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Detergent induced enhancement of antibody mediated flagellar agglutination of *Vibrio cholerae*

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Vibrio cholerae 01 and non-01 (NAG) strains are known to possess common flagellar (H) antigen which is a characteristic of the species (1, 2). However, the demonstration of the common H antigen by agglutination reaction using OH or H antiserum is not always possible (3). Only specialised treatment of the bacterial cell (4, 5) and potent antiserum (1) are required to demonstrate common flagellar (H) agglutination. In the present communication, we report that prior sensitisation of *Vibrio cholerae* with nonionic detergent (Triton X-100 or NP-40) solutions greatly enhances their agglutinability to H-antiserum.

Materials and methods: *Vibrio cholerae* 01 and non-01, *Vibrio parahaemolyticus* and *Escherichia coli* strains were isolated from stool cultures of patients with diarrhoea. All the strains were characterized by the conventional biochemical and serological tests. Strains were maintained as lyophilized or stab cultures and restored whenever needed. Crude flagella (CF) were prepared from a motile *V. cholerae* 01 strain (AD29, ElTor, Inaba) by essentially following the methodology of Eubanks *et al.* (6). Antiserum to CF was raised in rabbits by the intra-muscular immunization of CF with Freund's incomplete adjuvant. Five such immunizations were given at fortnightly intervals and serum was collected. Anti-O activity was removed by the treatment of the antiserum with appropriate quantities of heat killed (100 °C for 2 h) bacterial pellet until the absorbed serum failed to agglutinate heat killed AD29 strains or AD29 strains devoid of flagella (removed by shearing). The absorbed antiserum was used for the detection of H-antigen reactivity of bacterial strains in a slide test. For this, bacteria were grown on nutrient agar slant cultures (18 h) and one loopfull of bacteria was intimately mixed with one loopfull of the antiserum over a cleaned glass slide. Agglutination was noted with the naked eye after 30–45 sec of continuous agitation of the glass slide. H-agglutination was also carried out with bacterial cells previously treated with nonionic detergents *viz* Triton X-100 or NP-40 (BDH Chemicals Ltd., Poole, London). About two loopfulls of diluted detergent solution (1.5% Triton X-100 or 0.5% NP-40 in saline) were eventually taken over a cleaned glass slide and mixed with one large loopfull of bacteria. The mixture was kept for about 90 s following which 2 loopfulls of anti-H serum were again added to it. The suspension was agitated thoroughly for 30–40 s and the results were recorded as before. H-agglutination in the presence of 1.5% phenol-saline (PH test) was carried out as described by earlier workers (5).

Results and discussion: The H-agglutinability of 26 *V. cholerae* 01 and 20 non-01, 10 *V. parahaemolyticus* and 10 *E. coli* strains was tested with the H-antiserum and the results are presented in the table. Out of 26 *V. cholerae* 01 strains, 14 were directly agglutinable by the anti-H serum although phenol-saline treatment increased the number of agglutinable strains to 23. Interestingly, the same antiserum was able to agglutinate all of the 01 strains tested following treatment with either 1.5% Triton X-100 or 0.5% NP-40-saline solutions. Furthermore, detergent treatment also caused anti-H agglutination of all the 20 non-01 strains tested here in contrast to only 9 agglutinable following phenol-saline treatment. The anti-H serum, however, failed to agglutinate any of the *V. parahaemolyticus* and *E. coli* strains tested, even after detergent treatment, thereby establishing the species specificity of the H-agglutination reaction.

Slide agglutination of V. cholerae *01 and non-01,* V. parahaemolyticus *and* E. coli *strains by anti-H serum*[a]

Strains	Number studied	Saline	Phenol-saline	1.5% Triton-X-100-saline	0.5% NP-40-saline
V. cholerae 01	26	14(+), 12(−)	23(+), 3(−)	all(+)	all(+)
V. cholerae non-01	20	4(+), 16(−)	9(+), 11(−)	all(+)	all(+)
V. parahaemolyticus	10	all (−)	all (−)	all(−)	all(−)
E. coli	10	all (−)	all (−)	all(−)	all(−)

a, Pre-immune serum failed to show any agglutination. (+) positive agglutination; (−) negative agglutination.

The results presented here clearly establish that prior detergent treatment of *V. cholerae* strains greatly enhances their agglutinability by the anti-H serum. Furthermore, this treatment appears to give better results than those of phenol-saline treatment as recommended by other workers (5). It is suggested that H-antigenic determinants are probably present in cryptic forms in the native organism and detergent treatment induces better exposure of these antigens for more effective interaction with antibodies. The detergent-induced slide agglutination test described here may be adopted as a routine test for the identification of *V. cholerae* species of organisms in clinical laboratories.

