APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1986, p. 875–879
0099-2240/86/100875-05$02.00/0

Vol. 52, No. 4

Comparative Study of Expression of Hemagglutinins, Hemolysins, and Enterotoxins by Clinical and Environmental Isolates of Non-O1 *Vibrio cholerae* in Relation to Their Enteropathogenicity

KRISHNA DATTA-ROY,[1] KALYAN BANERJEE,[1] S. P. DE,[2] AND ASOKE C. GHOSE[1]*

Departments of Immunology[1] and Microbiology,[2] National Institute of Cholera and Enteric Diseases (Indian Council of Medical Research), Beliaghata, Calcutta—700 010, India

Received 18 February 1986/Accepted 8 July 1986

A comparative study was undertaken of clinical and environmental isolates of non-O1 *Vibrio cholerae* with respect to their hemagglutinating, hemolytic, enterotoxigenic, and enteropathogenic activities. Cell-associated hemagglutinin titers of the clinical and environmental isolates did not differ much, although the clinical isolates displayed higher cell-free hemagglutinin titers compared with those of environmental isolates. Culture supernatants of 61.5% (24 of 39) of clinical isolates showed hemolytic activity (≥10% lysis of rabbit erythrocytes), while only 33.3% (10 of 30) of the environmental group had such activity. Furthermore, hemolytic activities of the clinical isolates showed a good correlation with their cell-associated hemagglutinin titers which was not true for the environmental group. Culture supernatants of 45.8% (11 of 24) of the clinical and 20% (2 of 10) of the environmental isolates exhibited enterotoxigenic activity in the rabbit ileal loop assay. Such activity was mediated mainly by cholera toxin-like substances, although some of the isolates produced fluid-accumulating factors unrelated to cholera toxin. Experimental animal studies demonstrated that the enteropathogenic potential of the environmental isolates was significantly lower than that of the clinical group. Further analysis of our data showed that phenotypic expression of cholera toxin-like products by the non-O1 *V. cholerae* isolates was accompanied by their enteropathogenicity. The latter effect was also noted with some of the cholera toxin-negative isolates, particularly in those having high hemagglutinating and hemolytic titers.

Vibrio cholerae organisms of non-O1 serotypes are widely distributed in the aquatic environment and are free-living in nature (2, 28). Unlike *V. cholerae* O1, they do not usually have epidemic or pandemic potential (2). However, they have often been identified as the causative agents of sporadic cases (12, 16, 22) and localized outbreaks (1, 4, 29) of choleralike diarrhea which is sometimes accompanied by fever, blood, and mucus (4, 12). The severity of non-O1 diarrhea is of lesser magnitude compared with that of *V. cholerae* O1 (21). Apart from their diarrheagenic potential, these organisms have also been implicated in a few cases of extraintestinal infections (2, 12).

A good number of *V. cholerae* non-O1 isolates produce cholera toxin (CT)-like toxins (13, 19, 23, 31–33) although the majority do not. Apart from CT-like toxins, production of other kinds of factors responsible for fluid accumulation (FA) or enteritis by non-O1 strains has also been documented (11, 14, 15, 17, 18, 21, 22, 25, 26). Furthermore, they express certain other biologically active factors such as hemagglutinins (8, 10) and hemolysins (28) which may have some role in the expression of their pathogenicity. However, the relative importance of these putative virulence factors is still obscure. Moreover, it is not yet clear why clinical cases of diarrhea produced by non-O1 *V. cholerae* are rarely encountered despite the widespread occurrence of these organisms in the environment.

In the present communication, we made a comparative study of several clinical and environmental isolates of non-O1 *V. cholerae* with respect to their hemagglutinin, hemolysin, and CT-like toxin production capacities. We also tried to determine whether any of these properties either singly or in combination can be related to the enteropathogenic potential of the organisms as determined in the experimental animal models.

MATERIALS AND METHODS

Bacterial strains. The clinical strains of non-O1 *V. cholerae* used in this study were isolated in this Institute, essentially as pure cultures from the stool samples of diarrheal patients. Two of the clinical strains were kindly supplied by R. Sakazaki (Tokyo, Japan), while seven other strains were supplied by H. L. Smith (Philadelphia, Pa.). The environmental non-O1 *V. cholerae* strains were isolated from the water and sediments of local (Calcutta) ponds and the Hooghly River. All the strains were characterized by routine bacteriological tests carried out in the Microbiology Department of our Institute. Some of the strains could be serotyped by the antisera kindly supplied by R. Sakazaki. Strains supplied by H. L. Smith had already been typed in his laboratory.

