BACTERIAL PATHOGENICITY

Response of wild-type mutants of *Vibrio cholerae* O1 possessing different combinations of virulence genes in the ligated rabbit ileal loop and in Ussing chambers: evidence for the presence of additional secretogen

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Five wild-type mutant strains of *Vibrio cholerae* serogroup O1 that lacked the CTX virulence cassette, or contained a natural deletion of a virulence gene within the CTX virulence cassette, or possessed an additional virulence gene, along with a prototype toxigenic strain representing the El Tor classical biotypes were examined by in-vivo and in-vitro methods to determine their enterotoxic potential. The ability of whole cells and culture supernates of the strains to cause fluid accumulation in the rabbit ileal loop model revealed a pattern consistent with the presence of the various virulence gene(s), with those possessing the intact CTX virulence cassette being the most secretogenic. Culture supernates of strains without the CTX virulence cassette or the strain with an incomplete cassette were also able to evoke mild to moderate fluid accumulation in the rabbit ileal loop. Of the various media used, AKI and brain heart infusion broth appeared to support the production of a hitherto unknown secretogenic factor, because culture supernates of the non-toxigenic *V. cholerae* O1 strains showed higher fluid accumulation ratios when grown in these media than in the others. To confirm that the fluid accumulation elicited by these strains in the ileal loop was due to enterotoxin activity, the effect of supernate of the strains was examined in rabbit small intestine mounted on Ussing chambers. Increases in short circuit current and tissue conductance, as compared with the medium control, were observed even with the strains that did not possess the CTX virulence cassette, confirming their ability to disrupt the function of intestinal tissue. From these studies, it was concluded that strains of *V. cholerae* O1 devoid of the CTX virulence cassette were still able to elicit a secretory response in the ileal loop and displayed enterotoxic activity in an in-vitro experimental model.

Introduction

Cholera, an acute disease characterised by copious uncontrolled purging of 'rice-water' stools leading to severe electrolyte depletion, dehydration, acidosis, shock and, if left untreated, to death, is caused by *Vibrio cholerae* belonging to either the O1 or O139 serogroups [1, 2]. The clinical state of cholera is

principally attributed to an oligomeric protein known as cholera toxin (CT) that activates adenylate cyclase, resulting in increased levels of cyclic AMP leading to hypersecretion of salt and water [3]. A mélange of other putative toxins produced by *V. cholerae*, such as the El Tor haemolysin [4, 5], zona occludens toxin (Zot) [6] and accessory cholera toxin (Ace) [7] are believed to exacerbate the fluid secretion process and thereby contribute to the disease, but in ways not precisely discerned as yet. A dynamic 4.5-kb core region [8], termed the CTX virulence cassette [7], has now been identified in toxigenic *V. cholerae* O1 and O139 strains that, apart from carrying the CT genes (*ctxAB*), also carries the gene encoding a pilin-like

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intestinal colonisation factor [9], the gene encoding Zot [10] and the gene encoding Ace [7]. Most recent studies show that the structural genes for CT reside on the genome of a filamentous, lysogenic phage called CTX ϕ [11].

The development of a safe, attenuated, live oral recombinant cholera vaccine has been plagued with the problem of reactogenicity of the vaccine strains manifested as residual diarrhoea. Various attenuated *V. cholerae* O1 recombinant vaccine candidates have been constructed over the past decade. These strains were clearly incapable of causing severe diarrhoea, but were still capable of inducing unacceptable levels of mild diarrhoea in human volunteer studies [12, 13]. However, it must be mentioned that one recombinant *V. cholerae* O1 vaccine strain (CVD103.HgR) derived from the classical Inaba strain 569B has proved to be well tolerated and immunogenic in extensive trials in different countries [14]. A recent study examined a large collection of strains of *V. cholerae* of diverse origins with a battery of DNA probes specific for CT, Zot, Ace and E1 Tor haemolysin and was able to detect strains of *V. cholerae* O1 with natural deletion of the *ctxA* gene within the CTX virulence cassette [15]. The availability of these wild-type mutants of *V. cholerae* O1 provided the impetus for the present study. The objective was to assess the enterotoxicity of these wild-type mutants to see whether these strains were capable of disrupting the function of intestinal tissue and thereby determine if other secretogens are involved in the pathogenesis of *V. cholerae* O1. Such a study could provide useful insights into the well-noted reactogenicity of vaccine strains of *V. cholerae* O1 lacking known toxins, including CT.

