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Toxin production, adherence and protein expression by clinical *Aeromonas* spp. isolates in broth and human pooled ileostomy fluid

M. H. WILCOX*, A. COOK, I. GEARY AND A. ELEY

Department of Experimental and Clinical Microbiology, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX

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SUMMARY

The physiological behaviour of clinical *Aeromonas* spp. isolates was compared following culture in a conventional broth and human pooled ileostomy fluid (PIF). Protein expression was markedly affected by the growth medium, with an overall reduction in whole cell proteins in bacteria grown in ileostomy fluid. In addition, novel outer membrane proteins were produced in PIF but not in broth. The majority of *A. hydrophila* and *A. sobria* isolates produced toxin in both broth and PIF, whereas no cytotoxin positive *A. caviae* were found. Toxin titres were at least two doubling dilutions higher in 40% and 21% of *A. hydrophila* and *A. sobria* isolates, respectively, following culture in brain heart infusion broth compared with PIF. Bacterial adherence to Vero and A-549 cells was significantly more common in *A. hydrophila* (53%) and *A. sobria* (64%) than in *A. caviae* (15%) ($P < 0{\cdot}01$). We observed increased adherence by 6 aeromonas strains previously classified as adherence-positive, but not by 6 non-adherers, in PIF compared with brain heart infusion broth. The influence of growth medium on the expression of potential virulence determinants by *Aeromonas* spp. provides a rationale for the use of human ileostomy fluid in future *in vitro* studies, in order to simulate the nutrient conditions found *in vivo*.

INTRODUCTION

Aeromonas spp. are now widely accepted as causes of gastroenteritis, although there is a pronounced geographic variation in the incidence of infection [1–5]. The three phenospecies *Aeromonas hydrophila*, *Aeromonas sobria* (*A. veonii* biovar sobria) and *Aeromonas caviae* account for more than 80% of all clinical isolates, and it was originally believed that *A. hydrophila* and *A. sobria* were the main causes of diarrhoeal infections [6–9]. However, we and others have noted that *A. caviae* is a significant cause of gastroenteritis in infants, and indeed may be isolated more commonly in this age group than either *A. hydrophila* and *A. sobria*

* Correspondence and current address: Dr M. H. Wilcox, Clinical Microbiology and Public Health Laboratory, Level 6, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QW.

[4, 10–12]. Several studies have examined environmental and clinical aeromonads to determine the prevalence of potential virulence determinants, and in particular toxin production and cell adherence. Consensus findings indicate that toxin production (cytotoxin and enterotoxin) is more frequently expressed *in vitro* by *A. hydrophila* and *A. sobria* (*A. veronii* biovar sobria) than in *A. caviae* [6, 13]. Data from animal models have confirmed that the former species are generally more virulent [9]. However, recent evidence has contradicted these findings, and *A. caviae* has been observed both to produce cytotoxin and enterotoxin and to adhere to a range of cell types [12, 14, 15]. In one report it was noted that the choice of culture medium was critical to the results obtained in toxin assays [12]. The true prevalence of toxin production and cell adherence, which are well accepted virulence determinants in enteropathogens, by *A. caviae* therefore remains uncertain.

We have adopted a novel approach to the study of clinical *Aeromonas* spp., which involves the use of a biological fluid as a culture medium, to determine whether the expression of virulence determinants *in vitro* is more common when incubation conditions are employed which more closely reflect those found *in vivo*. Ala Aldeen and Barer examined the production of cytotoxin by 4 enteropathogen isolates cultured in specimens of human ileostomy fluid [16]. They found that toxin was expressed by *Escherichia coli* and *Vibrio cholerae*, but not by *A. sobria*. We have compared the growth, protein profiles, cytotoxin production and cell adherence of clinical isolates cultured in a conventional laboratory medium with human pooled ileostomy fluid (PIF).

MATERIALS AND METHODS

Bacterial strains

Sixty-one clinical *Aeromonas* spp. isolates were studied. They were recovered from faecal specimens obtained from patients with diarrhoea, submitted to the Bacteriology Departments at the Sheffield Children's and Royal Hallamshire Hospitals. The isolates were speciated by 4 phenotypic methods (conventional biotyping, suicide phenomenon and aesculin production, API 20NE strips, and outer membrane protein profiling), as described elsewhere [17, 18]. They comprised 26 *A. caviae*, 15 *A. hydrophila* and 14 *A. sobria* (*A. veronii* biovar sobria), while 6 isolates could not be speciated by these methods. In addition, toxin production was also examined in a further 17 reference strains which were kindly supplied by M. Altwegg, Institute of Medical Microbiology, Zurich, Switzerland (13 strains representing DNA hybridization groups 1–4, 5A, 5B, 6–12), and by T. Donovan, Public Health Laboratory, Ashford, Kent (4 laboratory reference strains of which 3 produce cytotoxin). Strains were stored at −70 °C until required and were maintained by sub-culture onto horse blood agar.

