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© EXPERIMENTAL STUDIES ON THE PATHOGENICITY OF *VIBRIO MIMICUS* STRAINS ISOLATED IN BANGLADESH

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Summary. *Vibrio mimicus*, a newly described species of the genus *Vibrio* has been isolated from stools of 14 patients with diarrhoea. Live cells of all the 14 strains tested caused accumulation of fluid in rabbit gut loops and diarrhoea in infant rabbits. Culture filtrates of all the strains caused increased capillary permeability in rabbit skin; however, five of the filtrates resembled cholera toxin in that they gave positive reactions in rabbit loops, chinese hamster ovarian and mouse adrenal cell monolayers and GM_1 ELISA tests and were neutralized by cholera antitoxin. None of the strains produced heat-stable toxin or possessed invasive capability as determined by Sereny's test. Thus, *V. mimicus* strains were divided into a group which produced a toxin immunobiologically similar to cholera toxin and the rest producing a heat-labile toxin unrelated to that of *V. cholerae*.

INTRODUCTION

A new species, *Vibrio mimicus*, has recently been described in the genus *Vibrio* on the basis of biochemical, antigenic and deoxyribonucleic acid relatedness (Davis *et al.*, 1981). This organism was isolated from shellfish, water and human diarrhoeal stools in different parts of the world including Bangladesh (Davis *et al.*, 1981). However, little is known about the pathogenic mechanisms of diarrhoeal illness caused by the organisms. The present study was undertaken to explore the pathogenic mechanism of diarrhoea caused by *V. mimicus*, using animal models, tissue culture systems and immunological assays.

MATERIALS AND METHODS

Subjects

Patients with diarrhoea attending the ICDDR,B Treatment Centre were included in the study. Cases were selected by faecal culture and included those showing pure or dominant growth of sucrose-negative *V. cholerae*-like colonies which did not agglutinate with cholera O-group 1 antiserum. In all cases there were no other known bacterial pathogens, intestinal parasites or rotavirus detected.

Microbiological examination

Stool specimens were microscopically examined for parasites immediately after collection. Primary culture included taurocholate tellurite gelatin agar (TTGA) (Monsur, 1961), thio-

sulfate citrate bile salt sucrose (TCBS) agar, MacConkey, deoxycholate citrate agar (DCA) and Campy-BAP medium (Blaser *et al.*, 1979). Presence of rotavirus was tested by the ELISA technique (Yolken *et al.*, 1977).

The suspected *V. mimicus* strains were subjected to the biochemical tests as suggested by Davis *et al.* (1981) following the methods described by Cowan and Steel (1974).

Enteropathogenicity tests

Production of heat-labile (LT) enterotoxin was tested in rabbit gut loops (De and Chatterjee, 1953; Annapurna and Sanyal, 1977), infant rabbits (Dutta and Habbu, 1955), by increase in capillary permeability of albino rabbit skin (Craig, 1965; Dubey and Sanyal, 1978), by cytotonic activity in chinese hamster ovarian (CHO) cell (Guerrant *et al.*, 1974) and mouse adrenal (Y1) cell (Sack and Sack, 1975) monolayers, and by GM_1 (Monosialosyl Tetraglycosyl Ceramide) Ganglioside enzyme-linked immunosorbent assay (ELISA) (Sack *et al.*, 1980). Heat-stable enterotoxin production was tested in rabbit gut loops after the culture filtrates were held at temperatures of 56° for 30 min, 65° for 15 min and 100° for 15 min (Agarwal and Sanyal, 1981), by the time course of fluid accumulation in rabbit loops (Sack, 1975) and by the suckling mice assay (Gianella, 1977; Sanyal, Saraswathi and Sharma, 1980). The culture filtrates for the above tests were made in Richardson's medium (Richardson, 1969). The invasiveness of the strains was tested in guinea pigs' eyes by the method of Sereny (1957) using inocula of about 10^7 colony forming units (c.f.u.). The minimal ileal loop reacting doses with live cells and culture filtrates were determined following the technique described earlier (Annapurna and Sanyal, 1977).