Materials and methods

Bacterial strains

Seven strains of *V. cholerae* O1 (GP5, GP6, GP12, GP71, GP156, V96 and VC20) were selected from our culture collection and used in this study. The choice of strains was dictated by the results of previous studies [15, 16] and the strains were selected to represent as many combinations as possible of the virulence genes as shown in Table 1. All the strains were biochemically characterised, including determination of polymyxin B sensitivity by methods described previously [17]. The strains were maintained as stabs in nutrient agar at room temperature and kept in the dark.

Detection of CT production

The test strains were grown in yeast extract peptone (YEP) [18] with shaking (120 rpm) at 37°C. The culture supernates of the *V. cholerae* O1 strains were examined for the presence of CT by a highly sensitive bead-ELISA [19]. Various dilutions of purified CT (Sigma) and uninoculated medium (negative control)

were run concurrently when the culture supernates of the test strains were assayed by the bead-ELISA.

Production of haemolysin

Brain Heart Infusion Broth (BHIB; Difco Laboratories, MI, USA) with NaCl 0.5% was used to grow the strains for determination of E1 Tor haemolysin production [20]. The strains were grown in 2 ml of BHIB in test tubes (10 × 13 cm) and also in 50 ml of BHIB contained in 250-ml Erlenmeyer flasks. Washed rabbit erythrocytes diluted to a final concentration of 1% in 10 mM phosphate buffer (pH 7.0) containing NaCl 1.3% were mixed with an equal volume of the culture supernate and incubated at 37°C for 1 h. The mixture was then centrifuged at 1000 *g* for 5 min and the amount of released haemoglobin in the supernate was measured spectrophotometrically at 540 nm.

Polymerase chain reaction assay

A multiplex polymerase chain reaction (PCR)-based assay was used to determine the presence of *ctxA* (encoding the enzymic subunit of CT) and *tcpA* (encoding the major structural subunit of TCP), while a single-format PCR assay was used for *toxR* (encoding the transcription activator ToxR), *zot* (encoding Zot) and *ace* (encoding Ace). Primer pairs were used to detect the following: a 301-bp fragment of *ctxA* [21], a 471-bp fragment of E1 Tor variant of *tcpA*, a 671-bp fragment of classical variant of *tcpA* [22], a 990-bp fragment of *toxR* [23], a 243-bp fragment of *zot* and a 284-bp fragment of *ace* [24].

The following were added to each 100 μ l of PCR mixture: 10 μ l of 10× PCR buffer (100 mM TAPS, pH 8.8; 500 mM KCl; 15 mM MgCl₂ and gelatin 0.1%), 2 μ l each of 2.5 mM dATP, dGTP, dCTP and dTTP, 100 pmol each of the primers and 2 U of *Taq* DNA polymerase (Takara Shuzo Co. Ltd, Japan). PCR was carried out in 0.5-ml thin-walled PCR tubes (Midwest, USA), with 42 μ l of the PCR mixture described above and 8 μ l of a Luria broth culture of the test strain, overlaid with mineral oil, heated at 94°C for 5 min and processed in a DNA thermal cycler (Biometra, Göttingen, Germany). PCR amplification was performed for 30 cycles and the cycling conditions for *ctxA*, *tcpA* and *toxR* were: denaturation at 94°C for 1 min, annealing at 60°C for 1.5 min and extension at 72°C for 1.5 min; for *zot* and *ace* the time for annealing and extension was 1 min each. A reagent blank (containing all the components of the reaction mixture and Luria broth instead of broth containing template DNA) and two *V. cholerae* O1 strains – one belonging to the E1 Tor biotype (VC20) and the other to the classical biotype (569B) – from our culture collection were run as controls. Amplified products from the PCR were electrophoresed on agarose 1.5% gels and were stained with ethidium bromide. ϕ X174-

