MICROBIAL PATHOGENICITY

Possible virulence factors involved in bacteraemia caused by *Aeromonas hydrophila*

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Summary. Eighteen strains of *Aeromonas hydrophila* from patients with bacteraemia were investigated for possible virulence factors. Cytotoxin and haemolysin were produced by all strains, whereas cholera toxin-like factor was produced by 33 % of strains only. Enterotoxin production was not detected. Haemagglutination of guinea-pig, fowl and rabbit erythrocytes was demonstrated by 83 %, 67 % and 61 % of strains, respectively. Fucose- and mannose-sensitive haemagglutinins were predominant. None of the strains agglutinated sheep erythrocytes. Extrachromosomal DNA was detected in 17 strains, 16 of which had a plasmid (3.6–5.1 MDa), the majority being between 4.6 and 5.1 MDa.

Introduction

Aeromonas hydrophila, a member of the family Vibrionaceae, is increasingly recognised as a pathogen of man and gives rise to both intestinal and extra-intestinal infections.^{1–3} The natural habitat of aeromonads is aquatic; the species can multiply in fresh and drinking water and give rise to infections in fish, turtles, alligators and other fresh water animals.⁴ The range of human extra-intestinal infections is from traumatic introduction of the micro-organisms into the skin, with resultant necrotising cellulitis, to opportunist systemic infections in immunocompromised patients.

Several potential virulence factors elaborated by *Aeromonas* spp. have been described. These include cytotoxin and haemolysin, which appear to be the same protein or very closely related,⁵ an enterotoxin, cholera toxin-like factor (CTF) and haemagglutinins. The pathogenic role of these factors remains unclear.

An earlier study³ that compared the exotoxin profiles of intestinal and extra-intestinal strains found haemolysin and cytotoxin to be produced by most strains, irrespective of their origin. Enterotoxin was produced by c. 10–20 % of enteric and wound isolates only. The production of CTF was most frequent in enteric isolates. Of the six bacteraemic strains studied, the haemolysin and cytotoxin were produced in combination in three strains and singly in the other three. Enterotoxin was not produced by any of these

strains and CTF by only one. These findings were interpreted with caution because numbers were small.

In this study, a further 18 bacteraemic strains of *A. hydrophila* were investigated to ascertain which of the virulence factors, if any, were involved in the pathogenesis of septicaemia.

Materials and methods

Eighteen strains of *A. hydrophila* from patients at the University Hospital, Kuala Lumpur, Malaysia were examined. All were isolated in biphasic blood culture media (bioMérieux) from patients with bacteraemia and identified by standard bacteriological methods⁶ as belonging to the genus *Aeromonas*. These strains were then confirmed and speciated by the API 20E system (bioMérieux). Although the API 20E does not reliably distinguish aeromonads, the property of inability to grow in the presence of NaCl 8 % and resistance to the vibriostatic agent O/129 excluded *Vibrio fluvialis*; non-production of gas from glucose excluded *A. caviae* and a negative aesculin hydrolysis test excluded *A. sobria*. In addition, the analytical profile index obtained by the API 20E system was ≥ 99.0 %, confirming that these strains were *A. hydrophila*. The strains were stored at room temperature on Tryptone Soy Agar (Oxoid) slopes during the course of the study. *Escherichia coli* WF5 (University of Surrey, UK) was used as the negative control in the detection of haemolysin, cytotoxin and enterotoxin. Cholera toxin (Sigma) 1 mg/L was used as the positive control for enterotoxin activity (vero cells) and CTF activity (ELISA).

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Patients' records were reviewed for data such as age, sex, underlying illness and other relevant predisposing factors such as burns and trauma. No attempt was made to obtain faecal isolates from these patients as this was a retrospective study.

Preparation of culture filtrates

Cell-free culture supernates were prepared with Brain Heart Infusion Broth (Oxoid) by the method of Cumberbatch *et al.*⁷ as described previously.³ The filter-sterilised cell-free supernates were stored at -20°C in small volumes and tested within 1 week of preparation.

