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CLINICAL FEATURES AND PATHOGENICITY OF O GROUP I NONAGGLUTINATING *VIBRIO CHOLERAE* AND OTHER VIBRIOS ISOLATED FROM CASES OF DIARRHEA IN DACCA, BANGLADESH

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On purely taxonomic grounds, the so-called NAG vibrios or non-cholera vibrios (NCV) clearly belong to the same species as the El Tor and classical biotypes of *Vibrio cholerae* (3, 20). There are very important differences, however, between members of the species. Most obvious is that some members are highly adapted human pathogens responsible for massive pandemics while others are, apparently water-dwelling saprophytes.

A biotyping scheme is needed which takes this into account. Possession of certain properties influences an organism's ability to propagate or maintain itself in the human small intestine, aquatic environment, or other potentially important ecological niches. These must be characterized and incorporated into the definition of appropriate biotypes. Since the potential for human pathogenicity is the crucial issue in discussions about the species *V. cholerae* now and in the foreseeable future, it seems especially appropriate that a greater effort be made to identify the nature of virulence characters among its members. With respect to the non-O-Group I agglutinating *V. cholerae* (or NCV or NAG vibrios) information concerning human pathogenicity is very incomplete. There is no doubt that some members of this group are human pathogens (1, 15, 24), nor that some produce an enterotoxin remarkably similar to cholera toxin (18, 25, 26, 27). Beyond this, however, the picture of this group is too badly blurred to identify patterns associated with a pathogenic or saprophytic mode of existence.

In the work reproted here, we attempted to define, in hospitalized patients, detailed clinical and laboratory features of disease due to non-O-Group I agglutinating *V. cholerae*. We have also been able to include cases associated with related species, such as *V. parahaemolyticus*, for which the diarrheal syndrome has not been clearly defined in Bangladesh. The *V. cholerae* non-O-Group I clinical isolates have been screened for biological activity in several animal models and for a number of specific virulence factors. Similar data about isolates from other sources in the world have also been included in the current analysis. Though incomplete, the results thus far reveal several virulence characters and patterns of potential clinical importance.

MATERIALS AND METHODS

All patients admitted to the Treatment Center of the Cholera Research Laboratory in Dacca, Bangladesh in November 1977 through April 1978 were screened. Treatment Center admissions consisted of the more seriously ill patients those judged to be moderately to severely dehydrated at the time they presented to the admitting phy

sician. The weight of each patient was measured at the time of admission and a stool specimen was collected by sterile rectal catheter for an immediate darkfield examination for vibrios (2). Patients whose stool contained organisms which possessed typical vibrio "shooting-star" motility that was not inhibited by the addition of Gardner and Venkatramen O-Group I antiserum were included in the study.

In many cases vibrio concentration in stool was insufficient for us to test motility-inhibition with specific antiserum. A 3-hour enrichment of stool in trypticase soy broth followed by darkfield examination was used to confirm the presence of non-agglutinating vibrios. All patients with suspected vibrio infection were tentatively admitted to the study until the results of the enrichment darkfield examination were known.

On admission, a history and physical examination were performed; stool was obtained by rectal catheter for microscopic analysis and culture; and a venous blood sample was drawn for measurement of electrolytes and specific gravity and for acute serology. A finger tip blood smaple was taken at 4 hours for specific gravity and a complete blood count. Intravenous fluid replacement was begun immediately after admission and maintained as needed. No antibiotics were given. Patients were kept in hospital for 24 hours after diarrhea had ended. Convalescent serum was obtained at 14 days.

For the detection of vibrios, stool samples were streaked to gelatine agar (GA), pH 8.5, and to taurocholate-gelatine agar (TTGA) (16) plates. Serial 10-fold dilutions of stool were spread plated on gelatine agar in order to determine vibrio concentration. A 6-hour enrichment culture of stool in alkaline peptone broth was also prepared and streaked to GA and TTGA plates. All plates were incubated overnight at 37°C. Gelatinase-positive colonies were screened by the string test (17, 22), oxidase test and a slide test for agglutination in O-Group I polyvalent antiserum. Selected isolates were streaked for purity on GA and stocks were prepared on heart infusion agar slants and covered with sterile paraffin oil. Isolates were taken from GA plates whenever possible to avoid using organisms exposed to bile salts-containing media. This was done in consideration of the fact that bile salts can act as a curing agent for plasmids (10, 23). Since we could not rule out the possibility that plasmids mediated improtant virulence facots in non-O-Group I *V. cholerae*, we wished to avoid selecting against them in the isolation procedure. Isolation from TTGA was necessary in less than 3% of cases.

Stool samples were also streaked directly onto MacConkey agar and SS agar, and enriched in selenite broth for detection of other bacterial enteric pathogens. *Salmonella* or *Shigella* sp. were confirmed by routine biochemical and serological tests. *Escherichia coli* isolates, in addition to routine biochemical confirmation, were tested for production of LT in the Chinese hamster ovary (CHO) cell assay (8) and for ST by the infant mouse assay (6). Two individual picks were grown in trypticase soy broth with 0.6% yeast extract (TSBY) in shake (100-120 cycles/min) at 37°C for 24 hours and tested in each assay.