Determination of CAHA titer. *V. cholerae* non-O1 strains were grown in nutrient agar slants for 18 h at 37°C. Cells were harvested by washing twice with cold normal saline and centrifugation (5,000 × *g*) and finally suspended in Krebs-Ringer-Tris buffer (KRT), pH 7.4. The concentration of the bacterial suspension was adjusted to 600 Klett units. The cell-associated hemagglutinin (CAHA) titer was determined against a mouse erythrocyte suspension (2.5% in KRT) as described by Dasgupta et al. (6). CAHA activities of some of the strains were also determined against chicken, rabbit, and human erythrocytes.

Determination of CFHA titer. Bacterial strains were grown in 50 ml of tryptic soy broth (Difco Laboratories, Detroit, Mich.) in 250-ml Erlenmeyer flasks for 18 h at 37°C while shaking. The culture was centrifuged at 14,000 × *g* for 30 min, and the supernatant was passed through a Millipore

* Corresponding author.

filter paper (0.45-μm pore size). Next, the cell-free culture supernatant was made 50% saturated with $(NH_4)_2SO_4$, and the mixture was kept overnight at 4°C. The precipitate was centrifuged at 16,000 × *g* for 15 min. The pellet was dissolved in the minimum volume of distilled water and dialyzed against KRT buffer. The dialyzed material was centrifuged, and the clear supernatant was appropriately diluted (10- to 15-fold) with the same buffer to give a protein concentration of 50 μg/ml. Hemagglutinin titers of these cell-free preparations were determined against a 2.5% mouse erythrocyte suspension as described previously (6). Cell-free hemagglutinin (CFHA) activities were also determined against chicken, rabbit, and human erythrocytes.

Determination of hemolytic activity. For the determination of cell-free hemolytic activity, bacteria were grown in brain heart infusion broth for 24 h at 37°C while shaking. The culture was centrifuged at 14,000 × *g* for 15 min, and the supernatant was collected. A 1-ml sample of this supernatant was mixed with 1 ml of 1% rabbit erythrocyte suspension in phosphate-buffered saline, pH 7.2. The mixture was incubated for 1 h at 37°C. Next, it was centrifuged at 200 × *g* for 2 min, and the supernatant was collected. The amount of lysis was determined by measuring the A_{540} of the supernatant. Finally, results were expressed as the percentage of lysis by comparing these optical density values with that of an identical erythrocyte suspension lysed (100%) with an equal volume of distilled water. Cell-free hemolytic activities of some of the non-O1 strains were also determined against chicken, mouse, and human erythrocytes in the same way as described above.

Determination of enterotoxic activity. Enterotoxin production capacity of the non-O1 *V. cholerae* strains was determined by the rabbit ileal loop (RIL) method (7) and the staphylococcal coagglutination (COA) test (20).

For the RIL test, bacteria were grown in brain heart infusion broth for 18 h at 37°C with shaking, and cell-free culture supernatant (1 ml) was injected into the ligated ileal loops of a rabbit. After 18 to 20 h, the rabbit was sacrificed and the accumulated fluid volume was measured. Results were expressed as the FA ratio (fluid volume in milliliters per loop length in centimeters). The culture supernatant of each of the bacterial strains was tested twice in two separate rabbits, and the mean of the two values was recorded.

For the COA test, COA reagent was prepared by essentially following the methodology of Ronnberg and Wadstrom (20). Rabbit antiserum against purified CT (Schwarz/Mann, Orangeburg, N.Y.) was raised in the laboratory and diluted fourfold to coat *Staphylococcus aureus* Cowan I cells. This dilution was found to be the optimum when prechecked with positive (purified CT at appropriate dilutions) and negative (uninoculated broth) controls. Non-O1 strains were grown in syncase broth (3) supplemented with lincomycin (100 μg/ml) at 30°C for 18 h without shaking (23). Cell-free culture supernatants were obtained by centrifugation at 14,000 × *g* for 30 min and passed through Millipore filter paper (0.45-μm pore size). The COA test was done on a glass slide by adding 25 μl of the culture filtrate to 25 μl of the COA reagent.