Cytotoxin and enterotoxin assay

The vero cell assay for the detection of cytotoxin and enterotoxin activity was performed as described previously.³ Cytotoxin activity was determined when $\geq 50\%$ cells were lysed when exposed overnight at 37°C to $50\ \mu\text{l}$ of cell-free supernate. Enterotoxin activity was detected when $\geq 50\%$ cells showed morphological cytopathic effects when exposed overnight at 37°C to $50\ \mu\text{l}$ of cell-free supernate heated at 56°C for 10 min to denature the cytotoxin.

Cholera toxin-like factor assay

The enzyme-linked immunosorbent assay (ELISA) method of Sack *et al.*⁸ modified by Miller *et al.*⁹ was performed as described previously.³ The results were read spectrophotometrically at 492 nm with a Dynatech MR600 Microelisa reader. A negative: positive cut-off value was calculated from the average values of control negative strains + 2 SD values.¹⁰

Haemagglutination assay

The haemagglutination assay was performed by the method of Crichton and Walker.¹¹ The presence of haemagglutinins was determined with fresh erythrocytes from sheep, rabbit, guinea-pig and fowl in the presence or absence of L-fucose (Sigma) 2% w/v and mannose (BDH Chemicals) 2% w/v. The erythrocytes were prepared by centrifuging 10 ml of blood collected in an equal volume of sterile Alsevier's solution. The resultant supernate was discarded, the packed cells were washed twice in sterile phosphate-buffered saline (PBS) and resuspended to 3% v/v in fresh PBS. For haemagglutinin production, the bacterial strains were grown on human blood agar (Oxoid) at 37°C for 24 h and harvested directly in PBS at a concentration of 10^{11} cfu/ml. Bacterial suspension ($25\ \mu\text{l}$) was added to $25\ \mu\text{l}$ of erythrocyte suspension and $25\ \mu\text{l}$ of PBS in a depression on a porcelain tile, rocked at ambient temperature for 10 min and observed for haemagglutination. Parallel tests were performed in which the PBS was substituted with $25\ \mu\text{l}$ of L-fucose or mannose 2% w/v. Haemagglutination

reactions were recorded as weakly (+), moderately (++) or strongly (+++) positive, or negative (–).

Plasmid analysis

Plasmids were isolated by the method of Kado and Liu.¹² Briefly, 1 ml of overnight culture incubated at 37°C was centrifuged for 10 min at 12000 rpm. The pellet was thoroughly resuspended in $100\ \mu\text{l}$ of lysing buffer (SDS 3%, 50 mM Tris, pH 12.6) and incubated at 65°C for 1 h with occasional mixing. Subsequently, $200\ \mu\text{l}$ of unbuffered phenol-chloroform (1:1 v:v) was added and the mixture was vortex mixed briefly before centrifugation for 5 min at 12000 rpm to separate the mixture. The upper aqueous phase was used directly on an agarose 0.6% gel prepared in Tris-borate-EDTA buffer (1x). The plasmids were electrophoresed through the gel at 130 V for 2 h in Tris-borate-EDTA buffer. Plasmids isolated from *E. coli* strain V517 were used as close circular DNA mol. wt. markers (7.5, 18.0, 26.0, 31.0, 11.5, 23.0, 29.5 and 34.0 MDa).

Results

The age and sex distribution of the 18 patients (table I) shows an equal distribution of the sexes; the age range was 17–72, the average being 43 years. All but one of the patients had a severe underlying illness or predisposing risk factor which could be categorised into three groups—leukaemia/lymphoma, seven (39%); trauma/burns, six (33%); and liver cirrhosis, four (22%). The case fatality rate was 72%.

All 18 strains of *A. hydrophila* produced haemolytic zones around the colonies on Ox Blood Agar (Oxoid), indicating the elaboration of a haemolysin. These strains were also found to produce cytotoxin. However, none elaborated any enterotoxin. CTF was produced by six strains, four of which were from patients involved in motor vehicle accidents or burns.

