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Toxin Production by *Vibrio mimicus* Strains Isolated from Human and Environmental Sources in Bangladesh

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Vibrio mimicus **has recently been isolated from aquatic environments of Bangladesh. A total of 125 of 300 environmental isolates, representing various biotypes, and 19 human isolates were tested for enteropathogenicity by using several models. Less than 1% of the environmental isolates and slightly more than 10% of the clinical isolates produced cholera toxin-like toxin. A significant percentage of the environmental isolates (25%) and of the human isolates (74%) induced fluid accumulation in ligated rabbit ileal loops. One environmental strain produced heat-stable toxin-like enterotoxin, whereas all of the human isolates did not.** ***V. mimicus*** **strains were divided into the following three groups on the basis of their activity in various toxin assays: (i) organisms which produce a heat-labile enterotoxin immunobiologically similar to cholera toxin, (ii) organisms which produce a heat-stable enterotoxin-like toxin, and (iii) organisms whose whole-cell cultures have some activity characteristic of heat-labile toxin (e.g., fluid accumulation in ligated rabbit ileal loops and positive permeability factor) but are not positive by the GM1 enzyme-linked immunosorbent assay. One isolate from this group was able to elicit these results with cell-free culture filtrates. There was no correlation of biotype with toxic activity of** ***V. mimicus*** **isolates.**

Vibrio mimicus, a newly described pathogen (5), has recently been isolated in Bangladesh from patients with diarrhea (3, 11) and from environmental sources (2). Previous investigators reported that a heat-labile toxin unrelated to cholera toxin (CT) or *Escherichia coli* heat-labile toxin (LT) and extracellular heat-stable enterotoxins (ST) were produced by clinical isolates of *V. mimicus* (8, 9, 11). Some clinical isolates have been found to produce toxins immunobiologically identical to CT (9, 14). However, the production of this so-called CT-like toxin by *V. mimicus* seems to be rare (8). As with non-O1 *Vibrio cholerae*, a minority of the isolates from both environmental and clinical sources produce CT-like toxin (8, 14, 15). It has been estimated that this enterotoxin is made by less than 10% of clinical and less than 1% of environmental isolates of non-O1 *V. cholerae* (16). However, the frequency of toxin-producing strains of *V. mimicus*, regardless of toxin type (LT, ST, or CT-like), is not well-documented (16), although in clinical isolates in Bangladesh, the rate appears to be high (12). This study reports the frequency of potentially virulent environmental and clinical isolates of *V. mimicus* from Bangladesh that express toxin and toxin-like activity by several assay methods.

MATERIALS AND METHODS

Bacterial strains. During the period of August 1984 to August 1985, 300 isolates of *V. mimicus* were isolated and identified from aquatic environments of Bangladesh. Isolates were selected for toxigenicity testing to cover the spectrum of biotypes we isolated. Biochemical profiles included tests for ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (β-galactosidase release), nitrate reduction, citrate utilization, gelatinase activity, growth in alkaline-peptone-water with 6% NaCl, string test results, and the production of hemolysin.

A total of 19 clinical isolates from diarrheal patients attending the treatment center of the International Centre for Diarrhoeal Disease Research in Bangladesh were included in the study. The criteria for selection was that the *V. mimicus* must have been isolated from the patient in the absence of known bacterial and parasitic pathogens and that the patient must also have been negative for rotavirus. All clinical isolates of *V. mimicus* were collected during the period of January 1980 to September 1985.

Finally, 20 freshwater prawn samples from different vendors at a local market were likewise cultured for *V. mimicus*, of which 8 were found to be positive.

Enterotoxin assays. Production of heat-labile enterotoxin was tested for by the ligated rabbit ileal loop (RIL) assay (6) and by an increase in capillary permeability in the skin of an albino rabbit (4). ST production was tested for by the suckling mouse assay (7). The activity of CT-like toxin was determined by the GM1 ganglioside enzyme-linked immunosorbent assay (ELISA) (10).

RIL test. RIL toxin determinations were accomplished by the procedure of De and Chatterjee (6). Whole cells (10^8 to 10^{10}) were inoculated into RILs after propagation at 37°C for 4 h in a shaker bath in T1N1 broth (1% Trypticase [BBL Microbiology Systems] plus 1% NaCl, pH 7.4). Eighteen hours after inoculation, the animals were sacrificed and the fluid contents of the ileal loops were measured. Results were expressed as the ratio of accumulated fluid volume (in milliliters) per loop length (in centimeters). The CT-producing *V. cholerae* 569B and an LT-negative *E. coli* K-12 strain 265 were used as positive and negative controls, respectively.

