BACTERIAL PATHOGENICITY

Enhancement of the virulence of *Aeromonas caviae* diarrhoeal strains by serial passages in mice

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Thirteen clinical *Aeromonas caviae* isolates from the faeces of 13 children with mild to severe diarrhoea were tested for enhancement of mouse lethality, adhesion ability, siderophore and cholera toxin cross-reactive (CTC) factor production, by four consecutive passages through mice by intraperitoneal injection of *A. caviae* suspensions. The passaged *A. caviae* strains were re-isolated from monomicrobial cardiac blood samples and inocula were prepared for the next passage. All *A. caviae* isolates possessed the ability to adhere to the mucosal epithelial surface of the rabbit small intestine. Serial passage in mice showed that the virulence of some isolates for mice was increased in terms of percentage mortality and a lowering of the LD50. For some of the isolates, but not all, serial passage appeared to increase siderophore production and adhesion to rabbit small intestinal cells. For the *A. caviae* isolates tested, increased values of the CTC factor were observed after passage. A clear correlation was observed between the lowering of LD50 and the enhancement of CTC factor production after passage in mice. These results indicate that the *A. caviae* isolates possessed virulence factors.

Introduction

The members of the genus *Aeromonas* are fish and human pathogens, causing fatal haemorrhagic septicaemias in the former, and gastro-enteritis as well as blood, wound and soft-tissue infections in the latter [1]. Mesophilic, motile *Aeromonas* spp. have been isolated from intestinal and extra-intestinal human infections and from the environment [1–4]. Three types of exotoxin – haemolysins, cytotoxins and the cytotoxic enterotoxin cholera toxin cross-reactive (CTC) factor, immunologically related to the cholera toxin – are produced [2, 5–10]. This CTC enterotoxin is capable of activating adenylate cyclase, resulting in an increased level of intracellular cAMP. *Aeromonas* enterotoxin fulfils the criteria for a cytotoxic enterotoxin [1]. *Aeromonas* strains secrete numerous extracellular proteins, including DNAase, RNAase, proteases, elastase, neuraminidase, lipase, phosphatase and fibrinolysin [11, 12]. *Aeromonas* proteases may enhance virulence, and have a broad specificity for many tissue proteins. The pathological effect of *aeromonas* pro-

teases is destruction of tissue proteins and loss of local tissue integrity [13].

A. caviae has been considered to be of low pathogenicity or non-enteropathogenic [2, 7, 14]. Gosling [15] showed that only one of 11 strains of *A. caviae* appeared toxigenic. The *A. caviae* strains studied by Janda *et al.* [16] were avirulent. Janda and Kokka [17] considered *A. hydrophila* and *A. veronii* biotype *sobria* to be inherently more pathogenic for mice than *A. caviae*.

Several reports have suggested that *A. caviae* strains did not produce any toxic material or that virulence mechanisms were unknown [5, 18, 19]. The enteropathogenicity of *A. caviae* is still questioned [20, 21] because conditions for the expression of enterovirulence have not been demonstrated consistently. However, this micro-organism has been recognised increasingly as a primary or secondary invader associated with gastro-enteritis in infants [21, 22]. *A. caviae* was rarely encountered as a cause of septicaemia [16].

To understand better the importance of certain virulence factors in the pathogenesis of gastro-enteritis caused by *A. caviae* and to judge whether they are linked to gastro-enteric disease, the influence of

consecutive passages through mice on mortality, LD50 values, siderophore activity, adherence ability and CTC factor production was determined.

Materials and methods

Bacterial strains

Thirteen isolates of *A. caviae* from 13 diarrhoeal faecal specimens, from 13 hospitalised children were studied. They were isolated in the absence of other well-recognised common bacterial enteropathogens and rotavirus. Each isolate was identified to the species level according to the criteria of other studies [18, 23–31]

Two hundred faecal specimens from 200 healthy controls were analysed for the presence of *A. caviae*. The age range of all hospitalised children and healthy controls was 2 months–2 years.

