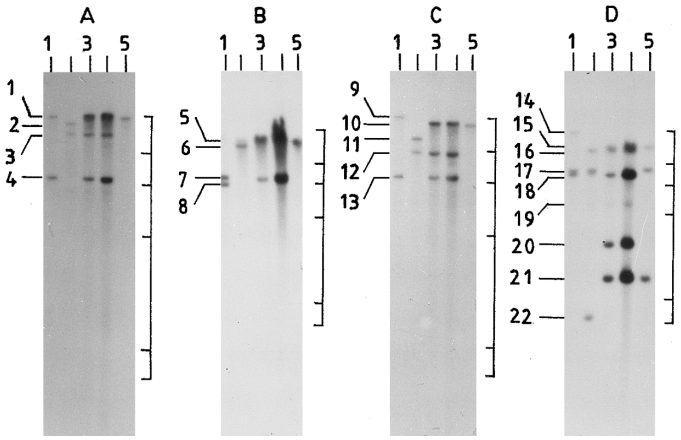


Figure 3. Top: Southern blot hybridization of *Ava*I (**A**)-, *Xba*I (**B**)-, *Pst*I (**C**)-, *Bgl*II (**D**)-, and *Xba*I+*Bgl*II (**E** and **F**)–digested *V. cholerae* O1 chromosomal DNAs with *ctxB* (**A**–**D**) and *ctx*+*RS1* (**E**, **F**) probes. **A**–**D**: Lanes 1, MAK757; 2, 569B; 3, VC1; 4, CO371; 5, CO471. **E**: Lane 1, CO371; 2, VC1; 3, VC56; 4, VC70. **F**: Lane 1, CO458; 2, CO387; 3, CO471; 4, CO810. Band designations correspond to schematic diagram below. Positions of λ-*Hin*dIII molecular size markers run on same gel are at right: top to bottom, 23.13, 9.41, 6.55, and 4.36 kb for **A**–**D** and 23.13, 9.41, 6.55, 4.36, 2.32, and 2.02 kb for **E** and **F**. Patterns for only few representative strains shown. Bottom: Schematic diagram (not to scale) of CTX genetic element, as deduced from Southern hybridization data above. Arrows and boxes correspond to RS1 and ''core'' of CTX genetic element, respectively, with hatched portion in boxes representing *ctx* operon. Letters correspond to bands in top section. Locations of *ctxB* and *ctx*+*RS1* probes are shown by filled boxes. Restriction site abbreviations: B, *Bgl*II; A, *Ava*I; P, *Pst*I; X, *Xba*I.