Vibrio isolates were subjected to the following confirmatory tests: Gram reaction and morphology, reaction on Kligler iron agar, growth characteristics in motility-indole-urea medium (9), Hugh-Leifson glucose, fermentation of sucrose, mannose, arabinose or mannitol in bromcresol purple broth, lysine or ornithine decarboxylase and arginine dihydrolase (Moeller method), halophilism shake culture test with 0, 3, 7 or 10% NaCl, growth at 4C, sensitivity to 0/129 vibriostatic compound using discs containing 10 and 150 mcg (21), and the Kanagawa test (Bacteriological Analytical Manual for Foods, 4th ed., AOAC, 1976). Antibiotic resistance was determined using the standard Kirby Bauer disc diffusion method (14).

Isolates were screened for biological activity in the following models: 1) experi-

mental infection in 10-day old New Zealand white rabbits using the technique of Dutta and Habbu (7); 2) virulence of whole cultures administered I.P. to young adult mice, lethal activity of filter-sterilized culture fluid; 3) rabbit ileal loop activity (5) of whole cultures and culture filtrates; 4) rabbit vascular permeability factor assay (4); 5) CHO and adrenal cell (19) assays; and 6) suckling mouse assay. TSBY cultures grown in shake flasks at 32°C and 100-120 cycles/min for 6 hours were used to prepare whole culture challenges. Filtrates for toxin assays were prepared from 18 hour shake flask cultures in the casamino acids medium of Kusama and Craig (12).

The adherence of isolates to isolated rabbit brush border cells and human group O erythrocytes (11) was evaluated using cells grown in TSBY shake flasks at 32°C for 16 hours. In the brush border adherence assay, vibrios and brush borders were incubated at 30°C for 30 min before the number of bacteria on each of 20 brush borders was counted.

RESULTS AND DISCUSSION

Over 700 patients were screened by darkfield microscopy. Direct darkfield examination of stool was not an efficient screening procedure by itself becuase vibrio concentration in too many stool specimens were at the borderline of detectability. It was necessary to admit tentatively all suspected vibrio cases, then confirm with a 3-hour stool enrichment and second darkfield examination.

Enrichment darkfield results agreed with bacteriological culture in 77 out of 81 cases in which one or both methods were positive (Table 1). This is better than 95% agreement. Sixteen isolates proved to be *V. cholerae* El Tor. These came from cases in which the enrichment culture still contained too few organisms to assess clearly the effect of O-Group I antiserum. In all, non-O-Group I agglutinating *V. cholerae* and related species were isolated in 74 cases, representing about 10% of the patients screened.

Table 1

Efficiency of Darkfield Examination Coupled with 3-hr Enrichment for the Detection of Non-O-Group I *V. cholerae*

	Bacteriological Culture	
Darkfield Result	Positive*	Negative
Positive	77	1
Negative	3**	483

Identification of isolates:

V. cholerae El Tor	16 (20%)
V. cholerae non-O-Group I	35 (44%)
V. parahaemolyticus	17 (21%)
Related organisms	12 (15%)

All 3 false negatives identified as *V. cholerae* El Tor.

Isolates fell into one of four well-differentiated groups: non-O-Group I *V. cholerae*, *V. parahaemolyticus*, Group F vibrios (13) and *Aeromonas hydrophila* subsp. *anaerogenes* (Table 2). Group F vibrios represent an intermediate cluster between the two genera *Vibrio* and *Aeromonas*. The string test was an effective differential method: 86% of *V. cholerae* and 71% of Group F vibrios were strongly positive while virtually the same proportion in the other two groups gave weak or negative reactions. Inhibition of growth in the presence of 0/129 vibriostatic compound was also a useful character. Production of lysine decarboxylase or arginine dihydrolase effectively separated *Vibrios* and non-*Vibrios*.

Table 2

Differential Characteristics of Vibrio-Like Isolates from Patients
(% of Isolates Showing Character)

	N	String Test	0/129 V.S.C.* Sensitivity 10 Mcg	150 Mcg	Lys**	Arg**	Heiberg Group		Growth % NaCl 0	7
V. cholerae Non-O-group I	37	Strong (86)	100	100	100	0	II V	(70) (30)	100	22
V. parahaemolyticus	22	Weak/Neg (92)	23	100	100	0	VII V	(91) (9)	14	100
Group F vibrios	7	Strong (71)	0	43	0	86	III	(100)	100	71
Aeromonas hydrophila subsp. *anaerogenes*	4	Negative (75)	0	0	50	100	I	(100)	100	50

0/129 Vibriostatic compound = 2, 4-diamino 6, 7 diisopropyl pteridine.
Lys = Lysine decarboxylase; Arg = Dihydrolase.

Heiberg grouping was almost completely exclusive with the exception of a few HG V *V. parahaemolyticus*. Growth in peptone containing 0 or 7% NaCl was useful in differentiating *V. parahaemolyticus*. In addition, all isolates identified as *V. parahaemolyticus* were tested for Kanagawa hemolysis and all were positive.