Mouse model of infection – LD50 studies

The relative virulence of the 13 *A. caviae* isolates was evaluated by LD50 studies. The 13 isolates were grown in casamino acids yeast extract medium (CYE), pH 7.5, at 37°C in a water bath with shaking for 10 h [32]. The cultures were centrifuged at 3000 *g* for 30 min. The bacterial cells were washed twice with sterile phosphate-buffered saline (PBS), pH 7.4, and resuspended in sterile saline to 6×10^9 cfu/ml ($A_{420} = 1.2$) (confirmed by plate counts made in duplicate). The relative virulence of the *A. caviae* isolates was evaluated by intraperitoneal injection into 8–10-week-old Swiss Webster mice, each weighing 18–20 g, of 1 ml of five serial two-fold dilutions of the bacterial suspension. Each isolate was injected into five groups of five mice. Control mice were inoculated with sterile saline. The inoculated mice were observed daily and deaths were recorded up to 72 h. After 72 h, the LD50 was calculated [33].

Passage through mice

All 13 *A. caviae* strains were passaged four times in mice by intraperitoneal injection. *A. caviae* was re-isolated from cardiac blood after each passage. Gross inspection for pathological changes was made.

Preparation of culture filtrates

All isolates were grown in CYE broth at 37°C for 10 h (log phase) in a water bath with shaking. The cultures were then centrifuged at 700 *g* for 30 min. The supernates were sterilised by filtration through 0.22- μ m pore-size filters (Millipore), stored at 4°C and tested in assays within 1 week of preparation [18, 34].

Preparation of cell lysates

Cells of *A. caviae* isolates cultured on trypticase soy agar plates for 24 h at 37°C were suspended in 0.02 M Tris-buffered saline (pH 7.5) containing lysozyme 0.5 mg/ml and EDTA 37.2 mg/L at 3×10^{11} cfu/ml. After incubation for 1 h at 37°C, the lysates were centrifuged at 700 *g* for 30 min. The supernates were sterilised by filtration through 0.22- μ m filters (Millipore), stored at 4°C and tested in assays within 1 week of preparation [35].

CTC factor assay

Cell-free supernates and lysates from the 13 *A. caviae* isolates were tested for CTC factor production by the GM₁ enzyme-linked immunosorbent assay (ELISA) [34, 35]. Briefly, 100 μ l of monosialoganglioside-GM₁ (Sigma) 50 μ g/ml in carbonate buffer (pH 9.6) were adsorbed to each well of a round-bottomed microtitration plate (VEB MLW Germany). After incubation for 24 h at 4°C the plates were washed three times with PBS containing CaCl₂ 100 mg/L, MgCl₂·6H₂O 100 mg/L, pH 7.4, bovine serum albumin fraction V (Sigma) 0.1% and Tween 20 (Sigma) 0.05% (PBS-BSA-Tween). Subsequently 100- μ l volumes of cholera toxin from *Vibrio cholerae* O1 (CT; Calbiochem Switzerland) 0.5–500 ng/ml were added in triplicate to the wells to create a standard curve. CT (100 ng/ml) was used as a positive control and uninoculated growth medium was used as the negative control. Culture supernates and lysates of *A. caviae* isolates were added in 100- μ l amounts to the wells; all samples were tested in triplicate. The plates were incubated at room temperature for 24 h. After three washes with PBS-BSA-Tween, 100 μ l of rabbit anti-CT serum (Calbiochem Switzerland) at a dilution of 1 in 640 was added to each well. After incubation for 24 h at 4°C, the wells were washed three times with PBS-BSA-Tween; 100 μ l of protein A-horseradish peroxidase conjugate (Sigma) diluted 1 in 1000 in PBS-BSA-Tween were added to detect bound immunoglobulins. After incubation for 1 h at 37°C, the wells were washed three times with PBS-BSA-Tween, and 100 μ l of the substrate 2,2'-azino-bis(3-ethylbenzthiazoline sulphonic acid) 0.1 mg/ml in 1 M phosphate-citrate buffer (pH 5.0) containing H₂O₂ 0.03% were added to each well. After incubation for 30 min at 37°C, the reaction was stopped by adding 150 μ l of 1 M H₂SO₄ to each well. The results were read as A₄₁₀ on a Sumal PE2 minireader (Carl Zeiss, Jena). The negative control of CYE broth gave a reading of 0 and wells with an A₄₁₀ of ≥ 0.1 were recorded as positive. The amount in nanograms of CT equivalent/ml of supernate and cell lysate was estimated from the CT standard curve.