Culture filtrate preparation. Strains of *V. mimicus* found to accumulate fluid in RIL were further studied. Supernatants of aerated cultures (180 rpm at 37°C for 18 to 24 h), grown in brain heart infusion broth (Difco Laboratories), were centri-

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TABLE 1. Results of RIL tests with live cells of *V. mimicus* isolated from different sources

Strain origin	No. positive/ no. tested (% positive)	Range of fluid accumulation in positive strains[a]
Environmental		
River water (surface)	9/40 (22)	0.57–2.22
River bottom (sediment)	6/17 (35)	0.57–1.50
Lake water (surface)	5/30 (17)	0.69–2.00
Lake bottom (sediment)	2/16 (12)	1.00–1.70
Pond water (surface)	0/9 (0)	
Plant root	9/13 (69)	0.51–2.30
Clinical		
Human diarrhea	14/19 (74)	0.79–2.07
Prawn	6/8 (75)	1.0–1.81
Positive-control *V. cholerae* (569B)		1.00–2.40

[a] Mean of fluid accumulation in loops of three rabbits with ≥0.50 interpreted as positive. Values shown are in milliliters per centimeter of gut.

fuged at 10,000 × *g* for 30 min and then passed through a sterile membrane filter (Millipore Corp.; pore size, 0.22 μm). The cell-free filtrates thus obtained were used with the skin permeability, suckling mouse, and GM1 ELISAs.

Skin PF. Vascular permeability factor (PF) was determined with albino rabbits of 1.5 to 2.0 kg. Hair was shaved from the back of the animal, and 0.1 ml of test, positive-, and negative-control materials were injected intradermally, approximately 2.5 cm apart. Each animal was able to accommodate approximately 40 tests. Two animals were used per filtrate, and samples were duplicated in each animal. Eighteen hours after inoculation, the animals were injected intravenously with a solution (2% in 0.15 M NaCl) of Evans blue dye at a concentration of 40 mg/kg of body weight. After 2 hours, allowing for permeation of the dye to take place, the induration and blueing of the lesions were recorded. The diameters of the zones of induration were measured to the nearest millimeter in two different directions (4).

Suckling mouse assay. Two- to four-day-old suckling mice were used for the ST assay (7). The milk-filled stomach of each mouse was inoculated with a 0.1-ml portion of the bacterial culture filtrate containing Evans blue (2%). Inoculated mice were held at 25°C for 4 h and then sacrificed with chloroform. The complete intestines were removed, and the ratio of the intestinal weight to the remaining body weight was determined to calculate the fluid accumulation ratio. Samples with a ratio greater than 0.083 were interpreted as ST positive (7).

GM1 ELISA. CT-like toxin was routinely assayed using the GM1 ganglioside ELISA described previously (10). We grew cultures in shaker flasks (180 rpm) of brain heart infusion medium for 24 h at 37°C. The culture filtrates were obtained for testing after centrifugation at 10,000 × *g* for 30 min.

RESULTS

Fluid accumulation with the RIL method with whole-cell cultures was found with 31 of 125 of environmental isolates of *V. mimicus* (24.8%), 14 of 19 clinical isolates (73.7%), and 6 of 8 prawn isolates (75%) (Table 1). However, bacteria-free culture filtrates of most of these strains failed to give positive reactions in RIL tests. Cell-free filtrates of one environmental and three clinical strains induced fluid accumulation in RIL.

All clinical isolates (19 of 19) and most environmental isolates (24 of 31) showed activity in the skin permeability test. Twelve environmental isolates and six isolates of human origin gave hemorrhagic lesions.

None of the clinical isolates produced ST-like enterotoxin as detected by the suckling mouse assay. One environmental isolate was positive by this method.

By GM1 ELISA, we found that only 1 of 125 environmental (0.8%) and 2 of 19 clinical (10.5%) strains of *V. mimicus* produced CT-like toxin (Table 2).

We found no correlation between biochemical characteristics and toxigenicity with our strains of *V. mimicus*.

DISCUSSION

Toxin production of *V. mimicus* has been reported almost exclusively from isolates of clinical origin. We examined clinical, as well as environmental, isolates of *V. mimicus* for their toxigenicity. We found that clinical isolates more frequently expressed toxic activity (74%) than environmental isolates did (25%). The significance of toxigenic or cytotoxic environmental strains is not known, and specific studies are required to elaborate their epidemiological importance and role in the pathogenesis of diarrheal disease. However, our hypothesis is that these environmental *V. mimicus* strains may play a significant role in diarrheal disease, as they represent a reservoir of organisms with virulence-associated characteristics.