Hae III (Takara Shuzo Co. Ltd) mol. wt marker was run concurrently.

Rabbit ileal loop assay

Challenge doses were prepared by growing the bacterial strains to mid-exponential phase in Tryptic Soy Broth (Difco) at 37°C followed by low speed centrifugation and resuspension to $c. 10^8$ bacteria/ml in phosphate-buffered saline (PBS, pH 7.2) for introduction into the ligated loops. Culture filtrates of the test strains, grown in various broths – i.e., AKI [25], YEP [18], BHIB, casamino acid-Yeast Extract medium with lincomycin 90 mg/L (CAYE-L) and syncase medium [26] – were obtained from the supernate which remained after centrifugation of the culture at 6000 *g* for 20 min in the cold.

Surgical procedures for the ligated intestinal loop test according to the method described by De and Chatterjee [27] were carried out on outbred New Zealand White rabbits of either sex weighing $c. 2.0$ – 2.5 kg. Briefly, laparotomy was performed on animals anaesthetised by intramuscular injection of ketamine (35 mg/kg body weight) and xylazine (5 mg/kg body weight). The small intestine was withdrawn and ligated at a distance of $c. 10$ cm from the ileocaecal region. Eight intestinal loops of 6–10 cm, separated by uninoculated segments of 1–2 cm, were made in each animal. Test loops were inoculated with 1-ml volumes of bacterial suspensions or culture supernates. Negative control loops received either PBS alone or the uninoculated medium, while the positive control loop received bacterial suspension of the prototype *V. cholerae* O1 biotype E1 Tor strain (VC20). Loops were replaced in the peritoneal cavity in the original position and the peritoneum was closed. After 18–20 h, the animal was killed with sodium pentobarbital and the abdomen was re-opened. The loops were taken out and the length of each loop in cm and the volume of the accumulated fluid in ml were measured. The extent of the fluid accumulation (FA) was expressed as the FA ratio.

Ussing chamber experiments

The test strains were cultured in 50 ml of AKI, YEP, BHIB, CAYE-L and syncase medium and incubated at 37°C with shaking (200 rpm). The cell-free culture filtrate obtained by centrifugation at 5000 *g* for 20 min and passage through a 0.22 μ m pore size disposable filter was lyophilised and resuspended in 15 ml of sterile double-distilled water. Ussing chamber experiments were performed with segments of small intestine from male adult New Zealand white rabbits (body weight 1.5–2.5 kg). Rabbits were anaesthetised by intramuscular injection of ketamine (35 mg/kg of body weight) and xylazine (5 mg/kg of body weight) and killed by air embolism. A 15-cm segment of distal ileum was teased loose from the mesentery, flushed

free of intestinal contents with Ringer's solution (115 mM NaCl, 25 mM NaHCO₃, 2.5 mM K₂HPO₄, 0.4 mM KH₂PO₄, 1.2 mM MgCl₂, 1.2 mM CaCl₂), opened along the mesenteric border, and stripped of muscular and serosal layers. Sheets of mucosa were then mounted on prongs in lucite Ussing chambers with an aperture of 1.13 cm² and bathed by freshly prepared Ringer's solution which was maintained at 37°C with water-jacketed reservoirs connected to a constant temperature circulating pump and gassed with O₂ 95% and CO₂ 5%. The potential difference and short circuit current (I_{sc}) were measured. The potential difference was allowed to reach a steady state. A 400 μ l volume of test supernate was added to either side of the mucosa. The changes in I_{sc} were recorded at 5-min intervals for the first reading and then at 15-min intervals up to 110 min. The non-parametric Kruskal-Wallis's test was applied to determine the statistical difference in variation in conductance of culture supernate of strains grown in different media.