1. Sakazaki, R., Tamura, K., Gomez, C.Z. and Sen, R. (1970) *Jpn. J. Med. Sci. Biol.*, **23**, 13-20
2. International Subcommittee on Vibrio Taxonomy (1972) *Int. J. Syst. Bacteriol.*, **22**, 123
3. Smith, H.L. (1974) *Appl. Microbiol.*, **27**, 375-378
4. Bhattacharyya, F.K. (1977) *Jpn. J. Med. Sci. Biol.*, **30**, 259-268
5. Pastoris, M.C., Bhattacharyya, F.K. and Sil, J. (1980) *J. Med. Microbiol.*, **13**, 363-367
6. Eubanks, E.R., Guentzel, M.N. and Berry, L.J. (1977) *Infect. Immun.*, **15**, 533-538

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Detergent induced agglutination test to identify *Vibrio cholerae* non-01 strains by any one serotype of *Vibrio cholerae* non-01 antisera

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Different serotypes of *Vibrio cholerae* non-01 (non-agglutinating or so called NAG) strains are known (1, 2). Antisera from rabbits injected with *V. cholerae* non-01 strains showed cross-reactions with heterologous strains (NAG vibrios), indicating that a common flagellar (H) antigen was responsible for the reactions (1, 3). However, it has also been reported that sera from rabbits injected with a single *V. cholerae* non-01 strain agglutinated only with the specific homologous strain and no cross-reactions occurred, indicating the absence of a common antigen present in the organisms tested (4). Despite these divergent results the established fact is that flagellar H antigen is common to all *Vibrio* species (5) but one serotype of NAG vibrio does not cross-react with any other serotype of NAG vibrio antisera. The present work clearly shows that each *V. cholerae* non-01 strain (NAG vibrio) will agglutinate (cross-react) with any one serotype of *V. cholerae* non-01 antisera, if the *V. cholerae* non-01 strain (live or killed) is previously treated with a non-ionic detergent (Triton X-100 or Nonidet P-40) solution in saline.

Materials and methods: *V. cholerae* 01, non-01, and *V. parahaemolyticus* strains were isolated from stool cultures of patients with diarrhoea. All the strains were accurately characterized by the conventional biochemical and serological tests (6). Motility of the *V. cholerae* non-01 strains was checked by the hanging drop in the grove slide and in sulphide indole motility (SIM) media. Haemagglutination characteristics of the *V. cholerae* non-01 strains were studied in a microtitre plate with red blood cells of human, sheep, rabbit and chicken. In the gut, the fluid accumulation performance of the *V. cholerae* non-01 strains was examined by the rabbit ileal loop experiment. Four different serotypes (12, 13, 23 and 42) of *V. cholerae* non-01 strains were intravenously injected into eight rabbits (each serotype to two rabbits) to raise four different serotypes of antisera. From the rabbits, four different serotypes of *V. cholerae* non-01 antisera were collected separately, sterilised by a 0.45 μm filter (Millipore Ltd., UK) and stored at $-20°C$ for subsequent use. Each of the antisera was titrated after inactivation by heating to 56°C for 30 min. The reciprocal titre of each antiserum was 2000. No cross-reactions with heterologous NAG vibrio strains were noted.

In the non-ionic detergent induced slide agglutination test, 18 h bacterial cultures from nutrient agar slant tubes were chosen. Two or three loops of non-ionic detergent, 1.5% Triton X-100 or 0.5% Nonidet P 40 (BDH, Poole) in normal saline were placed on a clean dry microscope slide. One loop of the vibrio strains (live or killed by 100°C for 2 h) was mixed intimately and 1 min was then allowed for the addition of two or three loops of any one of the raised *V. cholerae* non-01 (NAG vibrio) antisera or Inaba or Ogawa antisera (laboratory stocks). The suspension was agitated thoroughly for 30 to 40 s and resultant clumping was noted with the naked eye. Inaba or Ogawa antiserum was mixed with each of the vibrio strains (non-ionic detergent-treated, live or killed) to mark any case of cross-reaction. The agglutination test was also carried out, following the same procedure, with all strains previously treated with 1.5% phenol saline solution instead of non-ionic detergent treatment. For control, pre-immune serum was added to each untreated, non-ionic detergent treated or phenol saline treated, live or killed vibrio strain tested.

Agglutination of V. cholerae *01, non-01 and* V. parahaemolyticus *strains by* V. cholerae *non-01, Inaba and Ogawa antisera*[a]

Strains	Total no. studied	Treatment	*V. cholerae* non-01 antisera				*V. cholerae* 01 antisera	
			Serotype 12	Serotype 13	Serotype 23	Serotype 43	Inaba	Ogawa
V. cholerae non-01	94	Saline	2(+)* 92(−)	1(+)*93(−)	1(+)* 93(−)	1(+)* 93(−)	all(−)	all(−)
		PS	4(+) 90(−)	3(+) 91(−)	3(+) 91(−)	2(+) 92(−)	all(−)	all(−)
		TXS	all(+)	all(+)	all(+)	all(+)	all(−)	all(−)
		NPS	all(+)	all(+)	all(+)	all(+)	all(−)	all(−)
V. cholerae 01 Inaba	38	Saline	all(−)	all(−)	all(−)	all(−)	all(+)	all(−)
		PS	all(−)	all(−)	all(−)	all(−)	all(+)	all(−)
		TXS	all(−)	all(−)	all(−)	all(−)	all(+)	all(−)
		NPS	all(−)	all(−)	all(−)	all(−)	all(+)	all(−)
V. cholerae 01 Ogawa	36	Saline	all(−)	all(−)	all(−)	all(−)	all(−)	all(+)
		PS	all(−)	all(−)	all(−)	all(−)	all(−)	all(+)
		TXS	all(−)	all(−)	all(−)	all(−)	all(−)	all(+)
		NPS	all(−)	all(−)	all(−)	all(−)	all(−)	all(+)
V. parahaemolyticus	21	Saline	all(−)	all(−)	all(−)	all(−)	all(−)	all(−)
		PS	all(−)	all(−)	all(−)	all(−)	all(−)	all(−)
		TXS	all(−)	all(−)	all(−)	all(−)	all(−)	all(−)
		NPS	all(−)	all(−)	all(−)	all(−)	all(−)	all(−)

