

ENTEROTOXICITY OF *AEROMONAS HYDROPHILA*

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AEROMONAS HYDROPHILA has been isolated from animal excreta, sewage, soil, food, clinical specimens from man, and infections in cold-blooded marine and fresh-water animals (Hugh, 1970; Davis *et al.*, 1973; Annapurna and Sanyal, 1975). These data indicate that human beings in a community are being exposed continuously to this organism. In a preliminary communication Sanyal, Singh and Sen (1975) reported that some strains of *A. hydrophila* isolated from diarrhoeal and non-diarrhoeal subjects and water sources caused accumulation of fluid in ligated gut loops in rabbits. The present study was undertaken with a larger number of strains isolated from man and his environment to see (a) how many gave a positive result, (b) if they produced an enterotoxin, and (c) what histological changes occurred in the gut loop.

MATERIALS AND METHODS

Organisms. Fifty strains of *A. hydrophila* isolated in this laboratory during a community study of diarrhoeal diseases were used. They were isolated from children and adults with diarrhoea (7), healthy individuals (26), domestic animals (cow, buffalo, goat) and chickens (11), and water (6) from four sources, namely, the handpump of a shallow tube well, the tap of a piped filtered water supply, the river Ganges, and sewage (table I). They were characterised by the method described by Hugh (1970). The toxigenic strain of *V. cholerae*, no. 569B, was obtained from the Haffkine Institute, Bombay. All strains were maintained as stab cultures and did not undergo more than three subcultures.

Ileal loop test. This was done with live cultures, filtrates and crude toxin in ileal loops in adult rabbits (De and Chatterje, 1953) as described earlier (Sanyal, Singh and Sen, 1975). The inoculum was 1 ml of culture filtrate or crude toxin. Culture filtrate or crude toxin of *V. cholerae* 569B was the positive control, and Brain-Heart Infusion Broth (Difco) or isotonic saline was the negative control. Each test was done in three rabbits and 4–6 loops were usually prepared in each. The animals were killed after 6 h.

Passage of bacteria in ileal loops. One strain isolated from a case of diarrhoea and two from healthy individuals that gave no fluid accumulation in repeated tests were used. One ml of a 3–4 h nutrient broth culture diluted 1 in 10 in saline was injected into a loop. After 6 h the contents of the loop were removed aseptically and cultured on nutrient agar. Up to six colonies were isolated each time, cultured and inoculated into another loop until the strain gave a positive reaction.

Multiplication of bacteria in ileal loops. The method described by Sanyal, Singh and Sen (1975) was used with four strains isolated from a diarrhoeal case, a healthy individual, an animal and water from a hand-pump. A 3–4 h culture in nutrient broth was diluted 1 in 10 in isotonic saline and 1 ml was inoculated into each loop. Each experiment was done in three rabbits.

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Preparation of culture filtrates. Two ml of a 4–5 h peptone water culture were inoculated into 50 ml of Brain-Heart Infusion Broth in a 250 ml conical flask. The flasks were incubated at 37°C in a shaking water bath (120 oscillations per min.) for 18 h. The cultures were centrifuged at 4°C for 30 min. at 22 000 g, and the supernates were filtered through millipore filters of 0.45 µm average pore diameter and stored at 4°C.

Preparation of toxin. One hundred ml of basal medium (Finkelstein and Lankford, 1955, cited by Craig, 1966) in a 500-ml conical flask was inoculated from a stock culture and incubated at 37°C in a shaking water bath (120 oscillations per min.) for 18 h. The culture was centrifuged at 4°C for 30 min. at 22 000 g. The supernate was saturated to 90% with ammonium sulphate in an ice bath and centrifuged at 4°C for 30 min. at 22 000 g. The precipitate was suspended in 10 ml of distilled water and dialysed against it at 4°C with 5–6 changes of water. After estimating the protein content (Lowry *et al.*, 1951) the dialysed crude preparation was stored at 4°C.

