Toxin production by *Aeromonas* spp. from different sources

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Summary. One hundred and eleven isolates of *Aeromonas* from water and from human sources were identified to species level and tested for the production of cytotoxin. These results were correlated with the source of each isolate and, for those from human faeces, with the clinical history of diarrhoea. *A. caviae* predominated in water, comprising 16 of 32 isolates; only one isolate from water was *A. sobria*. In human faecal samples 21 of 76 isolates were *A. sobria*; this was a significant difference. Cytotoxin producing strains were significantly more common in patients with no known cause for their gut symptoms. It is concluded that gastro-enteritis caused by *Aeromonas* is related to species and to production of cytotoxin.

Introduction

Aeromonas spp. cause various diseases in man, including wound infections and septicaemia in immunocompromised patients (Abrams *et al.*, 1971; McCracken and Barkley, 1972; Washington, 1972). Most isolates have been from faeces and some studies have suggested that they are a cause of gastro-enteritis (Chatterjee and Neogy, 1972; Cumberbatch *et al.*, 1979; Gracey *et al.*, 1982; Pitarangsi *et al.*, 1982; Janda *et al.*, 1983) but others have refuted this possibility (Catsaras and Buttiaux, 1965; Bhat *et al.*, 1974; Millership *et al.*, 1983). Some strains may be more virulent than others and in this respect division of the genus into species would be a step towards identifying virulent isolates.

Recent studies (Janda *et al.*, 1984; Turnbull *et al.*, 1984) based on the work of Popoff and Véron (1976) have divided *Aeromonas* into three species—*A. sobria*, *A. hydrophila* and *A. caviae*. A DNA homology study confirmed this division although each species may contain three subspecies indistinguishable phenotypically (Popoff *et al.*, 1981). There is already some evidence that *A. sobria* is more likely to be a pathogen in man (Daily *et al.*, 1981; Watson *et al.*, 1985) and a relationship between species and enterotoxin production

detected in animal models has been demonstrated (Turnbull *et al.*, 1984).

Reports on the exotoxins of *Aeromonas* are very confused, particularly in relation to pathogenicity in the gut. Some workers have claimed the existence of a relatively heat stable cytotoxic enterotoxin (Ljungh *et al.*, 1977) and the gene coding for this product has been cloned (Chakraborty *et al.*, 1984). Others have been unable to detect any specific heat stable activity in cell culture (Cumberbatch *et al.*, 1979) although non-specific cell rounding effects were found.

There is no doubt about the existence of a heat-labile (56°C) cytotoxin. Most reports of animal studies have noted the heat lability of enterotoxin (Burke *et al.*, 1981; Johnson and Lior, 1981; Turnbull *et al.*, 1984) and it is quite likely that these assays have detected the cytotoxin. Further support for this view is provided by the work of Asao *et al.* (1984) who purified a haemolysin with a mol. wt corresponding to the β haemolysin of Ljungh *et al.* (1981). This substance had cytotoxic activity in Vero cells and enterotoxic effects in the infant mouse and rabbit ileal-loop tests. A second haemolysin, α , has also been described (Ljungh *et al.*, 1981) but it is only produced in the stationary phase of growth and little work has been done on it.

The aims of the following study were to correlate species with source for a large number of isolates of *Aeromonas*, and to examine cytotoxin production and relate species and toxin production to clinical history of human isolates.

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Materials and methods

Bacterial strains

One hundred and eleven isolates were examined. All but one were from north-east London or from the City of London. Twenty four were environmental isolates from taps, swimming pools and other chlorinated supplies; eight were from untreated pools and streams; and 79 were human isolates, all but three from faeces.