Ileostomy fluid

Approximately 2 l of ileostomy fluid were collected from each of 10 patients who had had ileostomies created because of inflammatory bowel disease. At the time of collection the patients were in remission and were not taking antibiotics. The fluids were frozen at −20 °C immediately after collection to prevent bacterial

growth. Each ileostomy fluid was tested for antimicrobial activity by filling a well cut in an agar plate seeded with *Staphylococcus aureus* NCTC 6571 and incubating overnight: any positive specimens were then discarded. An equal volume of sterile normal saline was added and mixed well in order to emulsify the semi-solid ileostomy fluid. To sterilize the mixture, pooled fluid was first passed through a large Seitz filter with a FCB grade filter pad (Carlson Ford, Ashton-under-Lyne, England), and then through a second filter containing a sterilizing grade filter. The filtrate was distributed aseptically into 500 ml volumes, and was checked for sterility by inoculation into blood culture bottles (in house) and incubation for 72 h. Sterile filtrate was stored at −20 °C until required and was then thawed at 37 °C. After thawing, 10 ml of filtrate was removed aseptically and the volume of 10 M or 1 M NaOH needed to adjust the pH of the fluid to 7·0 was noted. The volume of NaOH needed to achieve pH 7·0 in the remaining 490 ml was thus determined, and then added to the bottle. A further 10 ml was removed and the pH re-checked. This method was used to minimize the waste of ileostomy fluid as, due to an inability to sterilize the pH probe, the 10 ml aliquots had to be discarded. The resulting fluid was used throughout this study and is referred to as pooled ileostomy fluid (PIF).

Growth in ileostomy fluid and broth

The aerobic growth of 10 aeromonas strains was compared in PIF and brain heart infusion broth (Oxoid, Basingstoke, England) at both 30 °C and 37 °C. The 10 strains were randomly selected, but included representatives of each phenospecies. An initial inoculum equivalent to a concentration of $\sim 10^4$ c.f.u./ml was used, and viable counts were performed by a modified Miles and Misra technique in triplicate after culture for up to 48 h.

Protein profiles

Whole cell and outer membrane protein (OMP) profiles were examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, after culture of 3 of the strains used in the growth studies (one of each phenospecies was randomly selected) in broth and PIF at 37 °C, as described elsewhere [18]. A profile of the proteins which precipitated out of PIF during overnight incubation was obtained by centrifuging 25 ml of sterile fluid and then processing as with the whole cell preparations.

Cytotoxin assay

Vero cells and A-549 cells (a continuous human lung carcinoma line) were grown in 199 and BSC 1 media (Gibco, Middlesex, U.K.), respectively, and were seeded into 96 well polystyrene mictotitre trays. The trays were incubated at 37 °C in air with 5% CO_2 until almost confluent monolayers were obtained, at which point the growth medium was replaced with maintenance medium. Seventy-eight aeromonas strains were inoculated ($\sim 10^6$ c.f.u.) into 10 ml broth and PIF and were cultured at 37 °C for 18 h. Following centrifugation at 3000 ***g*** for 20 min, sterile filtrate was obtained by passing the supernatant through a 0·45 μ filter. Cytotoxin production was assessed by placing 0·1 ml of sterile filtrate into the first well of a row in a seeded microtitre tray. Doubling dilutions in maintenance

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medium were prepared up to 1 in 256, and sterile broth and PIF were included as controls. The trays were incubated overnight at 37 °C in air with 5% CO_2, and cytotoxicity was defined as $\geqslant 50\%$ rounding/detachment of cells. Cytotoxin positivity was defined as a reciprocal titre of $\geqslant 4$, and each strain was examined on at least two separate occasions.

Cell adherence assay

Adherence to Vero and A549 cells of clinical aeromonas isolates was examined using a modification of the method of Carrello and colleagues [19]. Briefly, log phase cultures were prepared by inoculating 0·5 ml of an overnight culture into 10 ml of broth or PIF and incubating for 3 h at 37 °C in air. Cultures were diluted in phosphate buffered saline (PBS) to $\sim 5 \times 10^6$ c.f.u./ml, and then 1 ml of each was added to semi-confluent cell monolayers grown on glass cover slips in Trac bottles (Sterilin, Staffordshire, U.K.). After incubation for 90 min at 37 °C in air with 5% CO_2, non-adherent bacteria were removed by washing 4 times with 2 ml PBS. The cells were fixed in 1 ml of a methanol/acetic acid mixture (3:1) for 5 min, stained with carbol fuchsin, and then mounted onto microscope slides.