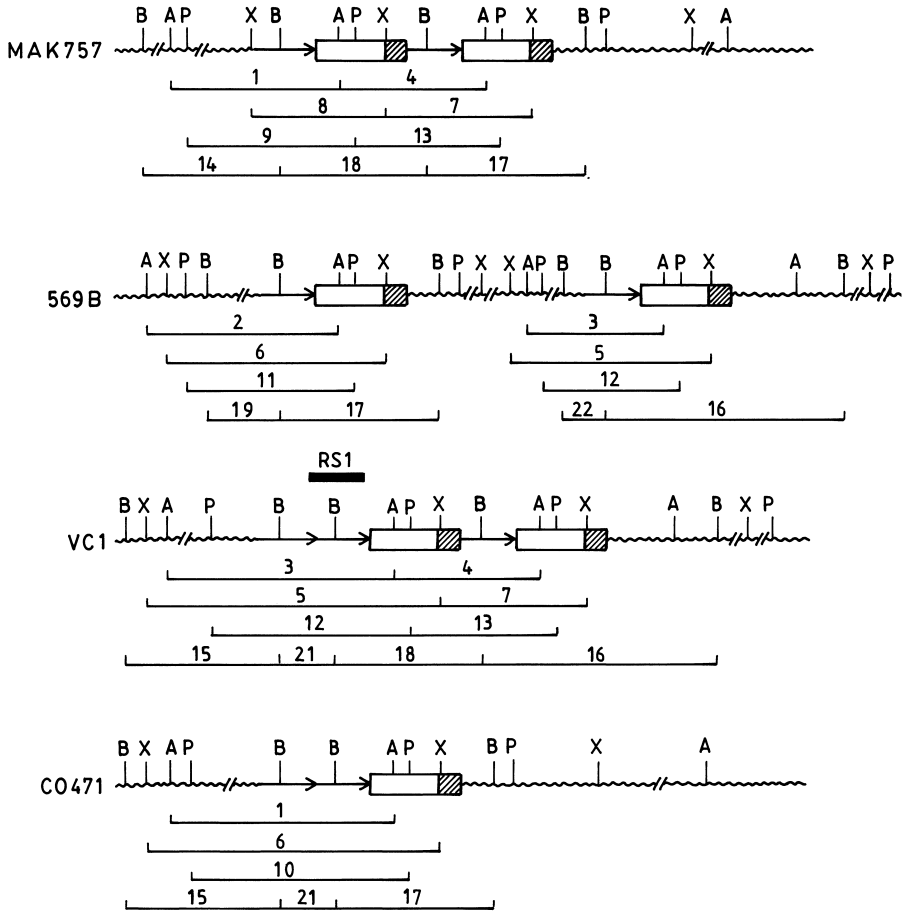


Figure 4. Top: Southern blot hybridization of *Ava*I (**A**)-, *Xba*I (**B**)-, *Pst*I (**C**)-, *Bgl*II (**D**)-, and *Xba*I+*Bgl*II (**E** and **F**)–digested *V. cholerae* O1 genomic DNA using *RS1* probe. Lanes: 1, MAK757; 2, 569B; 3, VC1; 4, CO371; 5, CO471. Band nos. correspond to schematic diagram at bottom. Patterns for only representative strains are shown. Positions of λ-*Hin*dIII molecular size markers run on same gel are indicated at right: top to bottom, 23.13, 9.41, 6.55, 4.36, 2.32, and 2.02 kb. Bottom: Schematic diagram of CTX genetic element (not to scale) as deduced from Southern hybridization data. Arrows and boxes correspond to RS1 and core region of CTX genetic element, respectively, with hatched portion in box representing *ctx* operon. Nos. correspond to bands in top section. Location of *RS1* probe is shown by filled box. Restriction site abbreviations: B, *Bgl*II; A, *Ava*I; P, *Pst*I; X, *Xba*I.

the derived structure, however, could not account for the 23-kb *Ava*I, 19-kb *Xba*I, and 21-kb *Pst*I bands (figure 4A–C, lane 3, bands 1, 5, and 10) and suggested the presence of another CTX element or a ''free'' RS1 elsewhere in the chromosome. The possibility of another CTX element was ruled out from the presence of only two bands in the Southern blot when the *ctx*B probe was used (figure 2A, lanes 3–9). Evidence in support of an independent RS1 came from the Southern hybridization experiments with *Bgl*II-digested DNA. Instead of four bands, as would be expected if no free RS1 element were present, a total of six bands—11.5 (doublet), 7.2, 5.2, 3.5, and 2.7 kb—appeared in the autoradiograph (figure 4D, lane 3, bands 15, 16, 18, 19, 20, and 21). It could be deduced that of these six bands, the 3.5-kb band and one other band (either a 11.5-kb or the 5.2-kb one) originated from the *Bgl*II cleavage of the independent RS1 element.

Further confirmation for this came from the fact that when the restriction endonuclease *Hin*dIII, which cuts outside the CTX genetic element [10], was used, two bands lighted up in the Southern blot with the RS1 probe (figure 5B, lanes 3 and

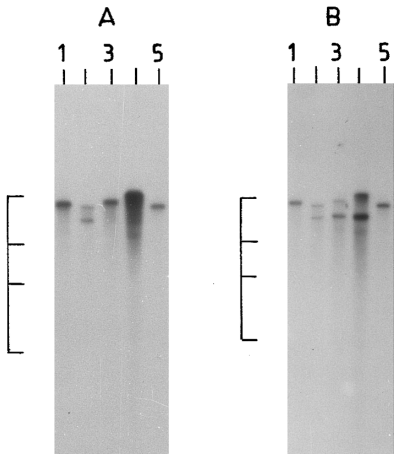


Figure 5. Southern blot hybridization of *Hin*dIII-digested *V. cholerae* O1 chromosomal DNA using *ctxB* (**A**) and *RS1* (**B**) probes. Lanes: 1, MAK757; 2, 569B; 3, VC1; 4, CO371; 5, CO471. Positions of λ-*Hin*dIII molecular size markers run on same gel are indicated at left: top to bottom, 23.13, 9.41, 6.55, and 4.36 kb.

4). In contrast, only one band appeared when the *ctx*B probe was used (figure 5A, lanes 3 and 4).