Siderophore detection and identification

The 13 *A. caviae* isolates were grown to stationary phase in Chelex 100 deferrated M9 medium [36]. Cell-

free culture supernates were examined for siderophore production by the universal chrome azurol assay [37]. The colorimetric tests of Arnow and Csaky were used to detect phenolate and hydroxamate-type siderophores [38, 39].

Adhesion assay

Adherence of *A. caviae* isolates to the epithelium of rabbit small intestine was assayed as described previously [40], but with one slight modification – the suspension of *A. caviae* cells was incubated with the intestinal sample for 1 h at 37°C. Non-adhering bacteria were removed from the sample by washing four times with Krebs-Ringer solution (KRT). KRT added to the rabbit small intestinal sample was the negative control. The fixed samples were dehydrated through a series of graded ethanol and acetone solutions and critical-point dried. They were coated with gold and examined by scanning electron microscopy (SEM) (Philips type 515). At a magnification of $\times 5000$ the number of bacteria per field was the adherence index.

Electron microscopy for detection of fimbriae [41]

The bacteria were grown statically in 2 ml of tryptic soy broth (TSB) containing yeast extract 0.6% for 24 h at 37°C. The cultures were centrifuged and the cells were washed twice with PBS, pH 7.8, and suspended in distilled water to $10^8 - 10^9$ cells/ml. Then 10- μ l volumes of the bacterial suspension, the negative stain (ammonium molybdate 2%, pH 6.8) and bacitracin 150 μ g/ml were mixed and 10 μ l of the mixture were applied to Formvar 200 mesh copper grids for 1 min. The excess liquid was removed and the grid was allowed to air-dry. The grids were examined with a JEM 1200 IIE transmission electron microscope and photographed.

Cell surface hydrophobicity

Each *A. caviae* strain to be tested was grown in CYE for 24 h at 37°C. The cultures were centrifuged at 3000 *g* for 20 min. The pelleted bacterial cells were resuspended in 2 mM sodium phosphate buffer, pH 6.8, to an A_{420} value of 1.0 ($10^8 - 10^9$ cells/ml).

Salt aggregation test (SAT) [42–44]. Briefly, 30 μ l of a range of concentrations of $(\text{NH}_4)_2\text{SO}_4$ in 2 mM sodium phosphate buffer, pH 6.8, from 4 M to 2 M were dispensed into wells of microtation plates and 30 μ l of the bacterial cell suspension were added to each well. The bacterial suspension in sodium phosphate buffer was used as a negative control. After thorough mixing, the plates were kept at room temperature for 24 h. The lowest concentration of ammonium sulphate that caused bacterial aggregation was recorded as the SAT value.

Bacterial adhesion to hydrocarbons test (BATH) [42, 43]. For each strain, 4 ml of bacterial suspension were added to each of two 13-mm diameter test tubes, and 4 ml of phosphate buffer were added to each of two other test tubes – control tubes. One ml of n-octane (Sigma) was added to both a test tube and a control tube, and 1 ml of n-hexadecane (Sigma) was added to the other assay tube and a control tube. The tubes were placed in a water bath for 10 min at 37°C to equilibrate, were shaken for 15 s and then incubated for 30 min at 37°C. After incubation, the aqueous layer was removed carefully and its A_{540} value was measured against a control of phosphate buffer. The percentage of partitioning in the hydrocarbon phase was calculated from the formula:

$$\frac{A_{540} \text{ (original suspension)} - A_{540} \text{ (aqueous suspension)}}{A_{540} \text{ (original suspension)}} \times 100.$$

Results

In the faecal specimens of 200 healthy controls, *A. caviae* was detected in only one specimen (i.e., 0.5%) from a child who had diarrhoea 1 month earlier. *A. caviae* was not present in the faeces of the remaining 199 healthy control children. Thirteen *A. caviae* isolates from 13 diarrhoeal faecal specimens were studied. The virulence of seven of them was examined after exponential and stationary growth in two culture media at 37°C.