Of the 57 cases for which a complete analysis is available so far, 8 (14%) were mixed infections (Table 3). Other pathogens included LT/ST *E. coli*, ST-only *E. coli*, *Shigella flexneri* or Shigella boydii. *Of the cases in which Vibrio* or *Vibrio*-like organisms were the only pathogen identified 20 (31%) had non-O-Group I *V. cholerae* and 21 (43%) had *V. parahaemolyticus*.

Table 3

Bacteriological Isolations from 57 Study Cases

	Single Pathogen		Total Infections	
	No.	%	No.	%
V. cholerae Non-O-Group I	20	41	25	44
Heiberg Group V	8	16.5	9	16
Heiberg Group II	12	24.5	16	28
V. parahaemolyticus	21	43	22	39
Group F vibrios	5	10	7	12
A. hydrophila subsp. *anaerogenes*	3	6	3	5

Table 4 compares clinical features of these cases. Only adult patients have been included in this table. A point not shown is that males and females are equally represented in the *V. cholerae* cases but males account for 17 (81%) of the *V. parahaemolyticus* cases. This difference is close to statistical significance (P = .06, Fishers Exact Test). Table 4 shows that almost all patients had vomiting. About one-quarter had muscle cramps; 71% of *V. cholerae* and 95% of *V. parahaemolyticus* cases reported abdominal pain. The frequency of fever was almost twice as great in *V. parahaemolyticus* cases (81%) as in *V. cholerae* cases (43%); the mean duration in the former was 14.7 hours compared to 9.1 hours in the latter.

Table 4

Clinical Features of Adult Cases with Single Pathogen

	V. cholerae non-O-Group I (N = 14)		*V. parahaemolyticus* (N = 21)		Related Organisms (N = 7)	
	No.	%	No.	%	No.	%
Vomiting	14	100	20	95	7	100
Muscle cramps	3	21	6	29	1	14
Abdominal pain	10	71	20	95	5	71
Temp. (>100° F axillary)*	6	43	17	81	3	43
Fever duration (hr)	9.1 ± 5.0**		14.7 ± 6.8		13.3 ± 6.1	

V. parahemolyticus vs other P < .05 Chi Square Test.

± 1 S. dev.

Table 5 presents selected admission laboratorv features of cases. The mean admission serum protein and the mean hematocrit value measured 4 hours after admission were normal. The mean total white count 4 hours after admission was elevated in all groups with the count in *V. parahaemolytucus* cases (21,100) significantly higher than the others. Most patients in each group had a shift to the left. Stool microscopic examination revealed less than 5 polymorphonuclear leucocytes per high powered field in 98% of cases with no difference between groups. Fecal red blood cells were observed significantly more often in *V. parahaemolyticus* cases; 10 cases (50%) had 6-10 or more RBC/hpf. Of these cases, 9 had a history of bloody or red stool. In the remaining 10 cases, only 2 reported a similar history. None of the *V. cholerae* cases reported bloody or red stool. Over 90% of all cases had parasitic infestation with no difference between groups.

Data in Table 6 shows that mean serum and stool electrolyte values in *V. cholerae* and *V. parahaemolyticus* cases are similar. Serum elelctrolyte values are essentially normal and stool electrolytes are similar to values reported elsewhere for diarrheas caused by enterotoxigenic *E. coli* and other agents. The data from cases due to related vibrio-like organisms, which is not shown, are also similar.

Table 5

Admisson Laboratory Features of Vibrio Cases

		V. cholerae Non-O-Group I (N = 14)	*V. parahaemolyticus* (N = 21)	Related Organisms (N = 7)	
Serum protein (Gm%)		8.1 ± 1.8*	7.5 ± 0.8*	7.4 ± 0.6*	
Hematocrit (%)**		37.9 ± 5.9	38.9± 5.3	34.4 ± 3.5	
WBC**		16,200 ± 4,300	21,100± 7,800	16,500 ± 7,100	p <.025†
Fecal leucocytes (No./hpf)	0-5	14	20	6	
	6-10	0	0	1	
Fecal RBC (No./hpf)	0-5	13	10	7	
	6-10	1	1	0	p = .002‡
	>10	0	9	0	

* N = 13, 19, 5 respectively.

** Measured 4 hours after admission.

† T test, *V. parahaemolyticus* vs others (grouped).

Fishers exact test, *V. parahaemolyticus* vs others (grouped in 2 x 2 contingency table (≤5 vs >5 RBC/hpf)

Table 6

Serum and Stool Electrolytes by Etiology

(Mean ± 1 S. Dev.)

		Na	K	Cl	HCO_3
Serum					
V. cholerae					
Non-O-Group I	14	135 ± 3	4.8± 0.6	100 ± 3	22.5± 3.3
V. parahaemolyticus	21	137 ± 3	4.3 ± 0.6	101 ± 4	25.0± 2.8
Stool					
V. cholerae					
Non-O-Group I	12	88 ± 33	21 ± 12	60 ±34*	33± 13*
V. parahaemolyticus	21	106 ± 24	21 ± 11	65 ± 19	41 ± 15**

N = 11.

