Enterotoxicity of clinical and environmental isolates of *Aeromonas* spp.

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Summary. Of 147 isolates of three species of *Aeromonas*, 54 were from clinical and 93 from environmental sources. When tested for enterotoxin production, most of the isolates (56%) caused accumulation of fluid in rabbit ileal loops (RILs). Although large proportions of clinical and environmental isolates of *A. caviae* (55% and 65%, respectively) elicited such a response in RILs, isolates of *A. hydrophila* and *A. sobria* produced significantly more fluid ($p < 0.05$). Furthermore, the environmental strains of *A. hydrophila* and *A. sobria* produced more fluid than the clinical isolates ($p < 0.05$). The strains of *Aeromonas* spp. that caused little or no fluid accumulation in the initial experiments became enterotoxin producers after 1–3 passages through RILs, regardless of their source, and showed gradual enhancement of fluid outpouring after each passage. The present study suggests that all strains of these species of *Aeromonas* are potentially enterotoxigenic, whether from clinical or environmental sources.

Introduction

Production of a heat-labile enterotoxin by clinical and environmental isolates of *Aeromonas* was first demonstrated in an adult rabbit ileal loop (RIL) model.^{1–3} Subsequently enterotoxin production by strains of *Aeromonas* was demonstrated in various other animal and tissue-culture assays.^{4–11} However, it was reported that, whereas clinical isolates of *A. hydrophila* and *A. sobria* produced enterotoxin,^{12–14} few or none of the environmental isolates did so.^{15,16} Other studies suggested that a smaller proportion of environmental strains of *A. hydrophila* and *A. sobria*, compared with those of diarrhoeal origin, are enterotoxigenic.^{17,18} Furthermore, strains of *A. caviae* from either clinical or environmental sources were reported to be rarely enterotoxin producers.^{9,10,19–21} Because these observations caused confusion about the enterotoxigenicity of *Aeromonas* spp., the present study was undertaken, with a large number of diarrhoeal and environmental isolates, to examine the enterotoxic activity of different species, and to correlate this with the sources of the isolates.

Materials and methods

Strains of *Aeromonas* spp.

We examined 147 strains (table), 54 isolated from cases of acute diarrhoea and 93 from environmental sources (water and fish). By the criteria of Popoff,²²

the strains were identified and classified into three species (*A. hydrophila*, *A. sobria*, *A. caviae*), according to their ability to hydrolyse aesculin, to ferment salicin, and to produce gas, acetoin and H₂S. The organisms were maintained in peptone agar stab cultures at room temperature and did not undergo more than three subcultures before the experiments for enterotoxin production.

Preparation of culture filtrates

Culture filtrates of the strains were prepared by the method of Annapurna and Sanyal.³ Briefly, 10 ml of Brain Heart Infusion Broth (BHIB; Difco) in a 50-ml conical flask was inoculated with 5–6 smooth separate colonies from overnight growth on nutrient agar (NA). The flasks were incubated at 37°C for 16–18 h in a shaking water bath with 80–120 oscillations/min. The cultures were centrifuged at 22 000 *g* for 20 min at 4°C, and the supernates were filtered through 0.22- μ m membrane filters (Millipore) and stored at 4°C. Culture filtrate was prepared from a strain only when live cells caused fluid accumulation in RILs either before or after passage (see below).

Enterotoxicity test

Live bacteria and culture filtrates were tested for enterotoxicity in the adult RIL model by the method of De and Chatterje²³ as adopted by Annapurna and Sanyal.² Bacteria grown in BHIB for 3 h were diluted 10-fold in the same medium and inoculated into RILs in doses of 1 ml containing 10⁵–10⁶ cfu. A BHIB-culture of the toxigenic strain 569B of *Vibrio cholerae*

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was used as a positive control and unseeded BHIB as a negative control. Culture filtrates (1 ml) were tested in the same way. Each test was done in three rabbits.

Passage in RILs

Successive passages of strains that caused little or no accumulation of fluid in the initial test were made in RILs as described by Sanyal *et al.*^{24,25} Briefly, each strain was cultured aseptically from an RIL on NA and incubated overnight; five or six colonies were inoculated into BHIB and incubated for 3 h, and 1 ml of diluted culture was inoculated again into an RIL. The process continued until a good response was obtained.

Results

In the initial tests on the three species of *Aeromonas* in RILs, live cells of the majority (56%) of the clinical and environmental isolates caused accumulation of fluid (table). Culture filtrates of these strains caused similar secretory response. With organisms from both sources, there were strain variations in the volume of fluid accumulation, and also variations between individual loops inoculated with the same strain (table).

Accumulation of fluid was caused by the majority of the strains of *A. caviae*—55% of clinical and 65% of environmental isolates; live cells and culture filtrates caused similar fluid accumulation. However, live cells of *A. hydrophila* and *A. sobria* of environmental origin caused significantly more fluid accumulation ($p < 0.05$) than those of *A. caviae* from either source (fig. 1); similar results were obtained with culture filtrates.

Furthermore, live cells and culture filtrates of *A. hydrophila* and *A. sobria* from environmental sources caused significantly more fluid accumulation ($p < 0.05$) than the clinical isolates (fig. 1); but with *A. caviae* no significant difference ($p > 0.05$) was found between strains of clinical and environmental origin.