Cultural conditions for optimum virulence

The effect of culture conditions on the virulence of the *A. caviae* isolates for mice was examined by the injection of washed cell suspensions. Cells from exponentially growing cultures in CYE and TSB $\times 2$ caused death – the LD50 being lower for isolates grown in CYE than in TSB $\times 2$. In contrast, cells in the stationary phase of growth, whether from CYE or TSB $\times 2$ cultures, were without effect on mouse mortality or were much reduced in virulence (isolates 378, 380 and 383) (Table 1).

Effects of passage in mice of A. caviae isolates upon virulence determinants

The effects of passage of *A. caviae* on mortality, siderophore and CTC factor production are presented in Table 2.

Bacteraemia occurred as evidenced by the culture of *A. caviae* from the cardiac blood of each 'squatting' mouse and dead mice after consecutive passages 24 h after inoculation.

All the *A. caviae* isolates produced siderophores. These were all of the phenolate type and none was of the

Table 1. Virulence (LD₅₀) for mice of seven clinical isolates of *A. caviae* grown in two different culture media and tested at two phases of growth

<i>A. caviae</i> isolate no.	LD50 (log ₁₀) 10 h*, 37°C		LD50 (log ₁₀) 18 h†, 37°C	
	CYE	TSB × 2	CYE	TSB × 2
375	9.85	0‡	0	0
376	9.6	9.6	0	0
378	9.5	9.7	0	9.9
379	9.4	9.7	0	0
380	9.3	9.3	9.5	9.7
383	9.4	9.6	0	9.6
384	9.4	9.7	0	0

CYE, casamino acid yeast extract broth; TSB × 2, glucose-free double-strength tryptic soy broth. *Exponential phase of growth. †Stationary phase of growth. ‡No mortality was observed.

hydroxamate type. Some isolates, e.g., 375, 376 and 380, produced greater and increasing amounts of siderophore with passage in mice, whereas others, e.g., 383, 384 and 388, continued to be poor siderophore producers despite passage.

CTC factor was not detected by ELISA in either supernates or lysates of isolates 383, 385 or 388. All the other isolates produced CTC factor, greater amounts being detected in supernates than in lysates. Generally, CTC factor levels increased with the number of passages in mice. For some isolates, e.g., 376, 378 and 379, CTC factor was associated with isolates at passage 0, whereas for others, e.g., isolates 375, 377 and 390, CTC factor was only detected after passage. Passage in mice caused some isolates, e.g., 380, 393, 376 and 377, to cause increased mortality and a decreased LD₅₀ for mice, whereas for other isolates, e.g., 383 and 385, passage did not substantially increase mortality or LD₅₀ for mice.

A correlation was found between CTC factor production, percentage mortality for mice and a decrease in LD₅₀. No such correlation was found between siderophore production and mouse mortality and LD₅₀.

The ability of the 13 isolates of *A. caviae* to adhere to the mucosal epithelial surface of rabbit small intestine was determined by SEM. The results are presented in Table 3 and Fig. 1.

All 13 *A. caviae* isolates were adherent strains. Five isolates (375, 378, 379, 380 and 383) showed enhanced adherence after passage in mice. All of these isolates except no. 383 manifested a correlation between the adherence index and decreased LD₅₀ value, and isolates 375, 378 and 380 manifested enhanced CTC factor production after passage in mice (Table 2). The adherence index of the other isolates did not increase with passage. Isolates 383, 385 and 388 that did not produce any CTC factor nor a decrease in LD₅₀ after passage, had a high adherence index, whereas others, e.g., 380, which produced high mortality, high values

of the CTC factor and a lower LD₅₀ values after passage in mice, had a low adherence index (Tables 2 and 3). In contrast, isolate 390 had a very high adherence index at passage 0, yet at this stage did not produce any CTC factor and caused low percentage mortality among mice (Tables 2 and 3).