N = 20.

Other data contrasting *V. cholerae* non-O-Group I and *V. parahaemolyticus* cases is shown in Table 7. *V. parahaemolyticus* was found only in adults while a significant proportion (30%) of *V. cholerae* cases occurred in children. Comparing only adult cases, the *V. cholerae* group had modestly elevated admission serum specific gravity (1.030). This was significantly different from the other groups 1.028. The duration of diarrhea before admission tended to be shorter in *V. parahaemolyticus* patients possibly because the higher incidence of bloody stool and fever led them to seek treatment sooner. Total duration of diarrhea was similar in both groups, but stool output was three times as great and IV requirement almost twice as great in *V. cholerae* cases. The mean stool pathogen count expressed as a log value was similar, representing a mean value of 3-4 x 10^8/ml.

Two findings are not shown in this table. First, the pediatric non-O-Group I *V. cholerae* cases tended to be more serious illnesses than adult cases. The mean total duration of diarrhea was 67 hours and mean stool value was 346 cc/Kg. Mean admission serum specific gravity was 1.028.

Second, 4-fold or greater agglutinating titer rises against the homologous isolate were seen in 5 of 9 paired sera from non-O-Group I *V. cholerae* cases, but in none of 9 *V. parahaemolyticus* patients. No patient in either group had a titer rise to O-Group I *V. cholerae*.

In the screening which has been done so far for properties that may contribute to virulence in non-O-Group I *V. cholerae*, a few have been identified that appear to be of clinical relevance. First, we have detected an enterotoxin in four of 14 isolates that is active in adrenal and CHO cell assays.[3] Some characteristics of this toxin will be described later. We are presenting the data in Table 8 to show that those patients with toxigenic isolates had more severe illness than those with nontoxigenic isolates. In the cases with toxigenic organisms, weight loss is greater, the admission serum specific gravity is significantly higher, and diarrhea lasts longer and is more profuse.

Four-fold or greater rises in serum gold-

Table 7

Comparative Features of *V. cholerae* Non-O-Group I and *V. parahaemolyticus* Adult Cases

	V. cholerae Non-O-Group I (N = 14)	*V. parahaemolyticus* (N = 21)	
Patients excluded due to age (≤ 10 yrs old)	6	0	P = .009*
Admission serum specific gravity	1.030 ± .004	1.028 ± .003	P < .001**
Duration of diarrhea (hr)			
Pre-admission	11.6 ± 8.9	7.6 ± 3.8	P < .06**
Total	42.1 ±31.4	36.8 ± 16.2	
Stool volume (cc/Kg)	60	20	P = .04†
IV volume (cc/Kg)	123	76	P = .05†
Stool pathogen count (log_{10} CFU/ml)	8.6 ± 0.7	8.4 ± 0.9‡	

* Fishers exact test. † Rank sum test.
** T test. ‡ N = 14.

Table 8

Comparative Features of Toxigenic and Nontoxigenic *V. cholerae* Non-O-Group I Cases

	(Mean Value ± 1 S. Dev.) Toxigenic (N = 4)	Nontoxigenic (N= 10)	
Weight loss (%)	5.6± 5.2	1.7± 1.6	P < .025*
Admission serum spec. gravity	1.0345 ± .0031	1.0290 ± .0033	P < .01*
Total duration of diarrhea (hr)	60 ± 40	36 ± 27	P < .10*
Stool volume (cc/kg)	103	41	P = .04**
IV volume (cc/Kg)	184	98	P = .01**
Serum titer rise			
Agglutinating	1/2	4/7	
Toxin neutralizing	2/3	0/8	P = .054‡

* T test. † Includes data from cases ≤ 10 yrs old.
** Rank sum test. ‡ Fishers exact test.

Table 9

Adherence Factors in Isolates from Study Cases

	Brush Border Assay No. Organisms/ Brush Border		Human Type O Red Cell Hemagg. Titer	
	N		N	
V. cholerae Non-O-Group I	18	13.2 ± 7.4*	16	3.9 ± 1.4
V. parahaemolyticus	18**	2.5 ± 1.8*	14	2.3 ± 1.9
Group F vibrios	5	3.9 ± 2.9	5	3.0 ± 1.2
Aeromonas hydrophila	3	18.1 ± 13.5	3	2.7 ± 0.6

(Mean Value ± 1 S. Dev.)

* $P < .001$, T test.

** Excludes 1 isolate with brush border adherence value > 30.

tinating titer against toxigenic or nontoxigenic isolates occur with equal frequency. Significant antitoxin titer rises, however, occur only in patients with toxigenic vibrios. Even with the very small numbers involved, this difference approaches statistical significance. Antitoxin titers were determined in adrenal cell neutralization assays. Neutralizing titers in the two positive sera rose against homologous non-O-Group I toxin, purified cholera toxin, and *E. coli* LT.