Determination of minimum reacting dose of culture filtrate and crude toxin in ileal loops. Culture filtrates (0.1–2.0 ml) were inoculated into different loops of each rabbit. The animals were killed after 6 h and the accumulation of fluid per centimetre of gut was measured. The minimal amount of filtrate causing maximal accumulation of fluid was regarded as the minimal reacting dose. One-ml volumes of different dilutions of the crude toxin in distilled water were tested in the same way and the minimal reacting dose was calculated. All experiments were done in triplicate.

Effect of temperature on culture filtrate. Culture filtrates were heated at different temperatures for various times in a water bath and 1 ml was inoculated into each loop. The animals were killed after 6 h and the reactions noted. Each culture filtrate was tested in three rabbits.

TABLE I

Fluid accumulation produced by live cultures of Aeromonas hydrophila in rabbit ileal loops

Source of strains		Number of strains tested	Number of strains positive	Fluid accumulated* and number of strains (in parenthesis)
Man	Diarrhoeal disease	7	6	0.5–1.0 (1) 1.0–1.5 (2) 1.5–2.0 (3)
	Healthy individuals	26	24	0.25–0.5 (6) 0.5–1.0 (11) 1.0–1.5 (7)
Animals	Cow	5	5	1.0–1.5 (5)
	Goat	4	4	0.5–1.0 (1) 1.0–1.5 (3)
	Buffalo	2	2	1.0–1.5 (2)
	Chicken	1	1	0.5–1.0 (1)
Water	Handpump	1	1	1.5–2.0 (1)
	Tap water	1	1	1.0–1.5 (1)
	River Ganges	2	2	1.0–1.5 (1) 1.5–2.0 (1)
	Sewage	2	2	0.5–1.0 (2)
Positive control (<i>V. cholerae</i> 569B)		1	1	0.8–1.5 (1)
Negative control (isotonic saline)		0 (none)

* Range of values (ml per cm of gut) in three tests.

Histopathology of ileal loops. Ileal loop tests were done with live cells and culture filtrates of two strains of *A. hydrophila* (nos. 2884 and 10 005) and *V. cholerae* 569B. Brain-Heart Infusion Broth provided the negative control. The rabbits were killed after 1 h, 2 h and 6 h and the reactions noted; each loop was immediately removed, cut through its entire length and placed in a Petri dish completely immersed in 10% formol-saline. A piece of normal gut measuring a few centimetres was removed and treated similarly. The tests were done in triplicate. Sections embedded in paraffin blocks were stained with haematoxylin-eosin, mounted in Canada balsam and examined under the microscope for histological changes.

RESULTS

Live cultures

Forty-seven of the 50 strains of *A. hydrophila* caused fluid accumulation in rabbit ileal loops (table I). The range of fluid accumulation per cm of gut varied from strain to strain. Most strains caused fluid accumulations comparable to those produced by live cultures of *V. cholerae* 569B. The three strains that gave no fluid accumulation (one from a case of diarrhoeal disease and two from healthy individuals) became positive after two serial passages in rabbit ileal loops and caused fluid accumulation comparable to that produced by *V. cholerae* 569B.

Four strains of *A. hydrophila* isolated from different sources caused no fluid accumulation when 10^2 bacterial cells were used as the inoculum; a small accumulation of fluid was produced with inocula of about 10^3 , and maximal accumulation (1 ml or more of fluid per cm of gut) with about 10^4 cells (table II). There was no increase in fluid accumulation when the inoculum was

TABLE II

Relationship between multiplication and production of fluid by strains of Aeromonas hydrophila in ileal loops

Strain number and source	Viable count of inoculum	Viable count per ml, 6 h after inoculation	Fluid accumulation (range in ml per cm of gut)
20 571 (diarrhoeal disease; man)	2.4×10^1	9.9×10^5	0
	1.3×10^2	1.5×10^7	0
	2.2×10^3	6.4×10^6	0.4-0.5
	2.3×10^4	5.3×10^9	1.0-1.2
7888 (healthy human being)	2.4×10^5	4.3×10^9	1.0-1.1
	1.3×10^2	1.5×10^7	0
	1.4×10^3	4.8×10^6	0.45-0.5
	3.2×10^4	6.3×10^9	0.9-1.0
12 192 (cow)	3.8×10^5	8.4×10^9	1.0-1.1
	1.4×10^2	3.3×10^6	0
	2.4×10^3	1.1×10^7	0.3-0.45
	1.8×10^4	7.8×10^8	0.9-1.0
150 (water; hand pump)	3.4×10^5	1.8×10^9	0.8-1.0
	2.0×10^2	5.6×10^6	0
	3.4×10^3	9.9×10^5	0.6-0.8
	2.3×10^4	5.1×10^9	1.2-1.5
	5.2×10^5	6.7×10^9	1.2