To evaluate the ability of the *A. caviae* isolates to attach to a surface, the hydrophobicity of the cell surface and the presence of fimbriae was determined, as was the enhancement of these properties by serial passage in mice (Tables 3 and 4). Eight original isolates, and those obtained after their passage in mice, had no fimbriae. Of these, five isolates (375, 378, 379, 380 and 390) were strongly or moderately hydrophobic by SAT and showed a lowering of LD₅₀, and three of them (375, 378 and 380) gave an enhanced value of the CTC factor after passage in mice. Isolates 383, 386 and 388 were weakly or not hydrophobic by SAT assay, but nos. 383 and 388 were moderately hydrophobic by BATH. These latter two isolates did not show a significant lowering of LD₅₀ and did not produce any CTC factor. Isolate 385 possessed weak hydrophobicity by SAT, but none by BATH, did not show significant lowering of LD₅₀ and did not produce any CTC factor even after passage in mice. Five isolates (nos. 376, 377, 384, 386 and 393) possessed fimbriae and with the exception of no. 386, exhibited weak or moderate positive hydrophobic properties by SAT or BATH assay, or both, produced CTC factor and showed lowering of the LD₅₀.

Only one isolate (377) became strongly hydrophobic by SAT after passage in mice; this isolate possessed fimbriae. Isolates 386 and 393 revealed the presence of fimbriae only after passage in mice. Only three strains became moderately hydrophobic by BATH (n-octane) after passage in mice. There was no correlation observed among the positive results by SAT and BATH (Table 3) and among the results of n-octane and n-hexadecane tests by BATH. Six isolates were positive in the BATH test when n-octane was used, but only two isolates were positive when n-hexadecane was used. The lowering of LD₅₀ and the production of the CTC factor did not correlate with hydrophobicity or the presence of fimbriae.

A scanning electron micrograph showing the adherence of some of the *A. caviae* isolates to the epithelium of rabbit small intestine is shown in Fig. 1.

Gross inspection of mice infected with the clinical isolates of *A. caviae* almost always revealed mice with raised fur, squatting and immobility. Diarrhoea and intestinal fluid accumulation was evidence of *A. caviae* enterotoxigenicity. Post-mortem examination of the alimentary tract from the stomach to the rectum of dead mice made immediately after death revealed distension, swelling, congestion and accumulation of yellow fluid.

Table 2. Effects of lethal passage in mice of clinical isolates of *A. caviae* on mortality, siderophore and cytotoxic toxin production