Determination of pathogenicity. (i) RIL method. Ligated ileal loops of rabbits were challenged with 0.5 ml of whole cultures of bacteria (10^9 cells per ml) grown in brain heart infusion broth for 18 h. The FA ratio was determined by the same procedure as described above.

(ii) The infant mouse model. Pathogenicity of some of the non-O1 *V. cholerae* strains was also determined in the infant mouse model (27). For this, laboratory-bred 4- to 5-day-old Swiss albino mice of both sexes were orally challenged with 0.1 ml of the bacterial suspension (10^9 cells per ml) in 0.1% Proteose Peptone (Difco Laboratories, Detroit, Mich.)–saline containing 0.01% Evan's blue. Bacteria were grown earlier in nutrient agar slant cultures for 18 h at 37°C and harvested. Groups of five mice were used for each bacterial strain. Results were scored by determining the number of diarrheagenic deaths in each group of mice within 24 h of challenge. A control group of mice received 0.1 ml of Proteose Peptone-saline with dye only. A hypertoxigenic strain of *V. cholerae* O1 (569B Inaba) was used as the positive control.

RESULTS

Hemagglutinating activity. CAHA titers of 32 clinical and 22 environmental *V. cholerae* non-O1 strains were determined against mouse erythrocytes. It was found that CAHA titers (reciprocal) varied considerably and ranged from ≤2 to 256. However, no marked difference could be noted between the clinical and environmental isolates with respect to their CAHA titers. Furthermore, comparable titers were obtained when some of the strains from both the groups were tested against chicken, human, and rabbit erythrocytes.

Seven clinical and three environmental isolates were also tested for their ability to produce CFHA. Interestingly, six of seven clinical and all three environmental isolates showed this activity against mouse erythrocytes, with titers ranging between 4 and 128 for the clinical isolates and 4 and 8 for the environmental samples. Comparable titers were obtained with rabbit erythrocytes. CFHA titers were, however, very weak or absent (titer of ≤4) for these strains against chicken and human erythrocytes.

Hemolytic activities. Hemolytic activities of 39 clinical and 30 environmental isolates were determined against rabbit erythrocytes. The hemolytic activities of strains belonging to both groups were found to vary considerably. However, a greater percentage of clinical isolates (61.5%) showed hemolytic activities above 10%, while only 33.3% of all the environmental isolates had such activities. Three clinical strains had very high hemolytic activities (80 to 100%), while none of the environmental strains belonged to this group. Hemolytic activities of some of these strains against erythrocytes of other species followed the order: rabbit > mouse > chicken > human.

Correlation of CAHA titers with hemolytic activities. CAHA titers of the 32 clinical and 22 environmental non-O1 isolates were plotted separately against their corresponding hemolytic activities (Fig. 1). Statistical analysis (5) of the data shows that the Spearman's rank correlation coefficient (r_ρ) between these two parameters for the clinical isolates is 0.58 and that this value is statistically significant ($P < 0.001$). On the other hand, r_ρ for the environmental samples is 0.36, which is not statistically significant ($P > 0.05$).

Enterotoxin production. (i) Culture supernatants of 11 of 24 (45.8%) clinical and 2 of 10 (20%) environmental strains gave positive ileal loop reactions (FA ratio, ≥0.3). However, FA ratios were comparatively higher for clinical isolates (ranging between 0.34 and 1.05 with a mean value of 0.59) than for environmental isolates (ranging between 0.40 and 0.45 with a mean value of 0.43). (ii) Culture supernatants of 9 of 24 (37.5%) clinical and only 1 of 10 (10%) environmental strains gave positive COA reactions. In all cases, the positive COA reaction could be blocked by prior incubation of the culture supernatants with rabbit anti-CT serum.

Pathogenicity test. A total of 24 clinical and 10 environmental strains were tested for their diarrheagenic potential in

the ligated ileal loops of rabbits (Table 1). All of the 24 clinical and 7 of 10 environmental strains showed positive loop reactions (FA ratio, ≥0.4). However, the reactivities of the clinical isolates were stronger than those of environmental ones, and this difference was statistically significant as determined by Student's t test ($0.05 > P > 0.01$).

Seven clinical and seven environmental non-O1 isolates were tested for their enteropathogenic potential in the infant mouse model (Table 2). The table also includes the serotypes, CAHA, CFHA, and hemolytic activities of these strains as well as their enterotoxin production capacities. These strains are also shown in Fig. 1 as filled circles. Of seven clinical isolates, six induced ≥60% diarrheagenic death within 24 h of challenge, whereas only two of seven environmental strains showed similar mortality rates. Mortality rates in mice challenged with the rest of the environmental strains varied considerably (0 to 40%).