The non-toxicogenic strains of *Aeromonas* (44% of the isolates) belonging to all three species became enterotoxin producers after 1–2 passages through RILs, regardless of their origin (table), though three environmental isolates (one of each species) required three passages before they caused marked fluid accumulation. The initially non-toxicogenic strains showed gradual enhancement of fluid outpouring after each passage: the findings with six representative strains (two of each species) are shown in fig. 2.

Discussion

The data of the present study indicate that not only the diarrhoeal isolates of *A. hydrophila* and *A. sobria* but almost equal proportions of environmental strains produce enterotoxin as judged by fluid accumulation in RILs, the classical model for detection of enterotoxic activity of bacteria. Moreover, most strains of *A. caviae* caused accumulation of fluid, whether they were of clinical or environmental origin (table). These data indicate that *A. caviae* produces enterotoxin and may cause diarrhoea in children, as suggested by its frequent isolation from the faeces of such cases in our study. Altwegg and Geiss¹⁸ and Namdari and Bottone²⁶ also observed a very high association of this species with diarrhoea in children.

However, our observation that *A. hydrophila* and *A. sobria* caused significantly more accumulation of fluid

Table. Accumulation of fluid in RILs inoculated with BHIB cultures of three species of *Aeromonas* from clinical or environmental sources

Species and source	Number of strains tested	Strains tested before passage in RILs		Strains tested after passage in RILs	
		Number (%) causing accumulation of fluid	Range of volumes of fluid (ml/cm of RIL)*	Number (%) causing accumulation of fluid	Range of volumes of fluid (ml/cm of RIL)*
<i>A. hydrophila</i>					
Clinical	5	3 (60)	0.5–0.7	2 (40)	0.7–1.2
Environmental	9	5 (56)	0.6–1.7	4 (44)	0.8–1.6
<i>A. sobria</i>					
Clinical	27	15 (56)	0.4–1.0	12 (44)	0.5–1.0
Environmental	33	14 (42)	0.5–1.3	19 (58)	0.6–1.5
<i>A. caviae</i>					
Clinical	22	12 (55)	0.4–0.9	10 (45)	0.5–1.2
Environmental	51	33 (65)	0.5–1.0	18 (35)	0.5–1.2
Positive control†			0.8–1.5		
Negative control‡			0.0–0.0		

*The stated range of volumes was observed in three RILs inoculated with each strain.

†BHIB-culture of strain 569B of *V. cholerae*.

‡BHIB.

Fig. 1. Enterotoxigenicity of clinical $\times$ and environmental $\parallel$ isolates of three species of *Aeromonas* in initial experiments before RIL passage. The bars on the columns indicate the range of fluid accumulation caused by different strains.

Fig. 2. Influence of RIL passage on volume of fluid accumulation caused by two representative strains of each species which were non-toxigenic when tested initially: *A. hydrophila* ○—○, *A. sobria* ●—●, *A. caviae* △—△.

than *A. caviae* suggests higher enterotoxigenic activity, which may explain the less frequent detection of enterotoxigenic activity in strains of *A. caviae*; but, in our study, a similar proportion of the strains of all three species produced enterotoxin (table), suggesting no species-specific difference in enterotoxigenic potential.

The environmental strains of *A. hydrophila* and *A.*

sobria caused significantly more accumulation of fluid than the clinical isolates (fig. 1). A probable explanation of this difference in the degree of enterotoxigenicity may be the observation that, compared with clinical isolates, strains of environmental origin produced quantitatively more constitutive chitinase, and some strains even elaborated inducible chitinase; both these properties correlated with higher enterotoxigenicity (unpublished data).

The large number of clinical and environmental strains that caused little or no accumulation of fluid in the initial experiments showed enhancement of their enterotoxigenic activity after only 1–2 passages through the susceptible rabbit ileum. Similar observations on the effect of passage through the gut of a susceptible host were made earlier,³ and also with many other organisms such as *V. cholerae* non-O1,²⁷ *Plesiomonas shigelloides*,²⁸ *V. fluvialis*,²⁹ *V. mimicus*,^{24,30} CT-gene-negative *V. cholerae* O1²⁵ and *Klebsiella pneumoniae*.³¹ Further, it is known that the so-called hypertoxigenic strain 569B of *V. cholerae* O1 also requires passage through rabbit gut for enhancement of toxin production; a mechanism of repression of toxin gene which becomes expressed on passage in a susceptible host may account for this phenomenon.³² There is every likelihood that toxigenicity of our fresh isolates may have decreased during 1–3 subcultures *in vitro* before the loop tests. It appears that repression-derepression of a virulence factor in a bacterial strain is a means of its adaptation to a particular micro-environment.

Strains of the three species of *Aeromonas* produced quantitatively more toxin after each passage (fig. 2). This may suggest that *Aeromonas*, being ubiquitous in our aquatic environment, may have easy access to the human intestine in a community with suboptimal hygienic conditions, and therein develop increased enterotoxigenicity. The present study demonstrates that strains of all three species are potentially enterotoxigenic regardless of their source.

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