The results clearly demonstrated that the structure of the CTX genetic elements and their chromosomal locations were different in strains of *V. cholerae* O1 isolated before and after the onset of the O139 epidemic. It was also seen that pre-O139, O1 strains except VC105 harbored a copy of a ''free'' RS1 element in the chromosome. These observations taken together with the ribotype data provided strong support for the notion that pre- and post-O139 strains of *V. cholerae* O1 arose out of two independent clones.

Presence of the lysogenic phage ϕO139 (also called K139) in pre- and post-O139 strains of V. cholerae O1. Hybridization analysis (data not shown) revealed that only 12 strains of the 61 in our collection harbored this phage. Of these, 6 belonged to group I and the other 6 belonged to group II. None of the group III strains, that is, strains isolated after the O139 outbreak, carried this phage (table 1).

Discussion

Several recent events in the epidemiology of cholera remain unexplained. Prominent among these enigmatic events are the sudden entry of the El Tor biotype of *V. cholerae* O1 into Latin America after 100 years of absence in that continent [20], the restrictive distribution of the classical biotype of *V. cholerae* O1 in southern Bangladesh [21, 22], the explosive emergence of *V. cholerae* O139 [23, 24], and the recent resurgence of the El Tor biotype of *V. cholerae* O1 in areas where serogroup O139 prevailed during the preceding year [2, 4]. With the availability of sophisticated molecular typing methods, these seemingly random events appear to fall into a pattern suggesting that multiple clones of *V. cholerae* O1 exist at any given time and that some clones are endowed with a property that enables them to flare into epidemics.

Using traditional typing systems, such as serogrouping, biotyping, and phage typing, it appeared that the El Tor biotype of *V. cholerae* O1 reappeared unaltered in Calcutta, after being temporarily displaced [2]. Pulsed-field gel electrophoresis, however, revealed a new clone of the El Tor biotype that was different from both the El Tor O1 serogroup that prevailed before the advent of the O139 serogroup in Calcutta and the four toxigenic clonal groups of the El Tor biotype of *V. cholerae* O1 [25] currently prevalent in the world [26].

In this study, the El Tor *V. cholerae* O1 strains isolated in Calcutta during different time periods were more rigorously characterized by recently developed molecular typing methods. When analyzed by the standardized ribotyping scheme proposed for *V. cholerae,* strains designated ribotype RI in this study were identical to ribotype 6a, displayed by the Indian subcontinent strains of 1992, in the collection examined by Popovic et al. [7]. Interestingly, ribotypes RII and RIII of this study did not match with any of the profiles presented in the standardized scheme [7], suggesting the evolution of two more ribotypes among the El Tor biotype of *V. cholerae.* Further analysis of the ribotyping data revealed that although RI was occasionally observed among strains isolated after June 1993 (group II and III strains), RIII was not seen among the strains isolated before November 1992 (group I strains) (table 1), thereby attesting to the novelty of this ribotype.

RFLP analysis of the CTX genetic element showed that with the exception of a few strains, all group II and III strains (strains isolated after June 1993) displayed CTX RFLP profiles distinct from those displayed by group I strains (isolated before the onset of the O139 epidemic) (table 1). An excellent correlation between the CTX RFLP patterns and ribotyping results could thus be seen.

One particularly interesting observation to emerge from this study was that even though the pre-O139 strains had a ribotype pattern already described [7], the location of the CTX genetic element in these strains was different not only from the post-O139 strains but also from all other El Tor strains examined thus far [10]. Also, none of the strains isolated after February 1994, that is, after the subsidence of the O139 outbreak, was lysogenic for phage ϕO139 [18, 27] (also called K139 [28]). All of these data taken together thus provided strong evidence that the El Tor biotype of *V. cholerae* O1, which reappeared after being temporarily displaced, belongs to a new clone distinct from those that existed earlier. Our experiments also revealed a certain degree of instability of the CTX genetic element in the chromosomes of the *V. cholerae* O1 El Tor strains, making it tempting to speculate that the chromosome of the El Tor biotype of *V. cholerae* O1 could be in a state of flux and that the emergence of new clones could be linked to this in a yet undetermined way.

In a recent paper, tantalizing evidence showed that the presence of the lysogenic phage K139 [28] (same as phage ϕO139 [18, 27]) could enhance the virulence properties of *V. cholerae*

O1. It was thus conceivable that the presence of this phage conferred some advantage to *V. cholerae* O1 strains that emerged after the advent of the O139 serogroup over those prevailing before the O139 outbreak. However, the absence of this phage in all group III strains ruled out this possibility. Our efforts at present are directed towards understanding the basis of the ''greater fitness'' of the new clone over the older one.

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