PS = 1.5% phenol in saline; TXS = 1.5% Triton X-100 in saline; NPS = 0.5% Nonidet P-40 in saline. (+) = positive agglutination; (−) = negative agglutination.
**Due to the same serotype. [a] Pre-immune serum failed to show any agglutination.*

Results and discussion: All the tested strains of *V. cholerae* were found to be motile. Out of 94 strains, 12 *V. cholerae* non-01 strains gave a positive reaction with the rabbit ileal loop experiment (i.e., 0.7 to 1.2 ml fluid accumulated per cm of ileal loop) and all tested *V. cholerae* non-01 strains showed prozone in the haemagglutination test. The Table shows the agglutination and cross-reactions for 189 bacterial strains (74 *V. cholerae* 01, 94 non-01 and 21 *V. parahaemolyticus*) with *V. cholerae* 01 or non-01 antisera. The detergent induced agglutination test was equally sensitive with all NAG vibrios irrespective of their observed characteristics such as motility, haemagglutination, and fluid accumulation in the ileal loop. When any one serotype of *V. cholerae* non-01 antisera was added to any one of the phenol saline treated vibrio strains, neither *V. cholerae* 01 nor *V. parahaemolyticus* strains were agglutinable, except for a very few *V. cholerae* non-01 strains. However, interestingly, each *V. cholerae* non-01 strain (live or killed) prominently agglutinated with any one serotype of *V. cholerae* non-01 antisera, if the cells had been previously treated with 1.5% Triton X-100 or 0.5% Nonidet P-40 in saline. This suggests that the non-ionic detergent solution caused better exposure of the cryptic antigenic determinants of the bacterial cells for more effective interaction with the antibodies present in the *V. cholerae* non-01 antiserum. In support of this view, results presented elsewhere clearly established that prior non-ionic detergent treatment of *V. cholerae* strains greatly enhanced their agglutinability by the anti H serum (7).

Since not only the live but also the killed non-ionic detergent treated *V. cholerae* non-01 strains agglutinated with any one of the *V. cholerae* non-01 antisera (the reaction was heat sensitive), the flagellar (H) antigen was not responsible for the agglutination reaction. Phenol or non-ionic detergent treated live *V. cholerae* 01 or *V. parahaemolyticus* strains did not exhibit the cross-reactions with Inaba or Ogawa or *V. cholerae* non-01 antiserum. On the other hand, *V. cholerae* non-01 antiserum gave an agglutination reaction only with the non-ionic detergent treated *V. cholerae* non-01 strains which thereby established the species specificity of the agglutination reaction and signified that common somatic antigenic materials were present in cryptic forms in all NAG vibrios.

This non-ionic detergent induced slide agglutination test could be used as a routine test in clinical and research laboratories for the rapid diagnosis of *V. cholerae* non-01 strains; this would greatly facilitate the planning of surveillance and control measures and epidemiological investigations.

1. Sakazaki, R., Tamura, K., Gomez, C.Z. and Sen, R. (1970) *Jpn J. Med. Sci. Biol.*, **23**, 13-20
2. Smith, H.L., Jr. (1979) *J. Clin. Microbiol.*, **10**, 85-90
3. Shimada, T. and Sakazaki, R. (1977) *Jpn J. Med. Sci. Biol.*, **30**, 275-277
4. Smith, H.L., Jr. (1974) *Appl. Microbiol.*, 27, 375-378
5. International Subcommittee on *Vibrio* Taxonomy (1972) *Int. J. Syst. Bacteriol.*, **22**, 123
6. Hugh,, R. and Sakazaki, R. (1972) *J. Conf. Public Health Lab. Directors*, **30**, 133-137
7. Dey, S.K., Kushari, J. and Ghose, A.C. (1983) *IRCS Med. Sci.*, **11**, 908

Med. Sci. Res., 1988; **16**, 689-690

Activity and stability of *Vibrio cholerae* L-asparaginase

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In *Vibrio cholerae* certain inducible enzyme activities are different in its toxigenic and non-toxigenic strains [1, 2]. Enzyme activity is enhanced on increasing the virulence of *V. cholerae* by serial passage in the rabbit ileal loop [3, 4], and also by the addition of exogenous cyclic adenosine-3',5'-monophosphate (cAMP) in a non-toxigenic strain [1, 2]. The enzyme of the non-toxigenic strain was also found to be unstable [4]. In this context we are still not aware of a report on L-asparaginase [EC 3.5.1.1.] in *V. cholerae*. The present study reports on the inducible L-asparaginase activity of the highly toxigenic, mildly toxigenic and non-toxigenic strains of *V. cholerae* as well as on the effect of exogenous cAMP on the enzyme production in the studied strains. We found that the stability of L-asparaginase of the three different toxigenic strains was different under the experimental conditions.

