Exotoxin profiles of clinical isolates of *Aeromonas hydrophila*

JAMUNA VADIVELU, SAVITHIRI D. PUTHUCHEARY and PARASAKTHI NAVARATNAM

Department of Medical Microbiology, University Hospital, Faculty of Medicine, University of Malaya, 59100 Kuala Lumpur, Malaysia

Summary. Eighty-six clinical isolates of *Aeromonas hydrophila* were studied for their ability to produce four exotoxins: a haemolysin active against rabbit erythrocytes, cytotoxin and enterotoxin detectable with Vero cell cultures, and the cholera toxin-like factor detected by an enzyme-linked immunosorbent assay. At least one exotoxin was produced by 80% of enteric and 96% of non-enteric isolates. The exotoxin profiles of non-enteric isolates were more restricted than those of enteric isolates, with haemolysin and cytotoxin producers preponderant. Although haemolysin and cytotoxin were produced by isolates from all sources, the enterotoxin and cholera toxin-like factor were more common amongst enteric isolates. The production of haemolysin and cytotoxin were closely related but the association between the enterotoxin and the cholera toxin-like factor was not significant.

Introduction

During the last two decades it has been recognised that *Aeromonas* spp. are significant pathogens in man. *A. hydrophila*, in particular, causes wound infections and septicæmia in immunocompromised patients.^{1,2} Recent studies have implicated *Aeromonas* spp. as an important enteric pathogen in man, both in children^{3,4} and adults.^{5,6} This wide range of effects suggests that complex pathogenic mechanisms may be involved, in which strains of *A. hydrophila* possess several virulence factors in different combinations.

Strains of *A. hydrophila* and *A. sobria* produce potential virulence factors such as cytotoxin,⁷ haemolysin (aerolysin),^{8,9} cytotoxic enterotoxin,^{5,10} and a cholera toxin-like factor (CTF)¹¹ which have been recognised as virulence factors in other enteropathogenic bacteria. Although these properties resemble those of enteropathogenic *Escherichia coli* and *Vibrio cholerae*, the role of these exotoxins in the pathogenesis of aeromonas infections remains unclear.

In this study the exotoxin profiles of *A. hydrophila* isolates from various clinical specimens were examined to elucidate the possible role of these toxins in the pathogenesis of aeromonas infections in man.

Materials and methods

Bacterial strains

Eighty-six clinical isolates of *A. hydrophila* from patients at the University Hospital, University of Malaya, Kuala Lumpur, Malaysia were examined.

They were from a variety of specimens including faeces from patients with diarrhoea, urine, pus, peritoneal dialysis fluid, blood, bile, throat swabs and tracheal secretions. The bacteria were isolated on ox-blood agar (Oxoid), deoxycholate citrate agar (BBL) and biotyped with the API 20E system.¹⁰ All strains were stored at 4°C on Tryptone Soy Agar (Oxoid) slopes during the course of the study. *E. coli* WF5 (University of Surrey) of environmental origin was used as a heat-labile enterotoxin (LT)-negative control.

Preparation of culture filtrates

Cell-free culture supernates were prepared by the method of Cumberbatch *et al.*¹² Bacterial strains were inoculated into Brain Heart Infusion Broth (BHI; Oxoid) and the cultures were incubated at 37°C with agitation at 100 rpm for 18–24 h. The broth cultures were then centrifuged at 12 000 *g* for 20 min, and the supernates were sterilised by filtration through 0.45-µm filter acrodiscs (Gelman). The cell-free culture supernates were stored at 4°C and tested in assays within 2 weeks of preparation.

Haemolysin assay

Haemolytic activity was assayed as described by James *et al.*¹³ Cell-free culture supernate (100 µl) was added to each well of a 96-well microtitration plate and incubated with an equal volume of rabbit erythrocyte 1% suspension in phosphate-buffered saline, pH 7.0, for 1 h at 37°C. Standards containing 0–100% lysed rabbit erythrocytes were prepared and all samples showing haemolytic activity ≥ 50% haemolysed erythrocytes were described as positive for the presence of haemolysin.