Results

Phenotypic and genotypic traits of the strains

Of the seven strains included in this study, five were wild-type mutants that lacked all the virulence genes in the CTX virulence cassette (GP5, GP6, V96), or contained internal deletion within the CTX virulence cassette (GP71), or, in addition to an intact CTX virulence cassette, also carried *sto* and produced NAG-ST-like toxin (GP156) [16, 28] as shown in Table 1. The prototype *V. cholerae* O1 strain of the E1 Tor biotype was represented by strain VC20 [29] and the classical biotype was represented by strain GP12, and these were used as the positive controls. Strains GP156, GP12 and VC20 produced CT when examined by the bead-ELISA (Table 1) and yielded the 301-bp *ctxA* amplicon; the other strains were negative for the factors in both assays. All the strains yielded the 990-bp fragment of *toxR* amplicon whereas only four of the seven strains (GP12, GP71, GP156, and VC20) yielded the 243-bp *zot* amplicon and 284-bp *ace* amplicon. Apart from strain GP6, all strains yielded the *tcpA* amplicon. In five strains (GP5, V96, GP71, GP156 and VC20), the *tcpA* amplicon was 471 bp, which was consistent with the E1 Tor variant of the *tcpA* gene. The size of the *tcpA* amplicon of strain GP12 was 621 bp, suggesting that the strain belonged to the classical biotype. Also, strain GP12 was sensitive to polymyxin B, a trait which is consistent with the classical biotype. All the strains carried the structural gene (*hlyA*) for the E1 Tor haemolysin; only strains GP71 and GP156 produced E1 Tor haemolysin when grown in 50 ml cultures, but none of these strains produced the haemolysin when grown in 2-ml cultures. Strain VC20, the prototype *V. cholerae* O1 strain of E1 Tor biotype, is genotypically *hlyA*-positive, but shows no haemolytic activity *in vitro* and this is consistent

Table 1. Genotypic and phenotypic traits of the *V. cholerae* O1 strains examined in this study

Strain no.	Place of isolation	Year of isolation	Serotype	Genotype									Heat-stable enterotoxin gene (<i>sto</i>)*	CT production (ng/ml)
				Presence of virulence genes by PCR						<i>tcpA</i>				
				<i>hlyA</i>	<i>toxR</i>	<i>ctxA</i>	<i>zot</i>	<i>ace</i>	E1	Tor	Classical			
GP5	Macao	1970	Inaba	+	+	—	—	—	+	—	—	—	—	
GP6	Macao	1970	Inaba	+	+	—	—	—	—	—	—	—	—	
V96	India	1990	Ogawa	+	+	—	—	—	+	—	—	—	—	
GP12	India	1971	Ogawa	+	+	+	+	+	—	+	—	—	++	
GP71	India	1974	Ogawa	+	+	—	+	+	+	—	—	—	—	
GP156	Australia	1979	Inaba	+	+	+	+	+	+	—	—	+	++	
VC20	Calcutta (India)	1992	Ogawa	+	+	+	+	+	+	—	—	—	++	

*Data on *sto* from references [16, 28].

with the characteristics of recent E1 Tor strains which are non-haemolytic or weakly haemolytic.

Enterotoxigenicity of whole cells examined by rabbit ileal loop test

Of the whole cell preparations of the seven strains examined, four (GP5, GP6, GP71 and V96) were non-secretogenic or the amount of fluid accumulated in the test loops of rabbits was negligible; one strain (GP12) was moderately secretogenic and two (GP156 and the positive control strain VC20) induced fluid accumulation consistent with that of a fully toxigenic strain of *V. cholerae* (Table 2).