Initial isolation with identification was done by methods previously described (Millership *et al.*, 1983; Millership and Chattopadhyay, 1985). Faecal specimens were enriched in alkaline peptone water and subcultured to xylose-deoxycholate-citrate agar or to bile salts-brilliant green agar. All specimens were also examined for *Salmonella*, *Shigella*, *Campylobacter* and, where indicated, for enteropathogenic *Escherichia coli*, *Staphylococcus aureus*, *Clostridium perfringens*, *C. difficile* and ova, cysts and parasites by standard methods. No virus studies were done. Water specimens were added to alkaline peptone water, or were filtered and the membrane filter incubated on bile salts-brilliant green agar. Identification to genus level was completed with API 20E trays and vibriostatic agent O129.

Species were defined by the results of up to 13 tests based on the scheme of Popoff and Veron (1976), (Barer *et al.*, 1986).

Toxin studies

Culture filtrates were prepared as described by Barer *et al.* (1986). Overnight shake cultures in tryptone-soya broth with yeast extract were centrifuged and filtered through a 0.45- μ m membrane filter (Millipore). Cytotoxic activity was studied in Vero cells.

Clinical histories

As much clinical information as possible was gathered whenever a faecal specimen yielded *Aeromonas*. For hospital patients, details were obtained from the notes

and by visiting the patient. When sample examination was requested by general practitioners or by the public health inspectorate, details were taken from the specimen request form and, where possible, further details were obtained from the patient's general practitioner.

Patients were then divided into four categories. Those who had had an episode of diarrhoea or vomiting (or both) within the previous 28 days (or if this information was not available, those whose sample was the first one submitted for investigation of these complaints) without any other cause for gastro-intestinal disease were placed in class A. Patients who had another cause, either infective or non-infective, were placed in class B. Those with no history of gastro-intestinal upset were in class C and when no history was available patients were placed in class D.

Statistical analyses were by χ^2 or Fisher's exact tests.

Results

Table I shows the numbers of each species isolated from human and environmental sources. *A. sobria* was rare in water samples but 28% of the isolates from faeces belonged to this species. The predominant species in water samples was *A. caviae*. However, there was little difference between the proportions of cytotoxin producers amongst strains isolated from the environment (28%) and from human sources (29%) ($p > 0.8$).

When human faecal isolates were grouped according to clinical history (table II), there was an excess of *A. sobria* (36%) from those patients with no other known cause for their diarrhoea compared with those with no symptoms (15%), but the difference was not significant ($0.2 > p > 0.1$). There were, however, significant differences between the proportions of patients in groups A, B and C with cytotoxin-producing strains in their stools ($\chi^2 = 6.3$, $df = 2$, $p < 0.05$). The largest difference was between group A (45% toxigenic) and group C (15% toxige-

Table I. *Aeromonas* species isolated from human and environmental sources

Species	Number of strains isolated from			
	human faeces	other human sources	chlorinated water	untreated water
<i>A. hydrophila</i>	9 (3)*	1 (1)	9 (7)	3 (1)†
<i>A. caviae</i>	42 (0)	1 (0)	11 (0)	5 (0)
<i>A. sobria</i>	21 (18)	1 (1)	1 (1)	0 (0)
<i>Aeromonas</i> group 4	4 (0)	0 (0)	3 (0)	0 (0)
Total	76 (21)	3 (2)	24 (8)	8 (1)

* Numbers in parentheses are numbers of toxigenic strains.

† One strain was not tested for toxin production.

Table II. Relationship of species and toxin production to clinical history in *Aeromonas* isolates from human faeces

Species	Number of strains isolated from patient group			
	A	B	C	D
<i>A. hydrophila</i>	3 (2)*	4 (1)	2 (1)	0 (0)
<i>A. caviae</i>	10 (0)	15 (0)	13 (0)	1 (0)
<i>A. sobria</i>	8 (8)	8 (5)	3 (2)	2 (2)
<i>Aeromonas</i> group 4	1 (0)	2 (0)	2 (0)	1 (0)
Total	22 (10)	31 (6)	20 (3)	3 (2)

A, B, C and D—see *Materials and methods*.