The ability of the strains to haemagglutinate was studied with sheep, rabbit, guinea-pig and fowl erythrocytes. Two of the 18 strains showed no haemagglutination; of the remaining 16, eight showed fucose- and mannose-sensitive haemagglutination with rabbit, guinea-pig and fowl erythrocytes only; one strain demonstrated the same pattern with guinea-pig and fowl erythrocytes and a further two strains with guinea-pig erythrocytes only. The remaining five strains exhibited variable patterns of haemagglutination (table II). None of the strains agglutinated sheep erythrocytes.

Plasmid profiles were obtained for 17 strains that had at least one plasmid (table I). Sixteen strains had at least one plasmid of between 3.6 and 5.1 MDa and the majority were 4.6–5.1 MDa. Three strains had additional plasmids; one strain had a ≥ 62 -MDa plasmid, one strain had a 2.85-MDa plasmid and a third had two plasmids of 2.95 and 2.6 MDa. Of the remaining two strains, one had a larger plasmid of ≥ 19 MDa and the other had no plasmids.

Table I. Age and sex distribution, predisposing factors, outcome, virulence factors and plasmid profiles of septicaemic strains of *A. hydrophila*

Patient no.	Age (years)	Sex	Predisposing factor	Outcome	Hly/Cyt	Ent/CTF	Plasmid (MDa)
1	68	F	Liver cirrhosis	D	+/+	-/-	4.6
2	54	M	Liver cirrhosis	D	+/+	-/-	4.8/2.85
3	61	F	Liver cirrhosis	A	+/+	-/-	5.1
4	50	M	Liver cirrhosis	A	+/+	-/-	4.8
5	72	F	Leukaemia/lymphoma	D	+/+	-/-	4.6
6	46	M	Leukaemia/lymphoma	D	+/+	-/+	5.1
7	24	M	Leukaemia/lymphoma	D	+/+	-/-	4.8
8	66	F	Leukaemia/lymphoma	A	+/+	-/-	4.6
9	59	F	Leukaemia/lymphoma	A	+/+	-/-	5.1
10	56	F	Leukaemia/lymphoma	D	+/+	-/-	4.8
11	17	F	Leukaemia/lymphoma	D	+/+	-/+	5.1
12	15	M	Trauma (MVA/Burns)	A	+/+	-/+	4.8
13	17	F	Trauma (MVA/Burns)	D	+/+	-/-	4.8/2.95/2.6
14	17	M	Trauma (MVA/Burns)	D	+/+	-/+	≥ 62/4.8
15	39	M	Trauma (MVA/Burns)	D	+/+	-/+	4.8
16	49	M	Trauma (MVA/Burns)	D	+/+	-/-	3.8
17	19	M	Trauma (MVA/Burns)	D	+/+	-/+	19
18	39	F	Post-partum death	D	+/+	-/+	—

D, died; A, alive; MVA, motor vehicle accident; Hly/Cyt, haemolysin/cytotoxin; Ent/CTF, enterotoxin/cholera toxin-like factor.

Table II. Individual haemagglutination patterns of five septicaemic strains of *A. hydrophila*

Strain no.	Rabbit			Guinea-pig			Fowl		
	C	F	M	C	F	M	C	F	M
1	1+	1+	1+	2+	2+	2+	1+	—	—
2	—	—	—	—	—	—	1+	1+	2+
3	—	—	—	1+	—	—	—	—	—
4	2+	1+	1+	3+	—	—	—	—	—
5	2+	1+	—	3+	—	—	1+	—	—

C, control (erythrocyte only); F, L-fucose; M, mannose.

Discussion

The ability of *A. hydrophila* to give rise to a wide range of infections in man indicates that complex pathogenic mechanisms that involve a range of protein toxins may be responsible. The wide natural habitat of the organism shows that it can adapt to different environments and suggests the presence of survival mechanisms.