DISCUSSION

Results presented in this study show that *V. cholerae* strains of non-O1 serotypes possess considerable amounts of CAHA, CFHA, and hemolytic activities. In fact, the CAHA titer ranges were comparable to those of *V. cholerae* O1 (ElTor) strains as reported earlier from this laboratory (6). It may be mentioned here that this is probably the first report which convincingly demonstrates the release of soluble hemagglutinin in the cell-free culture supernatants of non-O1 strains. However, CFHA activity showed some degree of species specificity and was demonstrable mainly against mouse and rabbit but not human or chicken erythrocytes. A high percentage of non-O1 strains also showed hemolytic activities toward rabbit and, to a lesser extent, mouse erythrocytes. Such activity was very weak, if present at all, against chicken and human erythrocytes. Similar results were also reported from the study of a non-O1 hemolysin by Yamamoto et al. (30), who, however, did not report any hemagglutinating activity in their system.

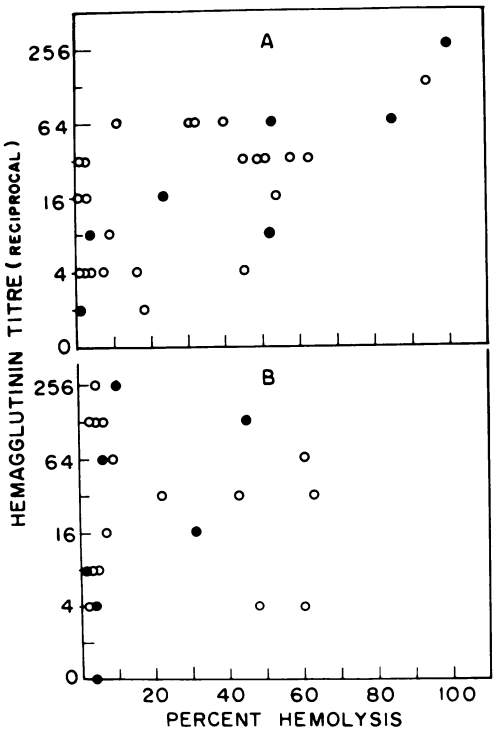

FIG. 1. Correlation between CAHA titers and hemolytic activities of 32 clinical (A) and 22 environmental (B) isolates of non-O1 *V. cholerae*. Filled circles represent isolates used for detailed study as presented in Table 2.

TABLE 1. Enteropathogenicity of whole cultures of non-O1 *V. cholerae* isolates in the RIL model

Source	No. of isolates[a]	No. of positive reactions[b]	FA ratio (ml/cm)		Statistical analysis[d]
			Range	Mean[c] ± SD	
Clinical	24	24	0.4–1.1	0.61 ± 0.18	$t = 2.28$
Environmental	10	7	0.4–0.5	0.46 ± 0.01	$0.05 > P > 0.01$

[a] The same isolates were used for these experiments as well as for studying enterotoxin production abilities in the RIL model and COA test.
[b] FA ratio, ≥ 0.4.
[c] Mean of positive reactions only.
[d] Done by Student's t test.

Results presented here (Table 2) failed to show any absolute correlation between CAHA and hemolytic titers and virulence of non-O1 isolates. This was true for isolates belonging to clinical as well as environmental groups. Furthermore, no significant difference in the CAHA titers between the clinical and the environmental isolates could be seen. However, a higher percentage of the clinical isolates (61.5%) had more than 10% of hemolytic activity compared with 33.3% of the environmental ones. Another interesting observation was that the CAHA titers of clinical isolates showed some correlation with their corresponding hemolytic activities, which was not true for the environmental group. The exact significance of these observations is not clear at present. *V. cholerae* O1 strains are known to express more than one kind of CAHA (8, 9). It is quite possible that the non-O1 strains also follow the same rule and that one of these hemagglutinins is probably related to its hemolytic activity. The clinical strains, for some reason yet unknown, may express more of the latter type of CAHA related to the hemolysin.