Materials and methods: Three *V. cholerae* classical Inaba strains, i.e., 569B (highly toxigenic) [5, 6], V63P$^-$ (mildly toxigenic [1, 2, 6], dependent on adenine and leucine for growth in minimal agar medium and resistant to streptomycin [6]) and CRCV$^-$ (non-toxigenic mutant of V63P$^-$ [1, 2, 6] were used in this study. All strains were obtained from the National Institute of Cholera and Enteric Diseases, Calcutta. They were serially passaged four times in the rabbit ileal loop (RIL) model [7] and the fluid accumulation (FA) ratio of each strain was noted. Before and after the fourth animal passage, each strain was checked for its respective identification characteristics [6, 8]. Each bacterial strain was grown in 150 ml inducing medium (TAYG medium) (tryptone (Difco Labs., Michigan) 0.2 g, L-asparagine 6.0 g, yeast extract (Difco Labs., Michigan) 1 g, L-glutamic acid 2.5 g, K_2HPO_4 2.5 g, $MgSO_4.7H_20$ 0.25 g, distilled water 1 l, pH 7.4) for 40 h at 30°C on a reciprocal shaker. Bacterial growth was measured in a Beckman spectrophotometer at A_{540}; 1.0 corresponded to 1.0×10^9 c.f.u/ml. The cultures were centrifuged ($6000 \times g$) for 20 min at 4°C and the cells (20 mg wet wt/ml) were suspended in 100 mM potassium phosphate buffer (pH 7.6). Each cell suspension was subjected to sonic treatment (Braunsonic, Melsungen, USA) at 2°C (10 kc, 10 min), centrifuged ($18\,000 \times g$) for 30 min at 4°C and the cell-free supernatant was used as the enzyme preparation. Cyclic AMP (Sigma Chemical Co., USA) or glucose was then exogenously added separately in TAYG medium and L-asparagine depleted TAYG medium. Each *Vibrio* strain was grown in each of these media (culture pH was maintained at 7.4) and enzyme preparation (or cell-free supernatant) was made separately as per above conditions and methods, for studying the effects of cAMP and glucose on the production of enzyme.

Enzyme preparations in the presence and absence of EDTA (Sigma Chemical Co., USA) and reduced glutathione (BDH Chemicals Ltd., UK), separately or combined, were kept at 0°C and 4°C to study their stability on different days. The L-asparaginase activity was assayed by direct Nesslerisation [9] with little modification. 1 IU of L-asparaginase activity is that amount of enzyme that liberates 1 μmol of ammonia in 1 min, under experimental conditions.

Table 1: Effect of exogenous cAMP and glucose on inducible L-asparaginase activity of V. cholerae *strains (IU × 10^2/mg protein; means ± SEM;* n = *6)*

Strain	Inducible medium	Inducible med. + 3 mM cAMP	Inducible med. + 0.5% glucose
569B (highly toxigenic)	27.34 ± 1.21[a]	27.63 ± 0.49[d]	2.94 ± 0.42[g]
V63P$^-$ (mildly toxigenic)	18.16 ± 0.98[b]	18.45 ± 0.24[e]	1.75 ± 0.33[h]
CRCV$^-$ (non-toxigenic)	3.94 ± 0.04[c]	14.76 ± 0.75[f]	0.00 ± 0.00[i]

Statistical comparisons : for a vs *b, b* vs *c, a* vs *c, f* vs *c, g* vs *d, h* vs *e, i* vs *f*, $p < 0.001$; *a* vs *d, b* vs *e, non-significant (Student's* t *test).*

Results and discussion: Before serial animal passage, the FA ratios and the enzyme activity of these strains were significantly lower than after the fourth animal passage but each strain exhibited its toxigenic pattern as expected. The identifying characteristics [6, 8] were, however, not changed and remained the same even after the fourth animal passage of each strain. In this study, all experiments were performed after the fourth serial animal passage of the strains. The FA values in the ligated loop of rabbits by 569B were 1.72 and 1.69 ml/cm, by V63P$^-$ were 1.06 and 0.76 ml/cm and by CRCV$^-$ 0.00 and 0.00 ml/cm, respectively, for whole cell cultures and cell free culture supernatants when used as separate toxin samples for each strain. Table 1 shows the L-asparaginase activity of the three strains. The enzyme production was significantly higher ($p < 0.001$) in 569B than that in V63P$^-$ and CRCV$^-$. Furthermore, the addition of exogenous 3 mM cAMP in TAYG medium increased the enzyme production of CRCV$^-$ up to approximately four-fold whereas no such significant alteration was seen in V63P$^-$ and 569B. The addition of 0.5% glucose (w/v) exogenously in TAYG medium greatly diminished the enzyme production (about 10-fold) of all three strains. A negative control for each of these experiments was performed separately with L-asparagine depleted TAYG medium, where no enzyme activity was detected. So these observations clearly reveal that in non-toxigenic *V. cholerae* (where the levels of cAMP are established to be four to five times less than that of toxigenic ones [1, 10]), the synthesis of inducible L-asparaginase is influenced by exogenous cAMP but this does not alter the enzyme production in toxigenic strains. It was also noticed that the enzyme production by the three strains was counteracted by glucose in the inducible medium (which is quite consistent with the findings of catabolite repression of glucose on L-asparaginase production in *E. coli* A-1 [11]), indicating thereby that in *V. cholerae* a certain physiological level of cAMP is necessary for full expression of the inducible L-asparaginase.