We have shown³ that cytotoxin and haemolysin were produced in combination in the majority of clinical isolates, suggesting that this may be an inherent property of *A. hydrophila*, although it was more prominent in the extra-intestinal strains. The production of cytotoxin and haemolysin by all 18 bacteraemic strains in this study supports this view, although the role of these factors in the pathogenesis of infection is unclear. One could speculate that the cytotoxin may play a role in severe, necrotising wound infections and bloody diarrhoea associated with this species.

We were unable to show enterotoxin production in the 18 bacteraemic strains and this corroborates our

earlier study.³ Although the gene that encodes enterotoxin production has been shown to be chromosomal,¹³ these bacteraemic strains showed no evidence of enterotoxin production, which suggests that these genes may be cryptic, absent or may be present on transposon(s) with reverse integration and expression of this property may not be necessary in the pathogenesis of bacteraemia.

Toxins similar to the cholera toxin have been found in enteric pathogens such as enterotoxigenic *Escherichia coli* and *Campylobacter jejuni*. There is a suggestion that the genes that occur on transferrable plasmids may be exchanged amongst organisms in the gut or the environment. Therefore, strains of *A. hydrophila* could have acquired the genes for CTF production. In this study, CTF was produced by 39% of strains, 71% of which occurred amongst strains isolated from patients with trauma or burns. This suggests that the organisms may have originated from an enteric source such as the patients' own gut flora, or from an environmental source during trauma. The absence of enterotoxin and CTF amongst the blood-culture isolates is in agreement with our previous findings and suggests that these toxins may not play a role in the pathogenesis of septicaemia due to *A. hydrophila*.

The ability to adhere to the intestinal mucosa is an essential early step in colonisation and development of an enteric infection. The role of mannose-resistant haemagglutinins (MR-HA) in colonisation by enterotoxigenic *E. coli* has been reported.¹⁴ Amongst *Aeromonas* spp., MR-HA have been found in diarrhoeic isolates, whereas environmental isolates were found to have mannose- and fucose-sensitive haemagglutinins (MS-HA and FS-HA).¹⁵ In this study, 89% of strains were found to possess

haemagglutinins, of which 69% possessed MS-HA and FS-HA, which suggests that these isolates may have originated from an environmental source. The organisms may have entered the bloodstream directly as a result of trauma or intravenous therapy. In two of these cases, MR-HA was detected (table II) that may have originated from the patients' gut flora. However, the role of these haemagglutinins in disease caused by *Aeromonas* spp. remains to be defined.

Genetic determinants of the haemolysin, cytotoxin and enterotoxin have been identified on three different segments of the *A. hydrophila* chromosome.¹³ However, the determinants for CTF have not been found. As in other organisms such as *E. coli*, the genes may be located on plasmids.¹⁶ Plasmids were present in 94% of strains of *A. hydrophila*, most of which seemed to have a conserved plasmid of 4.6–5.1 MDa. It is not clear whether the CTF gene may be located on this plasmid as not all strains with this plasmid produce CTF. Other factors such as adhesins and antibiotic resistance may be coded on this plasmid but this possibility was not investigated further. Three strains had additional bands, of which two strains had two plasmids and another three plasmids. It is possible that the smaller bands may have been products of shearing of the 4.8-MDa plasmid during preparation. Two of the strains had plasmids of 19 MDa and ≥ 62 MDa,

respectively, which may represent chromosomal contamination of the plasmid preparation.

This study supports our earlier findings³ that the production of cytotoxin and haemolysin are closely related and universal amongst all strains of *A. hydrophila* isolated from clinical specimens. One possibility is that, although all strains are able to produce haemolysin and cytotoxin, these factors may be less important in the pathogenesis of intestinal infection than the ability of the organism to invade and enter the bloodstream from intestinal or extra-intestinal infections. The production of CTF was commoner in strains from cases with trauma or burns that had been contaminated with water and soil. Hence the CTF genes may have been acquired from enteric pathogens in the environment. These strains did not produce enterotoxin, which is further evidence that the encoding genes may be cryptic or lost through the selection process in the pathogenesis of bacteraemia. The presence of a conserved plasmid suggests that it may be useful to investigate the nature of the genes present, which may give further information about the presence of virulence factors and haemagglutinins.

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