A high percentage of clinical isolates (45.8%) showed enterotoxin production activity in their culture supernatant which induced FA in the ligated ileal loops in rabbits. The fact that the majority of these reactions were mediated by the CT-like toxin is supported by the results of the COA test. Interestingly, supernatants of four clinical and one environmental isolates gave positive loop reactions but a negative COA test, thereby suggesting the presence of FA factors unrelated to CT. In fact, the culture supernatant of one (clinical) of these five isolates was subsequently found to produce heat-stable enterotoxinlike activity in the suckling mouse assay (K. Datta-Roy and A. C. Ghose, unpublished observation). Release of non-CT-like FA factor by non-O1 vibrios has already been documented by other workers (11, 14, 15, 17, 18, 21, 22, 25, 26), although its biochemical identity has yet to be clearly established. It was further noted that culture filtrates of two of the clinical isolates failed to induce FA in the RIL experiments, while giving positive reactions in the COA test. It is possible that supplementation of the culture medium with lincomycin, coupled with the higher sensitivity of the COA test (3), led to the detection of CT production by these hypotoxigenic strains.

That non-O1 strains can cause enteritis even in the absence of CT production is evident from our experimental animal studies. In fact, all the 24 clinical strains and a large percentage of the environmental strains (70%) induced FA in

TABLE 2. Expression of hemagglutinins, hemolysins, enterotoxins, and enteropathogenicity by some clinical and environmental isolates of non-O1 *V. cholerae*

Strain	Source	Serotype	COA reaction	% Hemolysis	CAHA titer	CFHA titer	RIL expt (FA ratio)[a]		Suckling mouse test mortality[b]
							Whole culture	Culture filtrate	
V_2	Clinical	37 (Sak)[c]	+	100	256	128	0.95	1.0	4/5
V_5	Clinical	6 (Sak)	+	1	2	0	1.1	1.05	3/5
10357	Clinical	2 (Sm)[d]	−	52	64	4	0.73	—[e]	3/5
9829	Clinical	11 (Sm)	−	23	16	ND[f]	0.65	—	2/5
9627	Clinical	34 (Sak)	+	52	8	ND	0.52	—	3/5
S_{22}	Clinical	34 (Sak)	−	40	64	ND	0.65	—	3/5
10259	Clinical	59 (Sm)	+	4	8	4	0.9	0.65	3/5
58N	Environmental	NT[g]	−	3	0	ND	ND	ND	0/5
N70	Environmental	6 (Sak)	−	45	128	ND	0.5	—	4/5
N75	Environmental	NT	−	31	16	ND	0.45	—	1/5
N38	Environmental	14 (Sak)	−	2	8	ND	—[h]	—	0/5
N76	Environmental	6 (Sak)	+	10	256	ND	0.4	0.45	3/5
N100	Environmental	52 (Sak)	−	3	4	ND	0.46	—	2/5
N5	Environmental	37 (Sak)	−	8	64	ND	—	—	1/5
Saline									0/5
569B Inaba		O1					1.5	1.1	5/5

[a] Mean values of FA ratios obtained from two different rabbits.
[b] Ratio of the number of dead/number of challenged animals.
[c] Sakazaki serotype.
[d] Smith serotype.
[e] FA ratio was considered to be negative (<0.3).
[f] ND, Not done.
[g] NT, Not typable by Sakazaki serotyping system.
[h] FA ratio, ≥0.4.

the ligated ileal loops of rabbits (Table 1). However, the diarrheagenic potential was significantly lower in the environmental group compared with the clinical group. These results agreed reasonably well with those obtained with the infant mouse model (Table 2), in which the diarrheagenic mortality rate was considerably higher for the clinical than for the environmental group. Spira and co-workers (21, 22) in their studies with non-O1 *V. cholerae* strains showed that a greater proportion of human isolates possessed enteropathogenic potential compared with isolates obtained from the aquatic environment. They also noted that enterotoxin production was not common among their environmental isolates.

The parameters which determine the enteropathogenic potential of *V. cholerae* non-O1 strains are yet to be defined. However, it is evident from the results presented in Table 2 that of the various parameters examined in this study, phenotypic expression of CT-like toxin by the non-O1 *V. cholerae* strains was accompanied by their enteropathogenicity. Such enteropathogenicity was also noted with some of the non-CT-producing strains, particularly in those having higher hemagglutinating and hemolytic titers. The precise role of the hemagglutinins and hemolysins in the pathogenic process remains to be defined. In a recent report, Spira et al. (24) suggested that colonization factors of non-O1 strains play a critical role in the determination of their pathogenicity and that environmental strains were more poorly colonizing than the clinical ones. Therefore, it would be of considerable interest to define more specifically the biochemical nature of such putative factors responsible for adhesion and colonization and to establish their relationship, if any, with the hemagglutinins or hemolysins or both. Such studies are under way.