TABLE III

The effect of temperature on the fluid accumulation produced by culture filtrates in ileal loops

Strain number	Heat treatment of culture filtrate		Range of fluid accumulated (ml per cm of gut)
	Temperature	Time	
2884	65	10	0 (0.8-1.0)*
	60	20	0 (0.8-1.0)
	60	10	0.8-1.1 (0.7-0.9)
	56	20	0.8-1.0 (0.9-1.1)
12 192-C ₂	65	10	0 (0.8-1.0)
	60	20	0 (0.8-1.0)
	60	10	0.9-1.2 (1.0-1.1)
	56	20	1.0-1.1 (0.8-1.0)

* Ranges for unheated filtrate given in parenthesis.

increased to 10^5 cells. The bacteria multiplied by about 10^3 - 10^5 in all the loops irrespective of the fluid accumulation produced.

Culture filtrates

The culture filtrates prepared from all of the 50 strains of *A. hydrophila* (three of them after serial passage in rabbit gut) gave positive reactions in ileal loops. Fluid accumulation varied from strain to strain; the amounts of fluid were similar to those obtained in tests with live cultures and there were no significant differences between strains from different sources. The majority caused fluid accumulation comparable to that of *V. cholerae* strain 569B.

Culture filtrates prepared from a strain isolated from diarrhoeal disease and one from a cow were tested in doses ranging from 0.1-2.0 ml. An inoculum of 0.25 ml produced fluid accumulation similar in range to that given by 2 ml of culture filtrate and 1 ml of culture supernate of *V. cholerae* 569B. Culture filtrates prepared from these two strains caused fluid accumulation after heating at 60°C for 10 min. or 56°C for 20 min. (table III); there was no fluid accumulation after the filtrates had been heated for 10 min. at 65°C or 20 min. at 60°C.

Crude toxin

The crude toxin prepared from a strain of *A. hydrophila* isolated from a case of diarrhoea caused fluid accumulation of 1.0-1.7 ml per cm of gut. An inoculum of 40 µg gave a maximal reaction. A similar preparation from *V. cholerae* 569B was the positive control and 120µg caused comparable fluid accumulation.

Histopathological changes

No significant difference was observed between the ileal loops exposed to live cells and culture filtrates of *V. cholerae* and *A. hydrophila* for up to 6 h

and the normal loops. The intestinal mucosa remained intact and there was no congestion in the blood vessels. Mild mononuclear leucocytic infiltration was noted in the loops exposed to *V. cholerae* and to a lesser extent in those exposed to *A. hydrophila* and in control loops. There was a reduction in the number of goblet cells in the intestinal mucosa of loops treated with both *V. cholerae* and *A. hydrophila* after exposure for 1 h.

DISCUSSION

The results of the present study of 50 strains of *A. hydrophila* isolated from man and his environment clearly indicate that the majority of strains are enterotoxic irrespective of the source of isolation. Most of the strains caused accumulation of fluid comparable to that of a toxigenic *V. cholerae* strain. The differences in fluid accumulation between strains may have been due to variation in the release of toxin. Three strains gave negative reactions in repeated tests and later caused accumulation of fluid after only two passages in ileal loops; this would seem to indicate potential enterotoxicity. The initial cultures were probably mixed populations containing a small proportion of toxigenic members and these probably increased in proportion during passage in the loops. However, this observation suggests that in a community where the organisms are in continuous circulation, the ratio of toxigenic to non-toxigenic organisms may be reduced under unfavourable conditions, and enhanced by passage through susceptible hosts.