Cytotoxin and enterotoxin assays

Vero cells at a concentration of 10^4 cells/well in microtitration plates were used for the detection of both the cytotoxin and enterotoxin.¹⁴⁻¹⁶

For the detection of cytotoxin activity, the cells were exposed for 18 h at 37°C to 50 µl of culture supernate. A positive response was recorded when $\geq 50\%$ of the cells were lysed. Enterotoxin activity was assayed by exposing the cells for 18 h at 37°C to 50 µl of culture supernate that had been heated at 56°C for 10 min to denature any cytotoxin present.¹⁷ A positive response was recorded when $\geq 50\%$ of cells showed morphological changes attributable to the cytopathic effect of the enterotoxin. Cholera toxin (CT) (Sigma) at a concentration of 10 ng/ml was used as a positive control for enterotoxin activity and culture supernate of *E. coli* WF5 was used as the negative control.

Cholera toxin-like factor assay

The enzyme-linked immunosorbent assay (ELISA) method of Sack *et al.*,¹⁸ modified by Miller *et al.*¹⁹ for the detection of CT, was used. Culture supernate (100 µl) that had been heated at 56°C for 10 min was added to GM1 (monosialoganglioside) adsorbed to 96-well Linbro microtitration plates (Flow Laboratories). After overnight incubation at room temperature, 100 µl of goat anti-CT (University of Surrey) at a titre of 250, and 100 µl of peroxidase-labelled mouse anti-goat immunoglobulins (Dako-Patt, Dako Ltd) were added sequentially to each well and incubated at 37°C for 1 h at each stage. *o*-Phenylene diamine dihydrochloride (Abbott Laboratories, USA) (100 µl) was added as substrate and incubated at 37°C for 20 min after which the reaction was stopped by adding 20 µl 8N H₂SO₄. The plates were read spectrophotometrically at 492 nm with a Dynatech MR600 Microelisa reader. A negative-positive cut-off value for each plate was calculated from the average values of negative control wells + 2 SD.¹⁶ CT (Sigma) at a concentration of 100 ng/ml was used as a positive control. Culture supernate of *E. coli* WF5 was used as a negative control.

Results

Table I shows the exotoxins produced by *A. hydrophila* strains isolated from different types of clinical specimen. Haemolysin production was demonstrated in 58–91% of strains and cytotoxin in 63–96% of strains. In both cases the greatest proportion of toxin producers was amongst wound isolates (21 out of 23 for haemolysin, 22 of 23 for cytotoxin). Production of CTF was found in 16–33% of strains, the highest proportion of producers was amongst stool isolates (12 out of 38). Enterotoxin production was detected in fewer isolates, 10–22% only.

A comparison of the production of exotoxins by

Table I. Exotoxin production by *A. hydrophila* isolates

Source	Total number tested	Number (%) of isolates that produced			
		haemolysin	cytotoxin	CTF	enterotoxin
Stool	38	22 (58)	24 (63)	12 (32)	5 (13)
Wound	23	21 (91)	22 (96)	5 (22)	4 (17)
Peritoneal fluid	10	8 (80)	8 (80)	3 (30)	1 (10)
Blood	6	5 (83)	4 (66)	1 (16)	0 (0)
Others	9	7 (78)	7 (78)	3 (33)	2 (22)
Total	86	63	65	24	12

isolates from enteric and non-enteric sources is shown in table II. Production of haemolysin and cytotoxin was commoner amongst the non-enteric isolates (85% each). Of the enteric isolates, only 58% produced haemolysin and 63% cytotoxin. However, production of CTF was more pronounced amongst enteric isolates (32%) than non-enteric isolates (25%). Enterotoxin production was not prominent amongst these isolates although there was an even distribution between enteric (13%) and non-enteric (14%) isolates. None of the isolates from blood produced enterotoxin.