<i>A. caviae</i> isolate no.	Passage no.	Mortality (%)	LD50 (log ₁₀)	Siderophore production	Cytotoxic toxin production* (ng/ml)	
					s	l
375	0 [†]	16	9.5	0.433	0	0
	1	32	9.3	0.559	0.3	0.2
	2	32	9.2	0.571	0.5	0.2
	3	32	8.8	0.717	0.4	0.2
	4	20	9.2	0.721	0.3	0.2
376	0	16	9.6	0.414	0.25	0.2
	1	12	9.2	0.695	0.2	0.2
	2	16	9.1	0.661	0.2	0.2
	3	28	9.4	0.576	0.5	0.2
	4	48	8.8	0.532	0.6	0.25
377	0	16	9.8	0.339	0	0
	1	24	9.4	0.372	0.3	0.2
	2	32	8.9	0.204	0.2	0.2
	3	32	9.2	0.335	0.7	0.2
	4	52	8.8	0.302	0.7	0.3
378	0	16	9.7	0.210	0.35	0.2
	1	40	9.2	0.332	0.3	0.2
	2	28	9.0	0.338	0.7	0.2
	3	48	8.7	0.453	0.3	0.2
	4	24	9.3	0.373	0.2	0.2
379	0	20	9.9	0.203	0.35	0.2
	1	48	8.9	0.263	0.2	0.2
	2	36	9.1	0.203	0.3	0.2
	3	20	9.6	0.261	0.3	0.2
	4	20	9.5	ND	0.2	0.2
380	0	12	9.3	0.361	0.3	0.2
	1	48	9.2	0.394	0.2	0.3
	2	48	9.2	0.368	0.6	0.4
	3	76	8.7	0.409	0.8	0.4
	4	76	8.0	0.520	0.8	0.5
383	0	28	9.3	0.048	0	0
	1	24	9.6	0.095	0	0
	2	24	9.5	0.049	0	0
	3	28	9.6	0.134	0	0
	4	ND	9.6	ND	0	0
384	0 [†]	24	9.4	0.071	0.2	0.2
	1	28	9.3	0.084	0.3	0.2
	2	40	8.8	0.096	0.6	0.3
	3	48	9.0	0.104	0.6	0.3
	4	56	8.9	0.227	0.8	0.3
385	0	12	9.7	0.243	0	0
	1	16	9.5	0.220	0	0
	2	12	9.6	0.311	0	0
	3	16	9.3	0.288	0	0
	4	24	9.1	ND	0	0
386	0	32	9.6	0.102	0	0
	1	32	9.6	0.133	0	0
	2	36	8.9	0.221	0.3	0.2
	3	40	9.3	0.253	0.2	0.2
	4	24	9.5	0.286	0.2	0.2
388	0	12	9.5	0.067	0	0
	1	16	9.4	0.069	0	0
	2	24	9.6	0.129	0	0
	3	16	9.7	0.165	0	0
	4	36	9.5	ND	0	0
390	0	12	9.5	0.273	0	0
	1	40	9.0	0.258	0.3	0.2
	2	44	9.0	0.196	0.3	0.2
	3	68	8.9	0.236	0.4	0.3
	4	52	9.2	ND	0.4	0.3
393	0	44	9.5	0.273	0.2	0.2
	1	44	8.9	0.296	0.4	0.3
	2	68	8.7	0.347	0.8	0.5
	3	52	8.6	0.378	0.9	0.6
	4	68	8.2	0.377	0.9	0.7
ATCC 15468	0				0	0
	1				ND	ND
	2				ND	ND
	3				ND	ND
	4				ND	ND

Mortality is given at the percentage of 25 mice that died after intraperitoneal injection; LD50 is expressed in log₁₀; siderophore production is given as the A₆₃₀ according to the CAS assay method [37].

*Ganglioside GM₁ anti-CT ELISA; values are expressed as CT-equivalent units (ng/ml) estimated from the CT-standard curve; s, Activity of culture supernate; l, Activity of cell lysate.

[†]The original isolates from hospitalised children (faeces samples).

The enhanced values after first passage and the highest values obtained for mortality, siderophore and toxin production and lowest LD50 values after serial passages are shown in **bold**; ND, not done.

Table 3. The effects of lethal passage in mice of clinical isolates of *A. caviae* on the adherence to the rabbit small intestinal surface, presence of fimbriae and cell surface hydrophobicity, in comparison with the lowest value of LD50 and the production of the CTC factor