In vitro 2 mM EDTA and 2 mM glutathione had no effect on enzyme activity. The results represented in Table 2 clearly show that 2 mM EDTA and 2 mM glutathione in combination could retain the enzyme activity of 569B much better at 0° and 4°C for 180 days (96% retention) and 32 days (81% retention), respectively, than EDTA and glutathione

Table 2: Stability of L-asparaginase activity of the V. cholerae *strains* (n = 4)

Strain	Addition (2 mM)	Relative L-asparaginase activity (%)[a]				
		Stored at 0°C (days)			Stored at 4°C (days)	
		10	30	180	8	32
569B (highly toxigenic)	None	6[illegible]	53	14	41	33
	EDTA	9[illegible]	87	79	85	73
	Glutathione	9[illegible]	81	66	70	61
	EDTA + glutathione	100	98	96	94	81
V63P⁻ (mildly toxigenic)	None	3[illegible]	16	0	6	0
	EDTA	9[illegible]	54	28	50	19
	Glutathione	8[illegible]	38	16	36	11
	EDTA + glutathione	9[illegible]	79	52	61	23
CRCV⁻ (non-toxigenic)	None	[illegible]	0	0	0	0
	EDTA	1	0	0	0	0
	Glutathione	1	0	0	0	0
	EDTA + glutathione	4	0	0	0	0

[a]During storage at 0° and 4°C the initial enzyme activity (IU × 10²/mg protein) at 27.65, 18.45, 14.76 for 569B, V63P⁻ and CRCV⁻, respectively, has been considered as 100% for each.

separately. In V63P⁻ the retention of enzyme activity by the above stabilizers in combination or separately was very poor in comparison with 569B, whereas in CRCV⁻ the enzyme was not at all stable even in the presence of stabilizers. This enzyme stability was checked in 100 mM potassium phosphate buffer, pH 7.6, as several other buffer systems with varying molarity and pH along with other stabilizing agents failed to give better results than those presented here. Even addition of 1 mM phenylmethylsulphonyl fluoride (Sigma Chemical Co., USA) to the supernatant immediately after preparation did not affect CRCV⁻ L-asparaginase stabilization, signifying that the enzyme inactivation was not due to the serine protease activity. In contrast, when the enzyme of 569B was kept at 10° to 12°C with EDTA and glutathione, it retained 40% of its activity after 10 days. From these results it is clear that glutathione offered some protection to the enzyme activity of 569B and V63P⁻ but EDTA was more effective in that respect probably by inhibiting metal ion dependent proteinases although the instability of the enzyme in CRCV⁻ is probably not due to protease activation as shown by the fact that protease activity in *V. cholerae* strains rises when virulence or toxigenicity increases [3]. Hence, this instability of the enzyme of the non-toxigenic strain was probably associated with the enzyme molecule which might be composed of easily dissociable (or unstable) structure. So, it appears that during *in vivo* synthesis of the enzyme of the toxigenic and non-toxigenic strains some conformational variations or modifications in the enzyme molecule might be responsible for this stability or instability of the enzyme.

These observations thus reveal the remarkable and stability of L-asparaginase of a highly toxigenic *V. cholerae*. Similar types of findings were also noti course of our experiments with another highly (classical Ogawa), mildly toxigenic (classical Og non-toxigenic (ElTor Ogawa) strain of *V. cholerae* presented here).

1. Chakraborty, M.K. and Ganguly, U. (1982) *IRCS Med. Sci.*, **10**
2. Chakraborty, M.K. and Ganguly, U. (1982) *IRCS Med. Sci.*, **10**
3. Sasmal, D., Guhathakurta, B. and Dutta, A. (1982) *IRCS M* 698
4. Roy, N.K., Das, G., Balganesh, T.S. *et al.* (1982) *J. Gen. Mic* 1927-1932
5. Mukherjee, S. (1978) *Methods Microbiol.*, **12**, 74-115
6. Dutta, A., Sasmal, D. and Pal, S.C. (1977) *Indian J. Mea* 770-776
7. De, S.N. and Chatterjee, D.N. (1959) *J. Pathol. Bacteriol.*, **66**, 5
8. Hugh, R. and Sakazaki, R. (1972) *J. Conf. Public Health La* **30**, 133-137
9. Whelan, H.A. and Wriston, J.C., Jr (1969) *Biochemistry*, **8**, 238
10. Ganguly, U. and Greenough, W.B. III (1975) *Infect. Im* 343-349
11. Barnes, W.R., Dorn, G.L. and Vela, G.R. (1977) *Ap Microbiol.*, **33**, 257-261

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Comparison of L-asparaginase activity in different species of *Vibrio*

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L-asparaginase (EC 3.5.1.1) activity was detected in *Vibrio cholerae* 01 and non-01, but not in other species, such as *V. parahaemolyticus, V. alginolyticus* and *V. anguillarum*. The hypertoxigenic strains of *V. cholerae* showed significantly higher enzyme activity ($P<0.001$) independent of their biotype and serotype, than those of less or nontoxin producers. The amount of the enzyme was relatively more in strains of *V. cholerae* 01 than non-01 and in those of biotype classical than ElTor. Four consecutive passages through rabbit ileal loops enhanced the enzyme as well as enterotoxic activities of the hypertoxigenic and less toxigenic strains, but not of the nontoxic ones. These observations suggest a correlation between inducible L-asparaginase activity and enterotoxigenicity in *V. cholerae*.

The antineoplastic activities of L-asparaginase (L-asparagine amidohydrolase EC. 3.5.1.1) have been well documented[1,2]. Most of the antilymphomic L-asparaginases have been found in strains of *Escherichia coli, Serratia marcescens, Erwinia carotovora, E. aroideae, Pseudomonas acidovoras* and *Ps. geniculata*. But the administration for a long duration of this enzyme from a single source causes an anaphylactic shock or neutralization of the drug effects[2,3]. Therefore, the discovery of new L-asparaginase serologically different but having similar therapeutic effects is highly desired. Antilymphomic L-asparaginase has been demonstrated in *Vibrio succinogenes*[3,4], but not in other species of the genus[5–7]. The present study was, therefore, undertaken to look for L-asparaginase activity in several species of *Vibrio* including *V. cholerae* 01 of biotypes classical and ElTor and non-01. An effort was also made to examine if there is any correlation between the enzyme and enterotoxic activities of the *V. cholerae* strains.