The exotoxin profiles of *A. hydrophila* isolates from clinical specimens are illustrated in table III. A wide range of exotoxin profiles was seen amongst enteric isolates, although eight isolates gave negative results in tests for all exotoxins studied. The non-enteric isolates were restricted to a few exotoxin profiles, although two of 48 isolates gave negative results in tests for all exotoxins studied. The commonest exotoxin profile in both enteric (12 of 38) and non-enteric (25 of 48) isolates was Hly⁺/Cyt⁺/Ent⁻/CTF⁻. The next most frequently occurring profile was Hly⁺/Cyt⁺/Ent⁻/CTF⁺ (6 out of 38 for enteric, 8 out of 48 for non-enteric). None of the isolates exhibited Hly⁺/Cyt⁻/Ent⁺/CTF⁺, Hly⁺/Cyt⁻/Ent⁺/CTF⁻, or Hly⁻/Cyt⁺/Ent⁺/CTF⁻ profiles. However, the Hly⁺/Cyt⁺/Ent⁺/CTF⁺ profile, indicating the presence of all four exotoxins studied, was only produced by two of 48 non-enteric isolates. The Hly⁻/Cyt⁻/Ent⁺/CTF⁻ profile, indicative of production of enterotoxin only was found in one isolate from stool and another from peritoneal fluid.

Table IV shows the relationship between the

Table II. Comparison of exotoxin production between enteric and non-enteric isolates of *A. hydrophila*

Exotoxin	Number (%) of isolates that produced toxin	
	enteric (n = 38)	non-enteric (n = 48)
Haemolysin	22 (58)	41 (85)
Cytotoxin	24 (63)	41 (85)
CTF	12 (32)	12 (25)
Enterotoxin	5 (13)	7 (14)

Table III. Exotoxin profiles of *A. hydrophila* isolates from enteric and non-enteric sources

Exotoxin profile				Number of isolates with the given profile				
				Enteric	Non-enteric			
					Wound	Blood	Peritoneal fluid	Others*
Hly ⁺	Cyt ⁺	Ent ⁺	CTF ⁺	0	1	0	0	1
Hly ⁺	Cyt ⁺	Ent ⁺	CTF ⁻	2	3	0	0	1
Hly ⁺	Cyt ⁺	Ent ⁻	CTF ⁻	12	14	2	6	3
Hly ⁺	Cyt ⁻	Ent ⁻	CTF ⁻	1	0	2	0	0
Hly ⁻	Cyt ⁺	Ent ⁺	CTF ⁺	1	0	0	0	0
Hly ⁻	Cyt ⁻	Ent ⁺	CTF ⁺	1	0	0	0	0
Hly ⁻	Cyt ⁻	Ent ⁻	CTF ⁺	2	1	0	1	0
Hly ⁻	Cyt ⁻	Ent ⁻	CTF ⁻	8	0	0	0	2
Hly ⁺	Cyt ⁻	Ent ⁻	CTF ⁺	1	0	0	0	0
Hly ⁺	Cyt ⁺	Ent ⁻	CTF ⁺	6	3	1	2	2
Hly ⁺	Cyt ⁻	Ent ⁺	CTF ⁺	0	0	0	0	0
Hly ⁺	Cyt ⁻	Ent ⁺	CTF ⁻	0	0	0	0	0
Hly ⁻	Cyt ⁺	Ent ⁺	CTF ⁻	0	0	0	0	0
Hly ⁻	Cyt ⁺	Ent ⁻	CTF ⁺	1	0	0	0	0
Hly ⁻	Cyt ⁻	Ent ⁺	CTF ⁻	1	0	0	1	0
Hly ⁻	Cyt ⁺	Ent ⁻	CTF ⁻	2	1	1	0	0
Total				38	23	6	10	9

Hly, haemolysin; Cyt, cytotoxin; Ent, enterotoxin; CTF, cholera toxin-like factor.