Another property we have begun to examine is adherence (Table 9). Thus far we have completed the rabbit brush border assay and haemagglutination of human type O red cells—both performed essentially as described by Jones and Freter. The mean hemagglutinating titer of the non-O-Group I *V. cholerae* is similar to that of the other three groups. The hemagglutinating titer of individual organisms did not relate to the organisms ability to adhere to brush borders. The difference between the mean number of vibrios per brush border with *V. cholerae* (13.2) and with *V. parahaemolyticus* (2.5) is highly significant. *Aeromonas hydrophila* also adheres strongly, while adherence in the group F vibrios is almost as poor as among *V. parahaemolyticus*.

A factor associated with brush border adherence in the non-O-Group I *V. cholerae* may be of clinical relevance if the relationship pictured in Figure 1 is confirmed. This figure shows a simple rank correlation between stool output in 18 cases and the brush border adherence of the homologous isolate. Severity of illness parallels the isolates successful adherence in the assay. The relationship is significant ($P < .05$; Spearman Rank Correlation Coefficient), and may reflect a colonization factor of some importance in human disease. Also interesting is the tendency of the few toxigenic isolates to cluster in the upper and right portion of the graph. The results so far are encouraging that clusters of identifiable virulence factors may be found that differentiate protential pathogens from those which are not in the non-O-Group I *V. cholerae*.

The clinical isolates described above as well as a number of non-O-Group I *V. cholerae* from other sources have been subjected to a battery of assays in order to characterize more fully their biological activity. Table 10 lists the source and number of non-O-Group I *V. cholerae* from other

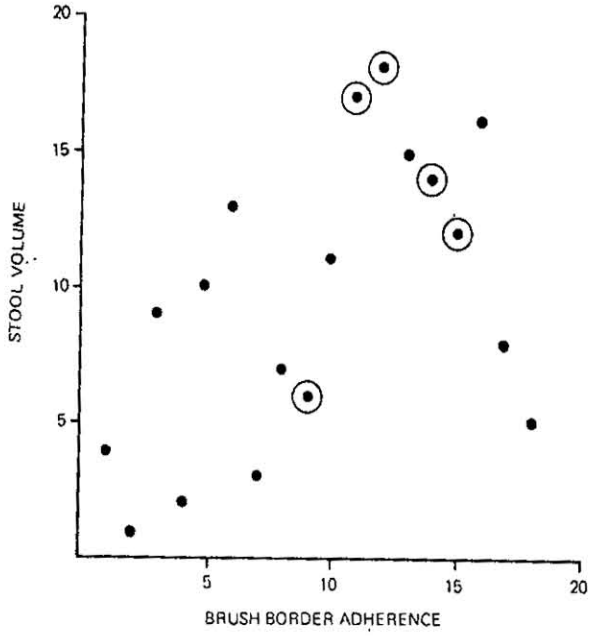

Figure 1. Rank Correlation of Brush Border Adherence with Severity of Diarrhea in *V. cholerae* Non-o-group I Cases (Circled Dots Represent Toxigenic Strains).

Table 10

Sources of Additional *V. cholerae* Non-O-Group I Strains Screened for Biological Activity

Country	Source	No. Examined
Bangladesh	CRL (1973 – 1977)	26
S. Africa	Robins – Browne	5
Sudan	Zinnaka	6
Czechoslovakia	Lee	3
England	"	8
Ghana	"	3
Algeria	"	4
Egypt	"	2
Guam	McIntyre	4
U.S.A.	Colwell	6

which have been screened along with these clinical isolates. Thirty of the 62 strains listed are from environmental sources, the rest are clinical isolations.

The methods used to test the isolates for bioactivity are presented in Figure 2. All procedures were standardized before the isolates were tested. Isolates were initially tested in duplicate in the infant rabbit model. Isolates which produced negative or variable results were retested until a clear cut result was produced. If after three passages isolates still failed to produce diarrhoea and death in the infant rabbit then they were considered to be negative in this model. Passaged strains were tested for their lethality for adult mice and for extracellular lethal toxin. They were then tested for ability to cause fluid accumulation in the ileal loop using whole culture and culture filtrates. Finally, filtrates were tested for skin permeability activity evidenced by blueing in the shaved back of rabbits, induction of fluid accumulation in the suckling mouse assay and induction of morphological changes in the CHO and adrenal cell lines.