It was observed that small amounts of fluid accumulated in the ileal loops with inocula of about 10^3 bacterial cells and inocula of 10^4 caused maximal reactions. The bacteria multiplied by about 10^3 – 10^5 in all the loops but accumulation of fluid took place only when the inoculum was 10^3 cells or more. With smaller inocula, no accumulation of fluid was noted even though the bacteria multiplied by about 10^5 . Fluid outpouring in ileal loops is considered to be due to liberation of an enterotoxic substance or substances by the organisms during multiplication in the intestine; this has been shown with *V. cholerae* (De and Chatterje, 1953); enteropathogenic *E. coli* (De, Bhattacharya and Sarkar, 1956), *Cl. perfringens* (Duncan and Strong, 1969) and *V. parahaemolyticus* (Sakazaki, Tamura, Nakamura, Kurata, Gohda and Kazuno, personal communication, 1974). The present study indicates that sufficient amounts of enterotoxic substances need to accumulate in the loop to induce fluid outpouring. The negative results with smaller inocula may have been due to the elaboration of less toxic substances during the 6 h of the experiment. The gradual increase in the range of fluid accumulation corresponded to the increase in size of inocula and indicated that a minimum of bacterial cells was necessary to cause maximal fluid accumulation. This was substantiated by the observation that further increase of the inoculum did not increase the range of fluid accumulation in the ileal loop.

We reported previously that cell-free culture supernates of *A. hydrophila* strains could cause accumulation of fluid in ligated ileal loops (Annapurna and Sanyal, 1975) and this has been confirmed by Wadström, Ljungh and Wretling

(1976). In the present study cell-free culture filtrates of all of 50 strains of *A. hydrophila* gave a positive reaction, similar to that of a toxigenic *V. cholerae* strain, indicating that an enterotoxic substance was produced during multiplication in the medium. Heating at 60°C for 20 min. and 65°C for 10 min. inactivated the biological activity of this substance. It could be precipitated with ammonium sulphate and was non-dialysable. This indicates that, like the cholera enterotoxin and *E. coli* heat-labile enterotoxin, the *A. hydrophila* enterotoxin is protein in nature.

Histological examination of ileal loops exposed to live bacterial cells and culture filtrates of *A. hydrophila* no. 884 for up to 6 h did not reveal significant changes other than a considerable reduction in the number of goblet cells. This change was noted even in specimens taken after exposure for 1 h and indicates a depletion of mucus from these cells as described in loops treated with *V. cholerae* (Norris and Majno, 1968; Yardley *et al.*, 1972). The mild mononuclear leucocytic infiltration observed in all the specimens treated with *A. hydrophila* and *V. cholerae* as well as in normal specimens might have been due to surgical manipulation (Moon, Whipp and Baetz, 1971).

While carrying out these experiments it was noted that culture filtrates of *A. hydrophila* nos. 10 005 and 5515, isolated from a buffalo and a non-diarrhoeal child respectively caused blanching of the ileal loop with loss of lustre immediately after inoculation. The phenomenon was observed repeatedly with these two strains but with no other. Accumulation of fluid was not affected. Histological examination of these loops after exposure for 6 h showed oedema at the tips and base of the villi and in the submucosal tissue; there was no oedema or congestion in loops treated for 1 and 2 h. Similar changes were not noted in tests with the other 48 strains. These observations suggest that a toxic factor was produced by these two strains which was different from the enterotoxin that caused fluid accumulation and further studies are needed to confirm this.

SUMMARY

Live cells and cell-free culture supernates of 50 strains of *Aeromonas hydrophila* isolated from diarrhoeic and healthy human faeces, drinking water, sewage, the river Ganges and faeces from domestic animals caused accumulation of fluid in ligated ileal loops of adult rabbits. The amount of fluid produced was comparable to that of a toxigenic strain of *Vibrio cholerae*. Three of the strains gave positive reactions only after two passages in ileal loops of rabbits. Inocula of about 10^3 viable cells and 0.25 ml of culture supernate caused fluid accumulation in the loops. The enterotoxic factor was inactivated at 60°C for 20 min. and 65°C for 10 min., was precipitated with ammonium sulphate and was non-dialysable; these results indicate the protein nature of the enterotoxin. An inoculum of 40 µg of crude toxin caused as much fluid accumulation as larger inocula. The only histopathological change in the loops was depletion of mucus from the goblet cells.

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