* Urine, bile and tracheal secretions.

production of haemolysin and cytotoxin. With the exception of isolates from blood, a large proportion of non-enteric isolates (77–92%) produced both haemolysin and cytotoxin. The production of either haemolysin or cytotoxin only, was found in 16% of enteric isolates and in 8% of the non-enteric isolates. However, amongst the non-enteric isolates, only one of 23 wound isolates and one of six blood-culture isolates produced cytotoxin but not haemolysin. Similarly, haemolysin production independent of cytotoxin production was found in two of six blood-culture isolates but not in any wound isolates. Thus, 50% of blood-culture isolates produced either exotoxin alone. Other non-enteric isolates did not express these exotoxins independently. The remaining 50% of blood-culture isolates produced both exotoxins in combination. However, as only small numbers of isolates from blood were studied,

these results should be interpreted with caution. Of enteric isolates, 31% gave negative results in tests for both exotoxins whereas a much smaller number of the non-enteric isolates gave negative results (5 of 48, 10%).

The relationship between the production of enterotoxin and CTF was examined (table V). Only a small proportion of isolates produced both exotoxins, two (5%) of 38 for enteric isolates and two (4%) of 48 for non-enteric isolates (including wound and urine isolates). Production of CTF alone occurred in 26% of enteric and 21% of non-enteric isolates. The production of enterotoxin alone occurred in 8% of enteric and 10% of non-enteric isolates. Therefore, generally, there was more often dissociation between production of CTF and enterotoxin (34% and 31% respectively for enteric and non-enteric isolates).

Table IV. Relationship between production of haemolysin and cytotoxin in 86 strains of *A. hydrophila*

Exotoxin profile	Number (%) of isolates with the given profile				
	Enteric	Non-enteric			
		Wound	Peritoneal fluid	Blood	Others
Hly ⁺ /Cyt ⁺	20 (52)	21 (92)	8 (80)	3 (50)	7 (77)
Hly ⁻ /Cyt ⁺	4 (11)	1 (4)	0	1 (17)	0
Hly ⁺ /Cyt ⁻	2 (5)	0	0	2 (33)	0
Hly ⁻ /Cyt ⁻	12 (31)	1 (4)	2 (20)	0	2 (23)
Total	38	23	10	6	9

See footnote to table III.

Table V. Relationship between enterotoxin and CTF production in 86 strains of *A. hydrophila*

Exotoxin profile	Number (%) of isolates with the given profile				
	Enteric	Non-enteric			
		Wound	Peritoneal fluid	Blood	Others
Ent ⁺ /CTF ⁺	2 (5)	1 (4)	0	0	1 (11)
Ent ⁻ /CTF ⁺	10 (26)	4 (16)	3 (30)	1 (17)	2 (23)
Ent ⁺ /CTF ⁻	3 (8)	3 (12)	1 (10)	0	1 (11)
Ent ⁻ /CTF ⁻	23 (61)	15 (68)	6 (60)	5 (83)	5 (55)
Total	38	23	10	6	9

See footnote to table III.

Discussion

Aeromonas spp. have been implicated as causative agents in wound infections, septicaemia and diarrhoea. The search for the mechanisms of pathogenicity has revealed that strains of *Aeromonas* produce a range of exotoxins. In this study, production of cytotoxin and haemolysin was prominent amongst isolates from various clinical sources and may be an inherent characteristic of *A. hydrophila* irrespective of source of specimen, but haemolysin and cytotoxin activity were more common in extra-intestinal isolates and may signify the importance of these exotoxins in the pathogenesis of such infections. Asao *et al.*⁹ demonstrated that the haemolysin also had cytotoxic activity, suggesting that these two exotoxins may be the same protein. However, in our study, 5–33% of isolates produced haemolysin alone and cytotoxin alone was produced by 4–17% of strains (table IV). This indicates that the haemolysin and cytotoxin may be two different proteins and raises the possibility that the severity of infection, or ability to cause tissue damage, may be related to the presence of both exotoxins.