Hydrophobicity									
Isolate no.	Passage no.	Adherence index (SEM)*	Fimbriae (TEM)	SAT	BATH (percent change in absorbance)		LD50 (log ₁₀)	CTC factor [†] (ng/ml)	
					n-octane	n-hexadecane		s	l
375	0 [‡]	15	—	Moderate	Not hydrophobic	Not hydrophobic	9.5	0	0
	3 [§]	25	—	Not hydrophobic	Not hydrophobic	Not hydrophobic	8.8	0.4	0.2
376	0	31	+	Moderate	Not hydrophobic	Not hydrophobic	9.6	0.25	0.2
	4	7.7	—	Weak	Not hydrophobic	Not hydrophobic	8.8	0.6	0.25
377	0	4.2	+	Weak	Moderate	Not hydrophobic	9.8	0	0
	4	2.8	+	Strong	Not hydrophobic	Not hydrophobic	8.8	0.7	0.3
378	0	10	—	Strong	Not hydrophobic	Not hydrophobic	9.7	0.35	0.2
	3	27	—	Not hydrophobic	Moderate	Not hydrophobic	8.7	0.3	0.2
379	0	2	—	Moderate	Not hydrophobic	Not hydrophobic	9.9	0.35	0.2
	1	22	—	Moderate	Not hydrophobic	Not hydrophobic	8.9	0.2	0.2
380	0	7	—	Moderate	Not hydrophobic	Not hydrophobic	9.3	0.3	0.2
	4	11	—	Weak	Not hydrophobic	Not hydrophobic	8.0	0.8	0.5
383	0	13	—	Weak	Moderate	Moderate	9.3	0	0
	2	22	—	Weak	Not hydrophobic	Not hydrophobic	9.5	0	0
384	0	7	+	Weak	Moderate	Not hydrophobic	9.4	0.2	0.2
	2	4	—	Not hydrophobic	Not hydrophobic	Moderate	8.8	0.6	0.3
385	0	31	—	Weak	Not hydrophobic	Not hydrophobic	9.7	0	0
	3	ND	—	Weak	Not hydrophobic	Not hydrophobic	9.3	0	0
386	0	10	—	Not hydrophobic	Not hydrophobic	Not hydrophobic	9.6	0	0
	2	6	+	Not hydrophobic	Not hydrophobic	Not hydrophobic	8.9	0.3	0.2
388	0	94	—	Not hydrophobic	Not hydrophobic	Not hydrophobic	9.5	0	0
	1	14	—	Not hydrophobic	Moderate	Not hydrophobic	9.4	0	0
390	0	115	—	Strong	Not hydrophobic	Not hydrophobic	9.5	0	0
	3	38	—	Not hydrophobic	Moderate	Not hydrophobic	8.9	0.4	0.3
393	0	7.4	—	Weak	Not hydrophobic	Not hydrophobic	9.5	0.2	0.2
	4	2.4	+	Weak	Not hydrophobic	Not hydrophobic	8.2	0.9	0.7

*Mean of 30 fields.

†CT-equivalent of units (ng/ml) in culture supernate (s) and cell lysate (l) in GM₁ ELISA. ND not done.

‡The original isolates from hospitalised children (faeces samples).

§Passage number. The LD50 value did not decrease after consecutive passages.

Discussion

Data about the role of *A. caviae* as an enteropathogen are conflicting [19]. It has been reported that although strains of *A. sobria* and *A. hydrophila* killed mice within 48 h when injected intraperitoneally, none of 11 *A. caviae* strains tested killed mice [45]. More recent investigations implicate *A. caviae* as an enteropathogen [5, 22, 46–49]. In the present study, all 13 isolates of *A. caviae* produced mortality in mice 24–48 h after injection.

Namdari and Bottone [22] noted that cytotoxin was produced by *A. caviae* in glucose-free TSB × 2 at 16 h because glucose repressed *A. caviae* cytotoxin production. The higher amounts of amino acids present in CYE medium may stimulate toxin production by *A. caviae* strains. Therefore, in the present study CYE was used for growth of *A. caviae* isolates to be used in the mouse model of infection. It has been shown that culture conditions have a decisive influence on the production of toxins by *A. caviae*.

The cytotoxic enterotoxin, CTC factor, was detected in *A. caviae* supernates and cell lysates and confirmed the enterotoxigenicity of the *A. caviae* isolates. This

caused diarrhoea and intestinal fluid accumulation in the mice. Although Shimada *et al.* [50] demonstrated the production of enterotoxin only by *A. hydrophila*, Potomsky *et al.* [51] reported that 24% of *A. caviae* strains reacted with antisera to cholera toxin by ELISA. Cytotoxic enterotoxin from *Aeromonas* spp. isolates caused fluid secretion in *Aeromonas*-associated diarrhoea [52].

Siderophore production has been suggested as one of the factors determining the virulence of a micro-organism [53, 54] and was demonstrated under iron-limiting conditions for all 13 *A. caviae* isolates. However, isolates produced varying amounts of siderophore. Chromosomal genes may control low-level production of siderophores whereas more virulent strains might harbour plasmids encoding for the production of other siderophores [55].