Neutralization of enterotoxin with cholera antitoxin

Attempts to neutralize the enterotoxin produced by *V. mimicus* strains were made in rabbits' skin following the methods of Craig (1965) and Dubey and Sanyal (1978) using their culture filtrates and pure cholera antitoxin (anti-CT) kindly supplied by Jan Holmgren and Ann-Marie Svennerholm, Institute of Medical Microbiology, University of Goteborg, Sweden. Initially, the culture filtrates of *V. cholerae* strain 569B were titrated in the skin of rabbits to find out the BD_7. Two-fold dilutions of the filtrate were inoculated in 0·1 ml amounts intradermally into different pre-marked sites on the clean, depilated dorsal surfaces of albino rabbits (1·5-2·0 kg body weight) in duplicate. Gel borate buffer (CH_3BO_3, 3·09 g; gelatin, 0·198 g; NaCl, 7·01 g; NaCl, 0·8 g; distilled water, 1 litre; pH 7·5) served as negative control. The presence of induration and necrosis was examined after 22 h and Pontamine sky blue in half normal saline was administered intravenously in a dose of 1·2 ml/kg of body weight. The blueing zone was measured 1 h after giving the dye and the results were expressed as the mean diameter of the reaction. The highest dilution of the culture filtrate giving a blueing diameter of 7 mm was taken as BD_7 and was mixed with equal amounts of anti-CT diluted serially two-fold in gel borate buffer, incubated in a water bath at 37° for 60 min and 0·1 ml quantities were tested in rabbits' skin in duplicate to find out the highest dilution of anti-CT that neutralized the BD_7 completely. The BD_7 of the 14 *V. mimicus* culture filtrates were found in a similar way and their neutralization was attempted with four times the dose of anti-CT that inactivated the BD_7 of *V. cholerae* 569B.

RESULTS

Bacteriology

All the 14 *V. mimicus* strains grew well on gelatin agar (GA), TTGA and TCBS media. The important biochemical characteristics of the organisms differentiating from *V. cholerae* biotype *eltor* strains were: they did not ferment sucrose and were negative for V-P, lipase and tartrate, all were sensitive to polymyxin B and did not agglutinate with *V. cholerae* O-group 1 antiserum.

Enteropathogenicity tests

Live cells of the 14 *V. mimicus* strains caused accumulation of fluid in rabbit gut loops (Table 1) and diarrhoea in infant rabbit models. Five strains caused fluid accumulation in loops and diarrhoea in infant rabbits comparable to that of *V. cholerae* 569B, whereas the remaining 9 strains gave lesser reactions. Culture filtrates of the strongly reactive 5 strains prepared in

TABLE 1

Results of rabbit loop tests with live cells and culture filtrates of Vibrio mimicus *strains (n=14).*

Strain designation	Rabbit ileal loop tests*			
	Live cells		Culture filtrates	
	No. positive/ No. of tests	Range of fluid accumulation (ml/cm of gut)	No. positive/ No. of tests	Range of fluid accumulation (ml/cm of gut)
X-9515	2/2	2·0-2·5	4/4	2·0-3·0
X-9529	2/2	2·0-2·5	4/4	2·0-3·0
X-9838	2/2	2·0-2·6	4/4	2·0-2·6
W-10497	2/2	2·0-2·0	4/4	2·0-2·5
W-10652	2/2	2·0-2·8	4/4	2·1-2·5
W-1724	3/5	0·6-2·0	0/4	0·0-0·0
X-6013	6/8	0·6-1·5	0/4	0·0-0·0
X-7858	3/4	0·5-2·0	0/6	0·0-0·0
W-11442	3/6	0·2-1·0	0/4	0·0-0·0
W-25156	4/7	0·6-1·4	0/4	0·0-0·0
W-26768	5/7	0·2-1·3	0/4	0·0-0·0
W-4459	2/2	0·5-0·5	1/6	0·0-0·3
W-7875	7/8	0·5-2·0	1/4	0·0-0·2
W-12739	6/7	0·5-1·0	1/6	0·0-0·2

* In each rabbit 6-9 loops were made. The inoculum of live cells per loop for all the 14 strains was about 10^6 c.f.u. However, in loops treated with culture filtrates of the first 5 strains the inoculum was 1·0 ml and for the remaining 9 it varied between 1·0-2·0 ml in different loops. Number of tests indicates the number of rabbits used.

Richardson's medium gave positive reaction in ileal loops, CHO and Y1 cell monolayers and GM_1 ELISA and filtrates of the other 9 strains gave negative reaction in the tests (Table 2). All the filtrates were positive in skin permeability tests with reciprocal BD_7 titres ranging between 160-320 for the first 5 strains and 4-20 for the others, indicating a 20-40 fold quantitative difference in toxin production. A dilution of 1:512 of anti-CT neutralized the BD_7 of *V. cholerae* strain 569B filtrate. In attempts to neutralize the BD_7 of all the culture filtrates four times as much anti-CT was used but only the above-mentioned 5 were neutralized. None of the culture filtrates caused accumulation of fluid in ileal loops after treatment at 56° for 30 min, 65° for 15 min or 100° for 15 min. Suckling mice assay with these preparations showed gut weight/body weight ratios less than 0·07. The time course of fluid accumulation in rabbit gut loops with 5 strains of *V. mimicus* showed a similar trend to that with *V. cholerae* 569B. The minimal loop reacting dose of live cells of these organisms was determined as 5×10^2 c.f.u. and that of culture filtrates as 0·25 ml containing about 22-25 μg of protein during the 18 h period of observation. None of the strains caused kerato-