Enterotoxigenicity of strains grown in different broths in the rabbit ileal loop test

Culture supernates of the test strains grown in five different broth media showed variations in their ability to induce fluid accumulation in the ligated loop between media, but followed a general overall pattern that reflected the trend shown with whole cells (Table 2). Again, the secretogenicity of the test strains in ascending order was V96, GP71, GP5, GP6, GP12, VC20 and GP156. A revealing finding was that, of the media used, AKI and BHIB appeared to support the

production of an unknown secretogenic factor, because all the test strains showed higher FA ratios than with other media. This included strains GP5, GP6 and V96, which by definition are non-toxicogenic *V. cholerae* O1 and should have been benign in the rabbit ligated loop.

Enterotoxigenicity of strains in Ussing chambers

Culture supernate of strains of *V. cholerae* O1 grown in different media were added to rabbit ileal tissue mounted between lucite chambers maintained under conditions of ionic, osmotic and electrical equilibrium to investigate whether the supernates induced an increase in *I*_{sc}. A general pattern was observed, with the fully toxigenic strain VC20 inducing the largest increase in *I*_{sc} irrespective of the medium used (Table 3). Interestingly, the other fully toxigenic strain (GP156) did not induce changes in *I*_{sc} consistent with that of the presence of CT and NAG-ST in all media. In contrast, strain GP12 – which is a fully toxigenic classical strain – evoked higher *I*_{sc} changes than strain GP156, and was more or less similar to strain VC20 in this respect. The broth medium used appeared to effect changes in *I*_{sc} with strain V96, the non-toxicogenic strain, showing fairly high *I*_{sc} changes in most media except syncase. Again, compared with the toxigenic strains, the non-toxicogenic strains GP5 and GP6 showed

Table 2. Fluid accumulating ability of the strains of *V. cholerae* O1 in rabbit ligated ileal loop

Strain no.	Fluid accumulation ratio (ml/cm)					
	Whole cells*	Supernate of strain grown in†				
		AKI	YEP	BHIB	CAYE-L	syncase
GP5	0	0.49	0.23	0.41	0.29	0.25
GP6	0.10 (0.18)	0.55	0.37	0.67	0.22	0.13
V96	0.19 (0.23)	0.31	0.07	0.53	0.13	0.26
GP12	0.41 (0.14)	1.16	1.21	0.96	1.10	0.85
GP71	0	0.47	0.11	0.60	0.11	0.22
GP156	1.13 (0.19)	1.18	0.85	1.33	1.48	1.23
VC20	1.04 (0.18)	1.12	1.17	1.1	1.42	1.26
Medium	...	0.09	0.25	0.26	0	0.13

*Values are mean (SE) of experiments performed in five rabbits.

†Values are mean of experiments performed in two rabbits.

Table 3. Variation in Isc in Ussing chambers after addition of 400 µl of culture supernates of different wild-type *V. cholerae* O1 strains grown in AKI, YEP, BHIB, CAYE-L and syncase to both mucosal and serosal sides of rabbit intestinal tissue recorded after 35 min and 110 min

Strain no.	Isc									
	AKI		YEP		BHIB		CAYE-L		Syncase	
	35	110	35	110	35	110	35	110	35	110
GP5	22 (12)	33 (8)	31 (21)	44 (4)	32 (23)	54 (11)	42 (20)	46 (13)	34 (9)	48 (13)
GP6	48 (17)	49 (12)	47 (17)	56 (3)	49 (29)	48 (16)	13 (9)	27 (12)	45 (31)	38 (21)
GP12	52 (15)	76 (11)	33 (15)	67 (0)	29 (17)	79 (24)	32 (14)	49 (9)	63 (18)	75 (18)
GP71	19 (13)	39 (9)	38 (16)	56 (4)	27 (26)	60 (28)	33 (21)	55 (26)	34 (12)	43 (9)
GP156	20 (2)	56 (2)	45 (24)	81 (26)	11 (3)	49 (4)	48 (16)	85 (28)	35 (16)	71 (22)
V96	35 (10)	63 (6)	23 (13)	52 (10)	31 (15)	69 (7)	30 (13)	57 (19)	21 (5)	12 (1)
VC20	39 (23)	80 (15)	22 (12)	91 (23)	43 (29)	75 (7)	56 (37)	90 (30)	36 (13)	84 (25)
Medium	14 (6)	17 (5)	11 (6)	21 (5)	23 (4)	11 (3)	5 (8)	5 (7)	6 (3)	2 (0)