These results indicate that *A. caviae* isolates may employ several strategies for securing host iron, for example, lysis of erythrocytes, digestion of haemoglobin and binding and assimilation of haem.

From this study it was confirmed by SEM that all the *A. caviae* isolates had the ability to adhere to

Fig. 1. Scanning electron micrograph showing adherence of *A. caviae* isolates 379, 383, 385 and 388 to epithelium of rabbit small intestine: (a) isolate 379 (magnification $\times 3250$); (b) isolate 383 ($\times 3250$); (c) isolate 385 ($\times 3250$); (d) isolate 388 ($\times 7000$).

epithelium of rabbit small intestine. Bacterial adherence is mediated by adhesin–receptor interactions. Adhesins are surface-attached bacterial proteins and interaction between adhesins and receptors is an important virulence factor for aeromonads [57]. Extracellular matrix components are one such possible attachment site and outer-membrane proteins are another [3, 58].

The fimbrial adhesins play a role during bacterial attachment to the intestinal mucosa *in vitro* and *in vivo*

[58]. In the present study, only five *A. caviae* isolates were shown to have fimbriae by TEM. Grewal *et al.* [59] have shown *in vitro* that formation of bacterial fimbriae was highly dependent on the presence of bile salts and a growth temperature of 37°C . Thus, the *in vivo* colonisation by bacteria of the human alimentary tract may be facilitated by fimbriae, because these conditions for the synthesis of fimbriae exist in the alimentary tract. A possible cause of the lack of fimbriae in some isolates of the study was the absence of bile salts in the culture medium. The adhesion of

Fig. 1. (Continued).

Table 4. Criteria of hydrophobicity

Test	Values	Results	
SAT	0.0–1.0 M	Strong	Positive test
	1.2–2.0 M	Moderate	Positive test
	2.2–4.0 M	Weak	...
	> 4.0	Not hydrophobic	...
BATH	> 50%	Strong	Positive test
	20–50%	Moderate	Positive test
	< 20%	Not hydrophobic	...

aeromonads to intestinal cells *in vivo* would be considered a crucial early step in the development of gastro-enteritis. Virulent strains of *A. caviae* are adherent to HEp-2 cells [20, 46] and all the 13 *A. caviae* diarrhoeal isolates in the present study adhered to the rabbit small intestinal surface, and various values of adherence index were obtained. Dickson and Koohmaraie [42] reported a similar observation. It

was found that the adherence index did not always increase with passage in mice and an increased adherence index did not always correlate with a decreased LD50 and raised CTC factor production.

Cell surface hydrophobicity has also been identified as an important factor for bacterial attachment to human epithelial cells [42], and in enabling bacteria to interact with animal cells and survive in the host [43]. All isolates tested possessed some degree of hydrophobicity; some showed strong hydrophobicity, whereas others showed moderate or weak hydrophobicity. The SAT method appeared to be suitable for assaying the hydrophobicity of pathogenic *A. caviae* isolates. The BATH n-octane method was more suitable for assaying hydrophobicity than the BATH n-hexadecane method. In the non-fimbriate diarrhoeal isolates examined, hydrophobicity may be the major factor responsible for adherence to epithelial cells.

In conclusion, the data presented in this study indicated that 10 of the 13 diarrhoeal isolates of *A. caviae* produced a factor that was antigenically similar to the cytotoxic enterotoxin of *V. cholerae*. The antigenic similarity of the CTC factor to the cholera toxin, intestinal fluid accumulation, and diarrhoeal symptoms in tested mice after intraperitoneal injection of a suspension of *A. caviae*, support the hypothesis that the CTC factor of *A. caviae* strains is a cytotoxic enterotoxin. These results advance the role of *A. caviae* as a gastrointestinal pathogen in the mouse model. After passage in mice, a correlation was found in the *A. caviae* isolates between the ability to increase production of the CTC factor, percentage mortality and lower LD50 values. The data on biological and clinical characteristics confirmed that *A. caviae* can participate in the pathogenesis of diarrhoea in children.

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