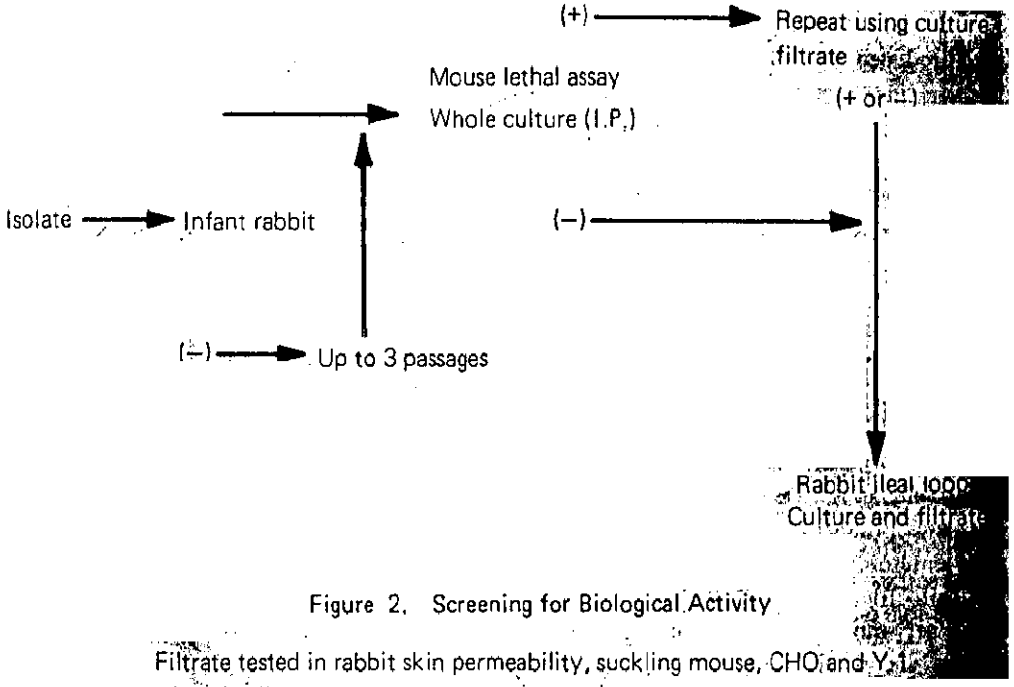

Figure 2. Screening for Biological Activity

Filtrate tested in rabbit skin permeability, suckling mouse, CHO and Y-1 adrenal cell assays.

A spectrum of biological activity was observed which fell into four basic patterns (Table 11). Isolates were characterized as being able to produce a cholera or CT-like toxin, a heat-stable or ST-like-toxin, enteritis without either toxin, or no activity in the biological assays. Out of 110 non-O Group 1 *V. cholerae*, 21 (19.1%) produced CT-like toxin. The concentration of toxin produced in shake flask cultures and measured by adrenal cell titration was one-tenth less than that produced by *V. cholerae* 569B but similar to levels produced by *V. cholerae* El Tor strains.

Table 11

Four Basic Types of Biological Activity Observed in Screening Assays

	N	Infant Rabbit	Ileal Loop Cult.	Ileal Loop Filt.	Rabbit Skin Permeability	CHO/Adrenal Cells	Infant Mouse
CT-like	21	Pos	Pos	Pos	Blueing	Pos	Neg
ST-like	2	Neg	Pos	Pos	Neg	Neg	Pos
Enteritis	73	Var	Var	Neg	Haemorrhagic lesion-var	Neg	Neg
Inactive	24	Neg	Neg	Neg	Neg	Neg	Neg

Two isolates both from the Chesapeak Bay, produced a toxin which was active in the suckling mouse assay and ileal loop. These strains were unable to cause disease in infant rabbits. Preliminary data which will be shown later suggest that this toxin is similar to the heat-stable toxin of *E. coli.*

Sixty-six percent of isolates had activity in the infant rabbit or the rabbit ileal loop when whole cells were used. But none produced demonstrable extracellular enterotoxin. The remaining 24 isolates were completely inactive in all assays shown. Results of the mouse lethal assay have not been shown, but virtually all isolates were lethal.

Isolates producing enteritis without detectable enterotoxin could be further subdivided into four categories as shown in Table 12. Forty-nine of these isoaltes produced diarrhea and death in the infant rabbit and fluid accumulation in the ligated ileal loop. Of these, culture filtrates from 7 isolates produced a haemorraghic lesion in the shaved back of rabbits. Isolates in Group III are inactive in the infant rabbit but positive in the ileal loop. Isolates in Group IV are ileal loop negative but cause disease in infant rabbits. The reason for these last two patterns is unclear. An isolate which cannot colonize the gut might be unable to produce diarrhoea in the infant rabbit whereas in the ligated ileal loop, colonization factors would not be as important. A positive infant rabbit reaction but a negative ileal loop, as in Group 4, may be explained by the older animals being refractory to the agent which initiates fluid secretion in the gut of infant rabbits. The brush border adherence assay does not differentiate Groups III and IV. For both, the mean number of vibrios adhering per brush border is about 11.

Table 12

Subgroups of Enteritis Type of Biological Activity

N	Infant Rabbit	Ileal Loop (Whole Cult.)	Rabbit Skin Haemorrhagic Lesion
42	Pos	Pos	Neg
7	Pos	Pos	Pos
11	Neg	Pos	Neg
13	Pos	Neg	Neg

Table 13 presents the patterns of biological activity in all of the isolates collected in our clinical study. Only 1/43 isolates in the non-O-Group I *V. cholerae* is completely without biological activity in the models tested. Seven isolates (16%) produce detectable CT-like toxin and the rest produce enteritis. None of the other species produce a CT-like toxin. Twenty *V. parahaemolyticus* isolates (74%) produce enteritis but only 57% of *A. hydrophila* and 20% of the Group F vibrios are active.