Values are mean (SEM) of four experiments.

lower changes in Isc over a period of time. Medium again played a role in changes in Isc with strain GP6 evoking hardly any change in Isc when grown in CAYE-L, but showing substantial changes in Isc, compared with the medium control, in all the other media used (Table 3).

The conductance values of culture supernates of different strains grown in different media are presented in Table 4. Conductance of a tissue is a useful index of the leakiness of the tissue – increased conductance indicating disruption of function of the tight junctions. Conductance was again influenced by the medium used and the least changes in conductance were observed in YEP and BHIB. For example, with the non-toxicogenic strains (GP5, GP6, V96), increases in conductance were observed when they were grown in AKI medium as compared with YEP and BHIB. In CAYE-L and syncase media, the two non-toxicogenic strains (GP5, GP6) did not evoke changes in conductance. This pattern in the changes of conductance was also consistent with all the other strains. However, the variation in conductance of the various strains grown in different media was not statistically significant.

Discussion

After more than 17 years of elegant and vigorous research on the development of oral recombinant

vaccine strains for cholera, it has become clear that the pathogenesis of cholera is not as straightforward as previously perceived. From the human volunteer studies with recombinant oral cholera vaccine strains [12], the involvement of another toxin in addition to those currently recognised or the process of colonisation, or both, have been attributed to be the cause of the mild to moderate residual diarrhoea [1, 12]. Interest in the ancillary secretogenic factors was further stimulated by the clinical observation of cholera-like diarrhoea caused by *V. cholerae* belonging to the non-O1 non-O139 serogroups. Although the percentage of such patients hospitalised at the Infectious Diseases Hospital in Calcutta is usually <4.9% [30], it presented an enigma as to how strains of *V. cholerae* devoid of the CTX virulence cassette were still able to cause a clinical condition very similar to cholera. To add to the mystery, non-toxicogenic strains of *V. cholerae* O1 associated with a cluster of cases of cholera were encountered recently in southern India [31]. All these events led to the design of the present study, to answer the basic question as to whether *V. cholerae* devoid of the CTX virulence cassette is enterotoxigenic.

Strains GP5, GP6 and V96 represented the non-toxicogenic strains of *V. cholerae* O1 in this study. Genotypically, the three strains were very similar, except that strain GP6 did not possess *tcpA*. Whole cells of the three non-toxicogenic strains were inert in the rabbit ileal loop assay, but culture filtrate of the three strains showed enterotoxic activity, especially

Table 4. Variations in conductance in Ussing chambers induced by culture supernates of the various *V. cholerae* O1 strains recorded after 95 min

Medium	Conductance (Ohm ⁻¹)*							
	GP5	GP6	V96	GP12	GP71	GP156	VC20	Medium control
AKI	66 ± 5	83 ± 33	62 ± 14	58 ± 0	50 ± 8	50 ± 8	63 ± 24	37 ± 16
YEP	31 ± 6	36 ± 1	45 ± 15	50 ± 0	35 ± 0	44 ± 0	39 ± 3	31 ± 8
BHIB	36 ± 5	56 ± 17	47 ± 8	45 ± 8	60 ± 5	32 ± 6	45 ± 11	42 ± 3
CAYE-L	48 ± 21	30 ± 6	35 ± 14	30 ± 12	25 ± 8	50 ± 14	56 ± 16	34 ± 21
Syncase	45 ± 5	45 ± 20	59 ± 18	60 ± 15	48 ± 5	47 ± 18	76 ± 15	40 ± 12