Material & Methods

Bacterial strains : Six *V. cholerae* 01 (of which 3 strains known[8,9] as non-toxigenic *viz.*, CRCV$^-$, EW_6 and ME_7), three *V. cholerae* non-01 and five *V. parahaemolyticus* (of which 2 were Kanagawa phenomenon positive and 3 Kanagawa phenomenon negative) strains were obtained from the National Institute of Cholera and Enteric Diseases, Calcutta, India. The strains of *V. alginolyticus* and

V. anguillarum were supplied by Dr R. Sakazaki (NIH, Tokyo, Japan). The remaining *V. cholerae* strains were isolated from stool samples of patients with diarrhoea and these strains were characterized by the conventional biochemical and serological tests[10] and also with the detergent induced agglutination tests by using (flagellar) H-antiserum[11] and *V. cholerae* non-01 antisera[12]. All the *V. cholerae* strains prior to and after passage were preserved by lyophilization or at -40°C.

Bacterial culture condition and enzyme preparation : Each strain was grown for 18 h at 37°C in nutrient agar slants and harvested by washing with normal saline and 0.5 ml of each suspension was separately inoculated to 150 ml defined inducible medium TAYG (standardised by the modification of TGY medium[13] as follows : tryptone, 0.2 g; L-asparagine, 6.0 g ; yeast extract, 1.0 g; L-glutamic acid, 2.5 g; K_2HPO_4, 2.5 g; $MgSO_4 . 7H_2O$, 0.25 g; in distilled water 1 litre; *p*H adjusted to 7.4) in 500 ml Erlenmeyer flasks to give an initial OD at A_{540} of 0.08. Cultures were grown for 40 h at 30°C on a reciprocating shaker (80 strokes/min; 8 cm stroke) for optimum inducible L-asparaginase production in this system. Each bacterial growth was assayed by measuring turbidity at 540 nm in a Beckman spertrophotometer (an A_{540} of 1.0 corresponded to 1.0×10^9 cfu/ml).

Each grown culture was centrifuged (6,000 × *g*) for 20 min at 4°C and cell pellet was washed twice with 0.1 *M* potassium phosphate buffer, *p*H 7.6 by centrifugation (6,000 × *g*) and finally resuspended in appropriate volume (20 mg wet wt of cells/ml) of the same buffer. The cell suspension was subjected to sonic treatment at 2-4°C for 10 min at 10 KC in 100 watts (by allowing intervals) by Braunsonic (Melsungen, USA) and centrifuged (18,000 × *g*) at 4°C. This cell-free extract was used as an enzyme preparation, made for each culture.

L-asparaginase assay : The enzyme of different strains was assayed by direct nesslerization method of Whelan *et al*[14], with some modifications. Here, 0.2 ml enzyme preparation was added to 1.6 ml of 0.5 *M* Tris-HCl buffer (*p*H 7.6) and the reaction was initiated by adding 0.2 ml of 0.1 *M* L-asparagine in the same buffer and allowed to proceed for 30 min at 37°C. Reaction was stopped by the addition of 0.5 ml of 1.5 *M* trichloroacetic acid and precipitated protein was removed by centrifugation (10,000 × *g*) at 4°C. The reaction mixture was diluted to 6.5 ml with deionized water and to it 0.5 ml of Nessler's reagent (Glaxo), was added, and kept for 16-18 min at 20°C before estimation of ammonia by determining the OD at 500 nm. Enzyme and substrate blank were included in all assays. Standard curve was prepared with ammonium sulphate. One L-asparaginase unit (IU) is defined as that amount of enzyme which liberates 1 μ mole of ammonia/min under the optimal assay conditions.

Determination of toxigenicity of V. cholerae strains :

(i) With live cells—The enterotoxic activity by live cells of *V. cholerae* was determined by the rabbit ileal loop (RIL) method[15]. For this bacterial cultures (18 h at 37°C) from nutrient agar slants were grown in peptone water medium (*p*H 8.4) for 4 h at 37°C, and 1 ml of live cells of bacterial suspension (10^7 cells/ml) was injected into the ligated ileal loops of a rabbit. In each

:ase; negative control loops were performed. After 20 to 22 h, the rabbit was sacrificed and accumulated fluid volume was measured. Results were expressed as the FA ratio fluid volume in milliliters/loop length in entimeters). The enterotoxic activity of ive cells of each of the strains was tested wice in 2 separate rabbits, and the mean alues were recorded.

(*ii*) With culture filtrate—Enterotoxin roduction capacity of the culture filtrate of *. cholerae* strains was also determined y the RIL experiment. The bacterial ultures in peptone-water (as mentioned arlier) were centrifuged at 14,000 × *g* for 0 min, and the supernatant was passed hrough membrane filters (Millipore) of .45 μm average pore size. One ml of this ulture filtrate was injected into the ligated eal loops of a rabbit and the FA ratio vas determined by the same procedure is described.

erial passage in RIL model: The three elected *V. cholerae* classical Inaba strains *iz.*, 569B, V63P⁻, and CRCV⁻) were erially passaged 4 times in RIL. From utrient agar slant growth (for 18 h at 37°C) acteria were inoculated to peptone water ıedium and grown for 4 h at 37°C. One l of whole culture (10^7 cells/ml) was in- cted to the ligated ileal loop of rabbit. tter 22 h, a small amount of accumulated aid from ileal loop was plated on nutrient gar and the strains were re-isolated and gglutinated with specific antiserum and ıe culture was re-grown and re-injected cording to above mentioned procedures r next passages. For one bacterial strain, separate rabbits were used for 4 serial assages. Finally, bacterial strain was isolated and confirmed by biochemical id agglutination tests[10–12].