We detected the presence of three different groups of *V. mimicus* on the basis of their activities in the different assay methods we used. The first group produced a toxin with activity and receptor specificity similar to that of CT which was called CT-like. Strains that produced CT-like toxin were positive by the GM1 ELISA, RIL test, and skin PF assay. One environmental strain, the only member of the second group, produced a toxin with activity in the suckling mouse model (7), which is used to determine the presence of ST. Heat inactivation per se was not performed by us. We considered this isolate to produce an ST-like toxin based on activity in the infant mouse alone. The production of ST-like toxin is of interest, in that several previous investigators had failed to detect ST production by *V. mimicus* isolates (11–13). However, Nishibuchi and Seidler (9) reported ST-like activity in suckling mice from a single isolate of *V. mimicus* isolated from an aquatic source in the United States. To our knowledge, this is the first report from Bangladesh of the identification of an environmental isolate of *V. mimicus* which produces this ST-like toxin. The third group we identified contained 39 isolates, all of which gave positive results in the RIL and PF tests but not in the GM1 ganglioside assay when whole-cell cultures were tested.

TABLE 2. Pathogenicity tests with culture filtrates of *V. mimicus* strains by various methods

Source	No. positive/no. tested			
	Skin PF	Suckling mouse assay (for ST)	GM1 ELISA (for CT-like toxin)	RIL test
Environmental (n = 125)	24/31[a]	1/31[a]	1/125	1/31[a]
Clinical (n = 19)	19/19	0/19	2/19	3/19

[a] A representative sample of all the phenotypes identified was tested.

Culture filtrates of 38 of 39 of these isolates failed to give positive results in RIL test and GM1 ELISA, thus reducing the likelihood of the presence of a free enterotoxin. However, the activity of this group was not exclusively cytotoxic, as the culture filtrates of one clinical strain were repeatedly able to give a positive result in RIL and PF tests but not in the GM1 ELISA.

The role of cell-associated toxins (cytotoxins) may be significant in diarrheal disease epidemiologically linked to *V. mimicus*. This needs clarification, in light of the markedly decreased number of positive RIL loops we obtained with cell-free culture filtrates.

Differences between the results of PF and RIL experiments likewise need examination. Activities associated with the skin permeability test are a result of the presence of diverse antigenic substances which may not be present in sufficient quantities to give a positive RIL result. The amount of these factors needed to give positive skin activity has been observed to be less than that required for ileal loop activity (1). However, PF and RIL activity are probably induced by more than one mechanism, at least one of which may be exclusive of the other. Our results clearly indicate that a majority of the RIL activity with our isolates is cytotoxic, and whole-cell cultures are necessary for RIL activity.

Overall, the frequency of RIL test-positive *V. mimicus* from our clinical isolates appeared to be markedly higher than that of environmental strains (74 versus 25%). This is in agreement with results obtained by other investigators (11, 16). The frequency of RIL test-positive *V. mimicus* strains from prawns (75%) was nearly identical to that seen with clinical specimens (74%). It appears that environmental isolates of *V. mimicus* that have RIL activity may be more prevalent than previously thought.

CT-like toxin production was rare with our isolates of *V. mimicus* (0.8%). These results are similar to those reported with non-O1 *V. cholerae* isolates (8, 16).

Our distinction between environmental and clinical strains of *V. mimicus* was based solely on their source of isolation. Environmental isolates were from aquatic sources, whereas clinical isolates were made from diarrheal stools of patients attending the International Centre for Diarrhoeal Disease Research treatment centers. In Bangladesh, there is much opportunity for fecal contamination of the aquatic environment. Therefore, our distinction between these strains must be viewed in this light. Fecal coliform counts to assess the level of contamination were not performed in this study, but another study performed at the same time in nearly the same waters showed significant levels of contamination (Zeaur Rahim, unpublished observation). However, our results do demonstrate marked phenotypic differences between our environmental and clinical isolates. The production of CT-like toxin, activity in RIL, and antibiogram patterns (2) were distinct between environmental and clinical sources, indicating biological differences between them.

We included the prawn toxigenicity data as an interesting point, as the rates of toxigenic activities of these isolates were similar to that seen with clinical isolates. It appears to us that prawns may represent an environmental source of *V. mimicus* human infections.

The true significance of *V. mimicus* as a human pathogen is not clear at present. The organism has been associated with human infections in both developed and developing countries, and environmental and clinical surveys identify it with increasing frequency. The results of this study suggest the ubiquity of this organism in the aquatic environment of Bangladesh. Our experiments were not undertaken to specifically address the mechanisms of pathogenicity of *V. mimicus* strains but rather to examine the properties of isolates we designated clinical and environmental in a variety of assays which reflect toxic and cytotoxic activities. Our findings suggest a homogeneity among strains within each category. While we are unable to state the epidemiologic significance of these two groups, there do appear to be environmental and clinical phenotypes which may be of help in the future in understanding the pathogenic potential of these organisms.

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