* Numbers in parentheses are numbers of toxin producing strain.

nic). All but four toxin-producing strains produced cytotoxin in titres or at least 400 and up to 10 000. Two strains with titres of < 100 were from patients in group C. Of the faecal isolates, 41% came from patients in group B who had other bowel pathology; 19% of these strains produced cytotoxin.

Nineteen isolates were found on direct plating and the results obtained with these strains are analysed in table III. Although the numbers are small it is notable that no cytotoxin-producing strains occurred in patients without gastro-intestinal symptoms in numbers detectable by direct plating.

Discussion

Aeromonads are not uncommon even in treated tap water (Burke *et al.*, 1984; Millership and Chattopadhyay, 1985) and it seems likely that faecal isolates are acquired from this source. The distribution of species amongst faecal isolates is, therefore, surprising given the excess of *A. caviae* in environmental samples. It suggests that *A. sobria* has a greater potential for colonising the gastro-intestinal tract. There is some experimental evidence to support this view; Watson *et al.* (1985) found that of a group of *Aeromonas* isolates the majority of strains invasive *in vitro* were *A. sobria*. Clearly colonisation must precede invasion.

A few cytotoxic strains of *Aeromonas* were found in healthy people but most were from persons with gastro-intestinal symptoms, particularly those who were previously healthy. The cytotoxin has been noted to have enterotoxin activity (Asao *et al.*, 1984) and it would appear that it is at least a marker of pathogenicity, whatever the role of any cytotoxic enterotoxin. The finding of toxigenic strains in

healthy people is not unexpected. Other enteropathogens, such as *E. coli*, require several virulence factors to produce disease (Lancet, 1983). It is also notable that two of the three strains in this category were poor toxin producers *in vitro*.

If the cytotoxin is a virulence factor it is necessary to explain the number of non-toxin producers from patients with no other explanation for their diarrhoea. There are several possible factors involved. Toxin production *in vitro* varies with the conditions of culture and of assay (Burke *et al.*, 1981; Ljungh *et al.*, 1981). A high proportion of patients with other bowel disease, in particular those due to other bacterial pathogens, has been noted amongst *Aeromonas*-positive patients (Shread *et al.*, 1981) and there is some evidence that *Aeromonas* occurs most often in water sources contaminated with other pathogens (Millership and Chattopadhyay, 1985). Bowel mucosa already damaged may be predisposed to colonisation by aeromonads that are not necessarily pathogenic.

The discrepancies in previous studies of the pathogenicity of *Aeromonas* may be explained by the methods of isolation used. Direct plating only detects *Aeromonas* when large numbers are present in a faecal specimen. Most enteric pathogens become the predominant bowel flora when they cause acute disease and it might be expected that only strains of *Aeromonas* causing diarrhoea will be found without use of an enrichment medium. None of the studies in which "enterotoxin" producing *Aeromonas* have been isolated almost exclusively from diarrhoeal stools have used an enrichment technique (Gracey *et al.*, 1982; Pitarangsi *et al.*, 1982; Janda *et al.*, 1983; Agger *et al.*, 1985). Although the numbers are small the re-analysis of our results in table III conforms to this pattern.

Table III. *Aeromonas* isolates from human faeces obtained by direct plating alone

Species	Number of strains isolated from patient group		
	A	B	C
<i>A. hydrophila</i>	0 (0)*	1 (0)	0 (0)
<i>A. caviae</i>	3 (0)	3 (0)	4 (0)
<i>A. sobria</i>	4 (4)	4 (2)	0 (0)
<i>Aeromonas</i> group 4	0 (0)	0 (0)	0 (0)
Total	7 (4)	8 (2)	4 (0)

A, B and C—see *Materials and methods*.

* Numbers in parentheses are numbers of toxin-producing strains.

Thus it appears that only some isolates of *Aeromonas* belonging to the species *sobria* and *hydrophila* are associated with diarrhoea. Epidemiological studies are unlikely to give a clear-cut answer to the question of whether *Aeromonas* is an

enteric pathogen or not in the absence of a detailed analysis of the virulence factors involved.

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