TABLE 2
Enteropathogenicity tests with Vibrio mimicus *strains.*

Rabbit ileal loop tests		Infant* rabbit tests	Skin PF	Neutralization of PF with anti-CT	CHO	Y1	GM1 ELISA	Suckling mice assay and heated culture filtrates in loops (ST)	Sereny's test
Live cells	Culture filtrates								
No. positive/ No. tested	No. positive/ No. tested								
5/5	5/5	+	+	+	+	+	+	—	—
9/9	0/9	+	+	—	—	—	—	—	—

* The inoculum of live cells per infant rabbit was about 10^8 c.f.u. for all the 14 strains and each strain was tested in duplicate rabbits.

conjunctivitis in guinea pigs' eyes within 72 h, whereas the positive control strain of *Shigella flexneri* always did so.

DISCUSSION

The *V. mimicus* strains formed colonies similar to *V. cholerae* on GA and TTGA and relatively small, smooth, regular, green colonies on TCBS due to their inability to ferment sucrose. These organisms possibly may be missed if only TCBS is used for isolation of vibrios as *V. cholerae* O1 and non-O1 usually form bright yellow colonies and *V. parahaemolyticus* grow as bright green, large, mucoid and sticky colonies. Use of a highly selective and differential medium like TCBS and of a less selective and non-differential medium is advocated for isolation of these vibrios. The organisms behaved like *V. cholerae* non-O1 of Heiberg's group V in their colonial morphology and carbohydrate fermentation properties; some other characters, however, were different and useful in distinguishing them from other related bacteria.

Live cells of all 14 strains caused accumulation of fluid in rabbit gut loop, indicating that they do liberate certain enterotoxic substance/s *in vivo*. Five of them, however, caused relatively more fluid accumulation than the remaining 9 strains. Similar results were obtained in 8-11 day-old infant rabbit tests in which the 5 strains caused profuse diarrhoea, whereas the other strains caused accumulation of fluid only in the large gut which usually occurs with less toxic strains of *V. cholerae* O1. The minimal loop reacting doses of live toxigenic cells of *V. mimicus* were comparable to the *V. cholerae* O1 strains. Clearly, there is a variable spectrum of toxicity amongst *V. mimicus* strains.

The observation that only the culture filtrates from 5 strains from the 14 strains caused accumulation of fluid in rabbit gut loop, was probably due to the fact that Richardson's medium is not suitable for good elaboration of toxin by all strains. It is known that if the live cells of certain organisms give positive ileal loop reactions, cell-free filtrates also do so when grown under suitable conditions. Moreover, the minimal loop reacting dose of live cells of the organisms being reasonably low, the positive reaction was most likely due to some enterotoxic substance liberated during multiplication *in vivo*. This argument appears to be valid because all the 14 culture filtrates caused increased permeability in rabbit's skin although quantitative differences were observed between the two groups. It indicates that a certain amount of enterotoxic substance was produced in *in vitro* cultures which was perhaps too low to cause fluid accumulation in rabbit gut loop. The organisms probably need some other medium to give a better yield of toxin.

The culture filtrates of the first group of 5 strains gave positive reactions in CHO, Y_1 and GM_1 ELISA tests, indicating that the enterotoxin is similar to cholera toxin and *E. coli* LT in its mode of action and receptor site. Skin permeability activity of these filtrates was completely neutralized by cholera antitoxin, suggesting their antigenic similarity as well. However, the other 9 culture filtrates were negative in the cell cultures as well as in GM_1 ELISA,

and their skin PF activity was not neutralized by cholera antitoxin. These observations may indicate that the second group of strains produces a different type of enterotoxic substance which also possesses skin PF activity but is not similar to that of cholera toxin. Recently, we demonstrated that certain *V. cholerae* O1 strains produce an enterotoxin not recognized previously which is heat-labile, protein in nature and differs from the known CT in antigenicity, receptor site, mode of action and genetic homology (Sanyal *et al.*, 1983). However, those strains elaborated the enterotoxin in Richardson's medium.

The enterotoxic substances in the culture filtrates of both groups of strains were heat-labile.

None of the strains proved invasive in Sereny's test and did not cause keratoconjunctivitis in guinea pigs' eyes.

The present study thus indicates that strains of the newly recognized species of *V. mimicus* may be divided into two groups based on their toxin production. One group produces heat-labile toxin immunobiologically similar to cholera toxin which is 20-40 times as much as the other group that elaborated a heat-labile toxin unrelated to that of *V. cholerae.*

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