ACKNOWLEDGEMENTS

We thank S. C. Pal, director of the National Institute of Cholera and Enteric Diseases, for his encouragement during the course of this study. We also thank A. Banerjee for supplying the environmental non-O1 *V. cholerae* isolates and G. B. Nair for providing us with the serotyping data of some of the isolates.

LITERATURE CITED

1. **Aldova, E., and K. Laznickova.** 1968. Isolation of NAG vibrios from an enteritis outbreak in Czechoslovakia. J. Infect. Dis. **118**:25–31.
2. **Blake, P. A., and R. W. Weaver.** 1980. Diseases of humans (other than cholera) caused by vibrios. Annu. Rev. Microbiol. **34**:341–367.
3. **Chakraborti, M. K., S. P. De, A. C. Ghose, N. C. Mukherjee, and S. C. Pal.** 1985. Comparative evaluation of toxin production by atypical *Vibrio cholerae* biotype ElTor strains from acute diarrhoea. Int. Res. Commun. Syst. Med. Sci. **13**:164–165.
4. **Dakin, W. P. H., D. J. Howell, R. G. A. Sutton, M. F. O'Keefe, and P. Thomas.** 1974. Gastroenteritis due to nonagglutinable (noncholera) vibrios. Med. J. Aust. **2**:487–490.
5. **Das, D.** 1980. Statistics in biology and psychology, vol. 1 and 2. Academic Publishers, Calcutta.
6. **Dasgupta, C., A. Sen Majumder, and A. C. Ghose.** 1983. Hemagglutination and adherence properties of *Vibrio cholerae*. IRCS Med. Sci. **11**:797–798.
7. **De, S. N., and D. N. Chatterjee.** 1953. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Pathol. Bacteriol. **66**:559–562.
8. **Faris, A., M. Lindahl, and T. Wadstrom.** 1982. High surface hydrophobicity of hemagglutinating *Vibrio cholerae* and other vibrios. Curr. Microbiol. **7**:357–362.
9. **Finkelstein, R. A., and L. F. Hanne.** 1982. Characterization and distribution of hemagglutinins produced by *Vibrio cholerae*. Infect. Immun. **36**:209–214.