After the 4th serial animal passage of the three strains in RIL, the attainable inducible L-asparaginase activity and the attainable toxigenicity of the strains were measured as described.

Data were compared using Student's 't' test.

Results & Discussion

L-asparaginase activity was found in *V. cholerae* 01 and non-01 but not in *V. parahaemolyticus*, *V. anguillarum* and *V. alginolyticus* strains (Table I). On the basis of enzyme activity in the strains of *V. cholerae* only, toxigenicity or virulence was determined by considering their fluid accumulation characteristics in RIL experiments by using live cells and culture filtrate as separate toxin samples. FA ratios of some strains in RIL were found more than 0.75 and 0.70 ml/cm with live cells and culture filtrate respectively and these strains were considered as highly toxigenic. Mildly toxigenic strains were those where FA ratios ranged between 0.35 to 0.74 ml/cm and 0.30 to 0.69 ml/cm with respect to live cells and culture filtrate respectively and the non-toxigenic ones had no fluid accumulation in ligated rabbit ileal loop. The enzyme activity of classical biovar was significantly higher than El Tor biovar, irrespective of two serovars Inaba and Ogawa of both highly toxigenic, mildly toxigenic and non-toxigenic strains of *V. cholerae* 01. The enzyme activity of *V. cholerae* 01 was also significantly higher ($P<0.001$) than that of *V. cholerae* non-01 in both the highly toxigenic and mildly toxigenic strains. The L-asparaginase activity in highly toxigenic strains was about two times higher than that in mildly toxigenic and six times higher than in non-toxigenic ones.

Table I. Comparison of L-asparaginase activity of *V. cholerae* strains and their FA ratio in RIL, before seria animal passage of strains

Strain	No. of strains studied	L-asparaginase activity IU/g wet wt of cells (mean±SE)	FA ratio in RIL experiment (mean value in ml/cm)	
			LC	CF
V. cholerae 01 and non-01				
Highly toxigenic				
Classical Inaba	6	18.63±0.227 A.a_1	0.97	0.94
Classical Ogawa	6	18.22±0.281 A.a_1	0.92	0.76
ElTor Inaba	6	14.57±0.125 A.a_2	0.95	0.84
ElTor Ogawa	4	14.54±0.102 A.a_2	0.90	0.75
NAG vibrio	1	11.72±0.030 A.a_3	0.82	0.72
Mildly toxigenic				
Classical Inaba	6	9.57±0.727 B.b_1	0.66	0.59
Classical Ogawa	5	9.38±0.196 B.b_1	0.58	0.49
ElTor Inaba	4	7.18±0.412 B.b_2	0.62	0.54
ElTor Ogawa	4	7.29±0.236 B.b_2	0.52	0.47
NAG vibrio	7	5.74±0.218 B.b_3	0.36	0.32
Non-toxigenic				
Classical Inaba	1	3.29±0.047 C.c_1	0.00	0.00
ElTor Ogawa	2	2.11±0.049 C.c_2	0.00	0.00
V. parahaemolyticus	5	0.00	—np	—
V. anguillarum	3	0.00	—	—
V. alginolyticus	3	0.00	—	—

LC, live cells; CF, culture filtrate; np, not performed; RIL, rabbit ileal loop. Mean values of L-asparaginase activity for each strain was performed in triplicate. $P<0.001$: A vs B, B vs C, A vs C A.a_1 vs A.a_2, A.a_3 vs A.a_1, A.a_2 vs A.a_3, A.a_3 vs B.b_3, B.b_3 vs C.c_2 ; $P<0.01$: B.b_1 vs B.b_2, B.b_2 vs B.b_3, B.b_1 vs B.b_3; $P<0.05$: C.c_1 vs C.c_2

Considering the highest L-asparaginase activity, three *V. cholerae* strains (classical Inaba), *e.g.*, 569B (highly toxigenic), V63P (mildly toxigenic), CRCV⁻ (non-toxigenic) were selected by (taking one representative from each toxigenic pattern) for successive serial passage (4 times) in RIL to find out the changes of the enzyme activity and toxigenicity, as it has beeen established that the 4th serial animal passage enhances the toxigenicity or virulence or a *V. cholerae* strain[16] and also activity of some other (*e.g.*, alkaline phosphatase, lecithinase protease) enzymes[17,18]. We also observed that after the 4th serial animal passage of the toxigenic strains the enzyme activity significantly enhanced ($P<0.001$), *e.g.*, 1.43 fold in the highly toxigenic and 1.88 fold

Table II. Variation of L-asparaginase activity and FA ratio, before and after animal passage of each of a highly toxigenic, mildly toxigenic and non-toxigenic strain of *V. cholerae*

Strain	L-asparaginase activity IU/g wet wt of cells (mean±SE)		FA ratio in RIL experiment (mean value in ml/cm)			
	Before passage	After 4th passage	Before passage		After 4th passage	
			LC	CF	LC	CF
V. cholerae						
Highly toxigenic						
569B (classical Inaba)	18.71±0.40	26.92±0.75*	1.04	1.00	1.72	1.69
Mildly toxigenic						
V63P− (classical Inaba)	9.91±0.47	18.65±0.39*	0.67	0.61	1.06	0.76
Non-toxigenic						
CRCV− (classical Inaba)	3.29±0.07	3.31±0.21†	0.00	0.00	0.00	0.00

LC, live cells; CF, culture filtrate. Each assay was performed in triplicate for each strain and gave reproducible results. *$P<0.001$; † non-significant

in the mildly toxigenic strain, and also the toxigenicity or virulence was found to increase in the form of FA ratio in ligated ileal loop when live cells and culture filtrate were used as separate toxin samples (Table II). However, in the non-toxigenic strain, neither such an increase in enzyme activity nor any fluid accumulation was observed.