Table 13
Biological Activity of Clinical Study Isolates

Species	Bio Activity				No. of strains examined
	CT-like	ST-like	Enteritis without CT or ST	Negative	
V. cholerae Non-O-Group I	7	0	35	1	43
V. parahaemolyticus	0	0	20	7	27
A. hydrophila. Subsp. anaerogenes	0	0	4	3	7
Group F vibrios	0	0	2	8	10

Table 14 shows the biological activity of non-O-Group I *V. cholerae* from different sources. The proportion of isolates producing enteritis is similar regardless of source. One-quarter of the clinical isolates from diarrheal patients and cases of other illness produce CT-like toxin. Ten of these isolates are stock cultures from past outbreaks and have maintained their toxigenicity for up to ten years. Environmental isolations are a richer source for organisms which are inactive in our model systems, 27% of environmental isolates were in this category. We hoped this would be the case since the widest possible range of phenetic characters must be represented or our attempt to define various biotypes of *V. cholerae* would not be meaningful.

Table 14
Biological Activity of *V. cholerae* Non-O-Group I Isolated from Different Sources

	Diarrheal patients (N = 72)		Non-diarrheal illness (N = 8)		Environmental (N = 30)	
	No.	%	No.	%	No.	%
CT-like	17	24	2	25	2	7
ST-like	0	–	0	–	2	7
Enteritis	49	68	6	75	18	60
Inactive	6	8	0	–	8	27

The spectrum of biological activity in each of the Heiberg groups represented in the sutdy is shown in Table 15. Toxigenicity, at least for CT-like toxin, is not limited to any one Heiberg group. What is not shown here, however, is that for a particular area at a particular time, alomost all toxigenic islates have occurred in the same Heiberg group. In Dacca druing the past year, all toxigenic isolates were in Group V. In contrast, the isolates from the Sudan outbreak of 1968 all belonged to Group I and those from Czechoslovakia in 1966 were in Group II. Thus, Heiberg grouping may be of some limited epidemiological value. We are unable to comment on serotyping since this was started recently and no data is available yet.

Table 15

Biological Activity of *V. cholerae* Non-O-Group 1 Belonging to Different Heiberg Groups

	Heiberg Group					
	(N = 21)		II (N = 64)		V (N = 25	
	No.	%	No.	%	No.	
ke	7	33	6	9	8	32
ke	0	–	0	–	2	8
itis	10	48	50	78	13	52
tive	4	19	8	13	2	8

The final table (Talble 16) contrasts the characteristics of the two enterotoxic activities we have detected in culture filtrates from non-O Group I *V. cholerae*. The CT-like toxins from 3 of the new clinical isolates are heat-labile and non-dialyzable. They elicit peak fluid accumulation at about 12 hours in both ileal loop and suckling mouse models. This was similar to the pattern of fluid accumulation following exposure to *V. cholerae* 569B culture filtrate. Y-1 adrenal and CHO cell activity can be neutralized by antitoxin prepared against purified cholera toxin. Antisera produced against the crude CT-like toxins neutralizes the activity of cholera toxin. Complete inhibition of activity occurs in either case and the tiration curves all have a single inflection point.

The ST-like toxin from two isolates from the Chesapeake Bay is not heat-labile, even at 100C for 30 minutes. It is dialyzable and, though not shown here, is precipitable by acetone and capable of passing through an Amicon AM-10 filter. Its action is much more rapid than that of cholera toxin in the ileal loop or suckling mouse. Peak fluid accumulation occurs by 4 hours. Its activity cannot be neutralized by anti-choelra toxin antiserum.

The CT-like toxin and ST-like toxin-producing isolates described here have been examined for plasmids by agarose gel electrophoresis. This work was done by Dr. Loré Anne McNichol in Dr. Rita Colwell's laboratory. Cleared lysates were run on 0.7% and 0.5% agarose gels and no plasmids were detected in any isolate. It does not seem likely, therefore, that the production of either toxin is plasmid-mediated.

Table 16

Comparative Features of CT-Like and ST-Like Toxins

	CT-like*	ST-like
Heat-labile (100C, 30 min)	Yes	No
(56C, 10 min)	Yes	No
Dialyzable	No	Yes
Peak fluid accumulation		
in ileal loops (hrs)	10–12	2–4
in suckling mouse (hrs)	12	4
Neutralized by anti-CT	Yes	
Antitoxin neutralizes activity of CT	Yes	

Tested in Y-1 adrenal cell assay.

Tested in suckling mouse assay.

In the preliminary results we have presented here, we can detect a number of patterns of biological activity that we believe would be very profitable to characterize further. The work done so far also appears to be a successful way of starting on the problem of biotyping the non-O-Group I *V. cholerae*, at least with respect to pathogenic potential. It is obvious, though, that much more work remains to be done.

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REFERENCES

1. Aldova, E., K. Lazonikova, E. Stepankova, and J. Lietova, 1968. Isolation of non-agglutinable vibrios from an enteritis outbreak in Czechoslovakia. J. Infect. Dis. *118*:25-31.