*Values are mean ± SE of four experiments.

when grown in AKI and BHIB. Likewise, a medium-dependent increase in Isc and tissue conductance were observed in the Ussing chambers experiment. Taken together, it could be summarised that the three non-toxicogenic strains possessed a medium-dependent enterotoxin factor. It appears that some component in AKI and BHIB triggers the production of the elusive secretogen. It is pertinent to mention here that certain clinical strains of *V. cholerae* non-O1 produced an extracellular factor when grown in AKI medium that evoked a rapid and dramatic cytotoxic response which manifested as cell rounding of CHO and HeLa cells without accompanying membrane damage [32]. The purified cytotoxin has shown impressive enterotoxin activity in the ligated ileal loop and is reportedly present in 81.3 and 100% of the toxigenic and non-toxicogenic strains, respectively, of *V. cholerae* O1 [33].

The magnitude of difference in Isc of the strains grown in different media was more or less similar in the Ussing chambers, although strains grown in AKI and BHIB showed greater fluid accumulation in the rabbit ileal loop test. This appears to be contradictory, but there could be a reason for this. The fluid accumulation in rabbit ileal loop is the end-product of the manifestation of an enterotoxin factor, while changes in Isc reflect either ion secretion (increase in Isc and potential difference) or perturbation in the paracellular junction (increase in tissue conductance) of ileal tissue. In other words, the results of the Ussing chamber experiments give an idea of the possible mechanism involved in inducing the secretogenic effect. In this case, it appears that the increased fluid accumulation in rabbit ileal loop observed with strains grown in AKI and BHIB may not be caused by an increase in Isc, but by a mechanism other than this which needs to be investigated.

The inertness of the whole cells of non-toxicogenic *V. cholerae* O1 in the rabbit ileal loop suggests that these strains possess an enterotoxin potential, but need appropriate external signals to stimulate production of the factor. It is now known that various environmental signals such as osmolarity, pH, temperature, amino acids, CO₂ and iron control the expression of co-ordinately regulated virulence determinants in *V. cholerae* and other bacteria (reviewed by Mekalanos) [34]. An alternative explanation is that the non-toxicogenic strains probably did not colonise and grow well in the rabbit ileal loops as compared with their toxigenic counterparts. Previous studies have documented that in-vivo growth of *V. cholerae* is greater for fully toxigenic strains than for poorly toxigenic strains [35, 36, 37]. It has been suggested that intestinal fluid secretion stimulated by CT creates an improved environment for bacterial multiplication [38]. Evidently, the supernates of non-toxicogenic strains of *V. cholerae* O1 examined in this study contain material that manifests enterotoxin activity in rabbit ileal loops and in Ussing chambers when grown in an

appropriate medium. We are unable to explain why whole cells of GP12 (the classical biotype strain) with an intact CTX virulence cassette were unable to evoke as much fluid accumulation as the prototype E1 Tor biotype strain, although culture supernate of this strain yielded data consistent with that of a toxigenic strain in rabbit ileal loops and in Ussing chambers.

Strain GP71 was chosen to assess the contributory effect of the natural deletion of the *ctxA* gene located within the CTX virulence cassette. The *ctxA* gene was clearly the most important constituent gene in the CTX virulence cassette in terms of evoking a secretory response. Strain GP71 was not secretogenic when whole cells were introduced in the ileal loop, but was secretogenic when supernates of the strain grown in broth were introduced into the ileal loop. Although these results appear conflicting, it is possible that in the broth some environmental signal may have triggered the production of an enterotoxin factor which did not happen when the cells were introduced directly into the rabbit ileal loop.

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