Our results clearly demonstrate that before serial animal passage of *V. cholerae* strains, the inducible L-asparaginase activity was significantly high in highly toxigenic strains as compared to the mildly toxigenic to non-toxigenic ones and this observation is consistent with that relating to other enzymes (*e.g.*, alkaline phosphatase, β-galactosidase and tryptophanase) in *V. cholerae*[17,19,20] also. After the 4th animal passage of the selected *V. cholerae* strains also, similar observation was made as the enzyme activity increased only in the toxigenic strains, along with enhancement of toxigenicity or virulence. Our results clearly establish that the L-asparaginase activity varies following a direct correlation with the toxigenicity or virulence of different strains of *V. cholerae* 01 and non-01.

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References

1. Broome, J.D. L-asparaginase. The evolution

of a new tumor inhibitory agent. *Trans NY Acad Sci* **30** (1968) 690.

2. Wriston, J.C. Jr. and Yellin, T.O. L-asparaginase. A review. *Adv Enzymol* **35** (1973) 185.
3. Capizzi, R.L. and Cheng, Y.C. Therapy of neoplasia with asparaginase. In : *Enzymes as drug*, J.S. Holcenberg and J. Roberts, Eds (John Wiley and Sons, New York) 1973 p 1.
4. Distasio, J.A., Niederman, R.A., Kafkewitz, D. and Goodman, D. Purification and characterization of L-asparaginase with anti-lymphoma activity from *Vibrio succinogenes*. *J Biol Chem* **251** (1976) 6929.
5. Imada, A., Igarasi, S., Nakahama, K. and Isono, M. Asparaginase and glutaminase activities of micro-organisms. *J Gen Microbiol* **76** (1973) 85.
6. Bilimoria, M.H. Conditions for the production of L-asparaginase 2 by coliform bacteria. *Appl Microbiol* **18** (1969) 1025.
7. Wade, H.E., Robinson, H.K. and Phillips, B.W. Asparaginase and glutaminase activities of bacteria. *J Gen Microbiol* **69** (1971) 299.
8. Datta, A., Sasmal, D., Datta, G.C. and Pal, S.C. Isolation and characterisation of a non-toxinogenic mutant of *V. cholerae* and possibility of a live oral cholera vaccine. *Indian J Med Res* **65** (1977) 770.
9. Mukherjee, S. Principles and practice of typing *Vibrio cholerae*. *Methods Microbiol* **12** (1978) 74.
10. Hugh, R. and Sakazaki, R. Minimal number of characters for the identification of *Vibrio* species, *Vibrio cholerae* and *Vibrio parahaemolyticus*. *Public Health Lab* **30** (1972) 133.
11. Dey, S.K., Kusari, J. and Ghose, A.C. Detergent induced enhancement of antibody mediated flagellar agglutination of *Vibrio cholerae*. *IRCS Med Sci* **11** (1983) 908.
12. Dey, S.K. Detergent induced agglutination test to identify *Vibrio cholerae* non-01 strains by any one serotype of *Vibrio cholerae* non-01 antisera. *IRCS Med Sci* **12** (1984) 326.
13. Kitto, G.B., Smith, G., Thiet, T.Q., Mason, M. and Davidson, L. Tumor inhibitory and non-tumors inhibitory L-asparaginases from *Pseudomonas geniculata*. *J Bacteriol* **137** (1979) 204.
14. Whelan, H.A. and Wriston, J.C. Jr. Purification and properties of asparaginase from *Escherichia coli* B. *Biochemistry* **8** (1969) 2386.
15. De, S.N. and Chatterjee, D.N. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. *J Pathol Bacteriol* **66** (1953) 559.
16. Smith, H. Studies on organisms grown *in vivo* to reveal the basis of microbial pathogenicity. *Ann NY Acad Sci* **88** (1960) 1213.
17. Roy, N.K., Das, G., Balganesh, T.S., Dey, S.N., Ghosh, R.K. and Das, J. Enterotoxin production, DNA repair and alkaline phosphatase of *Vibrio cholerae* before and after animal passage. *J Gen Microbiol* **128** (1982) 1927.
18. Sasmal, D., Guhathakurta, B. and Dutta, A. Changes in some enzymes of *Vibrio cholerae* on increasing the virulence by passage in rabbit ileal loop. *IRCS Med Sci* **10** (1982) 698.
19. Chakrabarti, M.K. and Ganguly, U. Comparison of B-galactosidase activity of toxigenic and non-toxigenic strains of *Vibrio cholerae* : Effect of cAMP. *IRCS Med Sci* **10** (1982) 846.
20. Chakrabarti, M.K. and Ganguly, U. Tryptophanase activity in toxigenic and nontoxigenic strains of *Vibrio cholerae*. *IRCS Med Sci* **10** (1982) 981.

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