Despite the increasing body of information on *A. hydrophila*-associated gastroenteritis, the production of enterotoxin and the nature of the putative enterotoxin has been controversial. Turnbull *et al.*²⁰ demonstrated fluid accumulation in the gut of infant mice suggesting the presence of an enterotoxin. Other workers have used Y1-adrenal¹⁰ and Vero cells²¹ in culture to establish the relationship between *A. hydrophila* enterotoxin and pathogenicity. In this study, enterotoxin was detected in tests with Vero cells in only a small number of isolates (table I). Furthermore, it was shown that amongst the isolates from faeces, CTF was found more often than enterotoxin, suggesting that CTF could be considered to be a marker of pathogenicity in *A. hydrophila* diarrhoea. However, if the genes responsible for CTF were similar to the LT genes of *E. coli*, which occur in single copy numbers and are present on transferrable plasmids,²² they could have been lost on subculture or storage. This may account for the lower number of CTF producers amongst faecal isolates examined in this study. However, in the pathogenesis of diarrhoea, additional factors such as colonisation factors and adhesins may need to be taken into account.

Chakraborty *et al.*²³ have cloned the gene responsible for the production of enterotoxin; it is a chromosomal factor and distinct from the cytotoxin, haemolysin and known *E. coli* enterotoxins. The presence of a factor in the culture supernate of *A. hydrophila* that cross-reacts with anti-CT indicates the production of a CTF that may be responsible for diarrhoea. The ability to produce this factor may be due to the presence of a plasmid-borne toxin gene acquired from other gut bacteria. This hypothesis would require confirmation by DNA hybridisation studies and comparison with truly environmental isolates. Enterotoxin production was not found

amongst blood-culture isolates, which suggests either the presence of factors in blood that may select for organisms that do not produce enterotoxin, or some unknown virulence factors that may be responsible for the invasive property of these strains.

It was evident from this study that non-enteric isolates displayed a more limited number of exotoxin profiles than enteric isolates. At least one type of exotoxin was elaborated by 80% of enteric and 96% of non-enteric isolates. The non-toxigenic isolates may have lost the toxin genes during culture or they may have been truly non-toxigenic. Haemolysin and cytotoxin production was found in isolates from both enteric and non-enteric sources whereas production of the enterotoxin and CTF were more prominent amongst enteric isolates, suggesting the possible importance of these exotoxins on their target sites.

In this investigation of the pathogenesis of aeromonas infections, tissue culture and serological assays were used to detect exotoxins. Further work is needed to elucidate, at the molecular level, the presence of the putative genes that code for protein exotoxins in isolates from various clinical and environmental sources.

This study was supported by a grant from Glaxo Malaysia Sendirian Berhad.