10. **Finkelstein, R. A., and S. Mukherjee.** 1963. Hemagglutination: a rapid method for differentiating *Vibrio cholerae* and ElTor vibrios. Proc. Soc. Exp. Biol. Med. **112**:355–359.
11. **Gyobu, Y., H. Kodama, H. Uetake, and H. Katsuda.** 1984. Studies on the enteropathogenic mechanism of non-O1 *Vibrio cholerae* isolated from the environment and fish in Toyama prefecture. Microbiol. Immunol. **28**:735–745.
12. **Hughes, J. M., M. D. Dannie, G. Hollis, M. S. Eugene, M. D. Gangarosa, and R. E. Weaver.** 1978. Noncholera vibrio infections in the United States. Ann. Intern. Med. **88**:602–606.
13. **Lahiri, A., R. K. Agarwal, and S. C. Sanyal.** 1983. Biological similarity of enterotoxin of *Vibrio cholerae* serotypes other than type 1 to cholera toxin and *Escherichia coli* heat labile enterotoxin. J. Med. Microbiol. **15**:429–440.
14. **Madden, J. M., B. A. McCardell, and D. B. Shah.** 1984. Cytotoxin production by members of genus Vibrio. Lancet **ii**:1217–1218.
15. **Madden, J. M., W. P. Nematouahi, W. E. Hills, B. A. McCardell, and R. M. Twedt.** 1981. Virulence of three clinical isolates of *Vibrio cholerae* non-O1 serogroups in experimental enteric infections in rabbits. Infect. Immun. **33**:616–619.
16. **McIntyre, O. R., and J. C. Feeley.** 1965. Characteristics of non-cholera vibrios isolated from caes of human diarrhoea. Bull. W.H.O. **32**:627–632.
17. **Nishibuchi, M., R. J. Seidler, D. M. Rollins, and S. W. Joseph.** 1983. Vibrio factors cause rapid fluid accumulation in suckling mice. Infect. Immun. **40**:1083–1091.
18. **O'Brien, A. D., M. E. Chen, R. K. Holmes, J. Kaper, and M. M. Levine.** 1984. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* 1 (Shiga)-like cytotoxin. Lancet **i**:77–78.
19. **Ohasi, M., T. Shimada, and H. Fukumi.** 1972. In vitro production of enterotoxin and hemorrhagic principle by *Vibrio cholerae*. Jpn. J. Med. Sci. Biol. **25**:179–194.
20. **Ronnberg, B., and T. Wadstrom.** 1983. Rapid detection by a coagglutination test of heat-labile enterotoxin in cell lysates from blood agar-grown *Escherichia coli*. J. Clin. Microbiol. **17**:1021–1025.
21. **Spira, W. M., and R. R. Daniel.** 1979. Biotype clusters found on the basis of virulence characteristics in non-O group I *Vibrio cholerae*, p. 440–457. *In* Proceedings of the 15th Joint Cholera Research Conference, U.S.-Japan Cooperative Medical Science Program. Japanese Cholera Panel, Toho University, Tokyo, Japan.
22. **Spira, W. M., R. R. Daniel, Q. S. Ahmed, A. Huq, A. Yusuf, and D. A. Sack.** 1978. Clinical features and pathogenicity of O group 1 non-agglutinating *Vibrio cholerae* and other vibrios isolated from cases of diarrhoea in Dacca, Bangladesh, p. 137–153. *In* Proceedings of the 14th Joint Cholera Research Conference, U.S.-Japan Cooperative Medical Science Program. U.S. Cholera Panel. NIH publication no. 80-200030. U.S. Government Printing Office, Washington, D.C.
23. **Spira, W. M., and P. J. Fedorka-Cray.** 1983. Production of cholera toxin-like toxin by *Vibrio mimicus* and non-O1 *Vibrio cholerae*: batch culture conditions for optimum yields and isolation of hypertoxigenic lincomycin-resistant mutants. Infect. Immun. **42**:501–509.
24. **Spira, W. N., P. J. Fedorka-Cray, and P. Pettebone.** 1983. Colonization of the rabbit small intestine by clinical and environmental isolates of non-O1 *Vibrio cholerae* and *Vibrio mimicus*. Infect.Immun. **41**:1175–1183.
25. **Stack, H. O., and I. Ciznar.** 1983. Biological activities of enterotoxin from *V. cholerae* non-O1. J. Hyg. Epidemiol. Microbiol. Immunol. **27**:189–195.
26. **Takao, T., Y. Shimonishi, M. Kobayashi, O. Nishimura, M. Arita, T. Takeda, T. Honda, and T. Miwatani.** 1985. Amino acid sequence of heat-stable enterotoxin produced by *V. cholerae* non-O1. FEBS Lett. **193**:250–254.
27. **Ujiiye, A., and M. Nakatomi.** 1968. Experimental cholera in mice. First report on the oral infection. Trop. Med. **10**:65–71.
28. **Wilson, R., S. Lieb, andA. Roberts.** 1981. Non-group I *Vibrio cholerae* gastroenteritis associated with eating raw oysters. Am. J. Epidemiol. **114**:293–298.
29. **World Health Organization.** 1969. W.H.O. Wkly. Epidemiol. Rec. **44**:10.
30. **Yamamoto, K., M. Atomani, T. Honda, Y. Takeda, and T. Miwatani.** 1984. Non-O1 *Vibrio cholerae* hemolysin: purification, partial characterization, and immunological relatedness to ElTor hemolysin. Infect.Immun. **45**:192–196.
31. **Yamamoto, K., Y. Takeda, T. Miwatani, and J. P. Craig.** 1983. Evidence that a non-O1 *Vibrio cholerae* produces enterotoxin that is similar but not identical to cholera enterotoxin. Infect.Immun. **41**:896–901.
32. **Zinnaka, Y., and C. C. J. Carpenter.** 1972. An enterotoxin produced by noncholera vibrios. Johns Hopkins Med. J. **131**:403–411.
33. **Zinnaka, Y., and S. Fukuyoshi.** 1974. Further observations on the NAG vibrio toxin, p. 61–81. *In* Proceedings of the 9th Joint Cholera Research Conference, U.S.-Japan Cooperative Medical Science Program, Grand Canyon, Ariz., 1973. U.S. Department of State Publication no. 8762. U.S. Department of State, Washington, D.C.