2. Benenson, A. S., M. R. Islam and W. B. Greenough III. 1964. Rapid identification of *Vibrio cholerae* by dark field microscopy. Bull. W. H. O. *30*: 827-831.

3. Colwell, R. R. 1970. Polyphasic taxonomy of the Genus *Vibrio*: Numerical taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus* and related *Vibrio* spp. J. Bacteriol. *104*:410-433.

4. Craig, J. P. 1965. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent choelra sera. Nature (Lond.) *207*:614-616.

5. De, S. N. and D. N. Chatterjee. 1953. An expenmental study of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Path. Bact. *66*:559-562.
6. Dean, A. G., Y-C. Ching, R. G. Williams and L. B. Harden. 1972. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. J. Infect. Dis. *125*:407-411.
7. Dutta, N. K. and M. K. Habbu. 1955. Experimental cholera in infant rabbits: A method for chemotherapeutic investigation. Brit. J. Pharmacol. and Chemother. *10*:153-159.
8 Guerrant, R. L., L. L. Brinton, T. C. Schnaitman and L. I. Gilman. 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive, in vitro assay for the enterotoxin of *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. *10*:320-322.
9 Hormaeche, E., and C. A. Peluffo. 1959. Laboratory diagnosis of *Shigella Salmonella* infections. Bull. W.H.O. *21*:244-277.
10 Inuzaka, N., S. Nakamura, M. Inuzaka, and M. Tomoeda. 1969. Specific action of SDS on the sex factor of *E. coli* K-12 Hfr strains. J. Bacteriol. *100*:822-835.
11. Jones, C. W., G. D. Abrams and R. Freter. 1976. Adhesive properties of *Vibrio choelrae*: Adhesion of isolated rabbit brush border membranes and hemagglutinating activity. Infect. Immun. *14*: 232-239.
12. Kusama, H. and J. P. Craig. 1970. Production of biologically active substances by two strains of *Vibrio cholerae*. Infect. Immun. *1*:80-87.
13 Furniss, A. L., J. V. Lee, T. J. Donovan. 1977. Group F, a new vibrio? Lancet. *2*:565-566.
14 Lennette, E. H., E. H. Spaulding, and J. H. Truant, eds. Manual of Clinical Microbiology. 2nd ed. 1974. Amer. Soc. Microbiology. Washington, D.C.
15. McIntyre, O. R., J. C. Feeley, W. B. Greenough III, A. S. Bennenson, S. I. Hassan, and A. Saad. 1965. Diarrhea caused by non-cholera vibrios. Am. J. Trop. Med. Hyg. *14*:412-418.
16. Monsur, K. A. 1961. A highly selective gelatin-taurocholate tellurite medium for the isolation of *Vibrio cholerae*. Trans. Royal Soc. Trop. Med. Hyg. *55*:440-442.
17. Neogy, R. M. and A. C. Mukerji. 1970. A study of the "string" test in *Vibrio* identification. Bull. W.H.O. *42*:638-641.
18. Ohashi, M., T. Shimada and H. Fukumi. 1972. *In vitro* production of enterotoxin and hemorrhagic principle by *Vibrio cholerae*, NAG. Jap. J. Med. Sci. Biol. *25*:179-194.
19. Sack, D. A. and R. B. Sack. 1975. Test for enterotoxigenic *Escherichia coli* using Y-1 adrenal cells in miniculture. Infect. Immun. *11*:334-336.
20. Sakazaki, R. G., C. Z. Gomez and M. Sebald, 1967. Taxonomical studies of the so-called NAG vibrios. Japan. J. Med. Sci. Biol. *20*:265-280.
21. Shewan, J. M., W. Hodgkiss and J. Liston. 1954. A method for the rapid differentiation of certain non-pathogenic asporogenous bacilli. Nature *123*: 208-209.
22. Smith, H. L. 1970. A presumptive test for vibrios: the "string" test. Bull. W.H.O. *42*:817-818.
23. Tomoeda, M., M. Inuzaka, N. Kubo and S. Nakamura. 1968. Effective elimination of drug resistance and sex factors in *Escherichia coli* by sodium dodecyl sulfate. J. Bacteriol. *95*: 1078-1089.
24. Yajnik, B. S. and B. G. Prasad. 1954. A note on vibrios isolated in Kumbh Fair, Allahbad. Indian Med. Gazette *89*:341-349.
25. Zinnaka, Y. and C. C. J. Carpenter, Jr. 1972. An enterotoxin produced by noncholera vibrios. Johns Hopkins

Med. J. *131*: 403-411.

26. Zinnaka, Y. and S. Fukuyoshi. 1974. Further observations on the NAG vibrio toxin. *In* Proceedings of the Ninth Joint Cholera Research Conference, U.S.-Japan Cooperative Medical Science Program, Grand Canyon, Arizona, October 1973. Dept. of State Publication No. 8672.

27. Zinnaka, Y., S. Fukuyoshi and Y. Okamura. 1973. Some observations on the NAG vibrio toxin. *In* Proceedings of the Eighth Joint Choelra Resarch Conference, U.S.-Japan Cooperative Medical Science Program, Tokyo, August 1972.