References

1. McCracken AW, Barkley R. Isolation of *Aeromonas* species from clinical sources. *J Clin Pathol* 1972; **25**: 970–975.
2. Washington JA. *Aeromonas hydrophila* in clinical bacteriologic specimens. *Ann Int Med* 1972; **76**: 611–614.
3. Gracey M, Burke V, Robinson J. *Aeromonas*-associated gastroenteritis. *Lancet* 1982; **2**: 1304–1306.
4. Janda JM, Bottone EJ, Skinner CV, Calcaterra D. Phenotypic markers associated with gastrointestinal *Aeromonas hydrophila* isolates from symptomatic children. *J Clin Microbiol* 1983; **17**: 588–591.
5. Pitarangsi C, Echeverria P, Whitmire, R *et al.* Enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*. Prevalence among individuals with and without diarrhea in Thailand. *Infect Immun* 1982; **35**: 666–673.
6. Goodwin CS, Harper WES, Steward JK, Gracey M, Burke V, Robinson J. Enterotoxigenic *Aeromonas hydrophila* and diarrhoea in adults. *Med J Aust* 1983; **1**: 25–26.
7. Donta ST, Haddow AD. Cytotoxic activity of *Aeromonas hydrophila*. *Infect Immun* 1978; **21**: 989–993.
8. Wretling B, Mölby R, Wadström T. Separation of two hemolysins from *Aeromonas hydrophila* by isoelectric focusing. *Infect Immun* 1971; **4**: 503–505.
9. Asao T, Kinoshita Y, Kozaki S, Uemura T, Sakaguchi G. Purification and some properties of *Aeromonas hydrophila* hemolysin. *Infect Immun* 1984; **46**: 122–127.
10. Ljungh A, Popoff M, Wadström T. *Aeromonas hydrophila* in acute diarrheal disease. Detection of enterotoxin and biotyping of strains. *J Clin Microbiol* 1977; **6**: 96–100.
11. Campbell JD, Houston CW. Effect of cultural conditions on the presence of a cholera toxin cross-reactive factor in culture filtrates of *Aeromonas hydrophila*. *Curr Microbiol* 1985; **12**: 101–106.
12. Cumberbatch N, Gurwith MJ, Langston C, Sack RB, Brunton JL. Cytotoxic enterotoxin produced by *Aeromonas hydrophila*: relationship of toxigenic isolates to diarrheal disease. *Infect Immun* 1979; **23**: 829–837.
13. James C, Dibley M, Burke V, Robinson J, Gracey M. Immunological cross-reactivity of enterotoxins of *Aero-*

- monas hydrophila* and cholera toxin. *Clin Exp Immunol* 1982; **47**: 34–42.
14. Speirs JI, Stavric S, Konowalchuk J. Assay of *Escherichia coli* heat-labile enterotoxin with vero cells. *Infect Immun* 1977; **16**: 617–622.
 15. Giugliano LG, Mann GF, Drasar BS. Response of mammalian cell lines to the toxins of *Escherichia coli*. *J Med Microbiol* 1982; **15**: 531–539.
 16. Vadivelu J, Dunn DT, Feachem RG *et al.* Comparison of five assays for the heat-labile enterotoxin of *Escherichia coli*. *J Med Microbiol* 1987; **23**: 221–226.
 17. Wadström T, Ljungh Å, Wretling B. Enterotoxin, haemolysin and cytotoxic protein in *Aeromonas hydrophila* from human infections. *Acta Pathol Microbiol Scand* 1976; Sect B **84**: 112–114.
 18. Sack DA, Huda S, Neogi PKB, Daniel RR, Spira WM. Microtiter ganglioside enzyme-linked immunosorbent assay for vibrio and *Escherichia coli* heat-labile enterotoxins and antitoxins. *J Clin Microbiol* 1980; **11**: 35–40.
 19. Miller CJ, Drasar BS, Feachem RG, Hayes RJ. The impact of physico-chemical stress on the toxigenicity of *Vibrio cholerae*. *J Hyg* 1986; **96**: 49–57.
 20. Turnbull PCB, Lee JV, Miliotis MD *et al.* Enterotoxin production in relation to taxonomic grouping and source of isolation of *Aeromonas* species. *J Clin Microbiol* 1984; **19**: 175–180.
 21. Millership SE, Barer MR, Tabaqchali S. Toxin production of *Aeromonas* spp. from different sources. *J Med Microbiol* 1986; **22**: 311–314.
 22. Black RE, Merson MH, Rahman ASMM *et al.* A two-year study of bacterial, viral, and parasitic agents associated with diarrhea in rural Bangladesh. *J Infect Dis* 1980; **142**: 660–664.
 23. Chakraborty T, Montenegro MA, Sanyal SC, Helmuth R, Bulling E, Timmis KN. Cloning of enterotoxin gene from *Aeromonas hydrophila* provides conclusive evidence of production of a cytotoxic enterotoxin. *Infect Immun* 1984; **46**: 435–441.
-