The adherence of 61 clinical isolates cultured in broth was compared with Vero and A549 cells in parallel, and 3 cover slips for each strain were examined. Adherence was defined as a ratio of attached to non-attached bacteria of greater than one, having counted at least 100 attached bacteria on each cover slip. In addition, the adherence of 12 aeromonas strains (6 adherers and 6 non-adherers, randomly selected) to Vero cells was compared following culture in broth and PIF in parallel. The strains were coded and were examined in 3 separate experiments each on 3 cover slips, and readings were made by 2 observers. For these experiments, the mean numbers of attached bacteria per cell were calculated after counting at least 10 cells in different fields on each cover slip.

RESULTS

Growth and protein profiles

PIF was able to support the growth of all aeromonas strains examined. Viable counts of bacteria cultured in brain heart infusion broth and PIF were similar (Fig. 1), although for some strains the growth rate was slower at 30 °C than at 37 °C. Incubation for longer than 24 h was associated with reduced cell viability, particularly following culture in PIF. This phenomenon was seen after incubation at 30 °C and/or 37 °C in different strains (data not shown). For these reasons, in further studies strains were cultured at 37 °C overnight. Marked differences were observed in the whole cell and OMP profiles of bacteria cultured in broth compared with PIF (Fig. 2*a* and 2*b*). In general, there was a gross reduction in protein expression in PIF compared with broth, which was most clearly seen in the whole cell protein profiles. In addition, novel proteins were produced in PIF but not in broth. There was evidence that cells bound to some of the proteins present in PIF during incubation. Bands representing proteins of approximately 12 kDa and 33 kDa were present in the profiles of both bacteria cultured in PIF and also in an extract prepared from a precipitate of PIF itself.

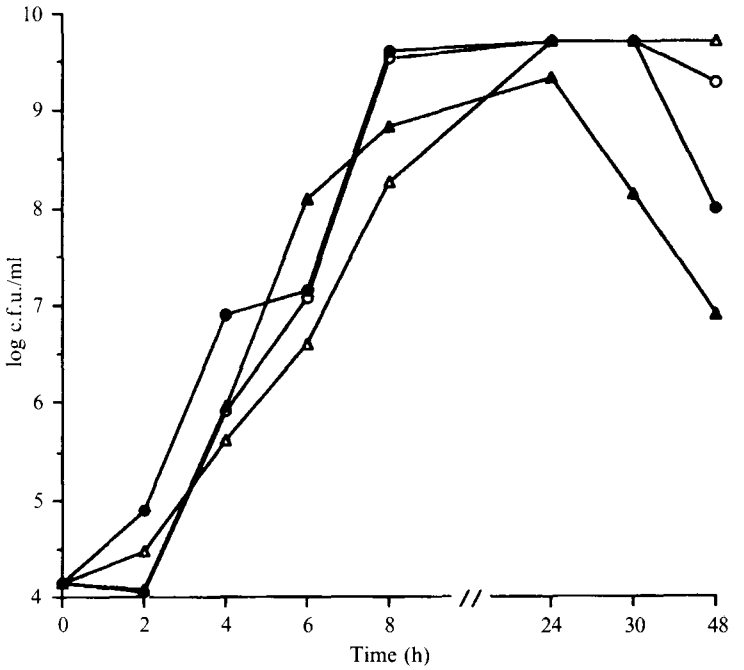

Fig. 1. Comparison of growth profiles of *A. hydrophila* in broth and pooled ileostomy fluid at 30 °C and 37 °C. ○——○, Broth at 30 °C; △——△, PIF at 30 °C; ●——●, broth at 37 °C; △——△, PIF at 37 °C.

Cytotoxin production

When Vero cells were compared with A549 cells, toxin production was detected at 1–2 dilutions higher following culture in both media (data not shown). Also, preliminary studies indicated that the supernatants prepared from bacterial cultures in 50 ml PIF contained toxin at most one dilution higher than when 10 ml PIF was used; to conserve PIF, 10 ml volumes were therefore used for toxin experiments. The results of the cytotoxin assay using Vero cells are shown in Table 1. The majority of *A. hydrophila* and *A. sobria* isolates produced toxin in both broth and PIF, whereas no cytotoxin positive *A. caviae* were found. Median toxin titres produced by *A. sobria* were fourfold higher than *A. hydrophila* in both media. Toxin titres were markedly higher following culture in brain heart infusion broth than in PIF. For example, 40% and 21% of *A. hydrophila* and *A. sobria* isolates respectively, produced at least two doubling dilutions more toxin in broth. The greatest differences in toxin production between the two media were observed in *A. hydrophila*, with 6 strains observed to have titres at least eightfold higher in broth than in PIF (Fig. 3). Five strains produced less than 1:4 toxin in PIF (and so would be classified as non-toxin producers by the definition used in the present study), but were toxin positive following culture in broth. The cytotoxin producing reference strains which we examined were positive in our assay when cultured in broth; the cytotoxin-negative reference strain was negative for cytotoxic activity in this assay. Of the 13 DNA hybridization group (HG)

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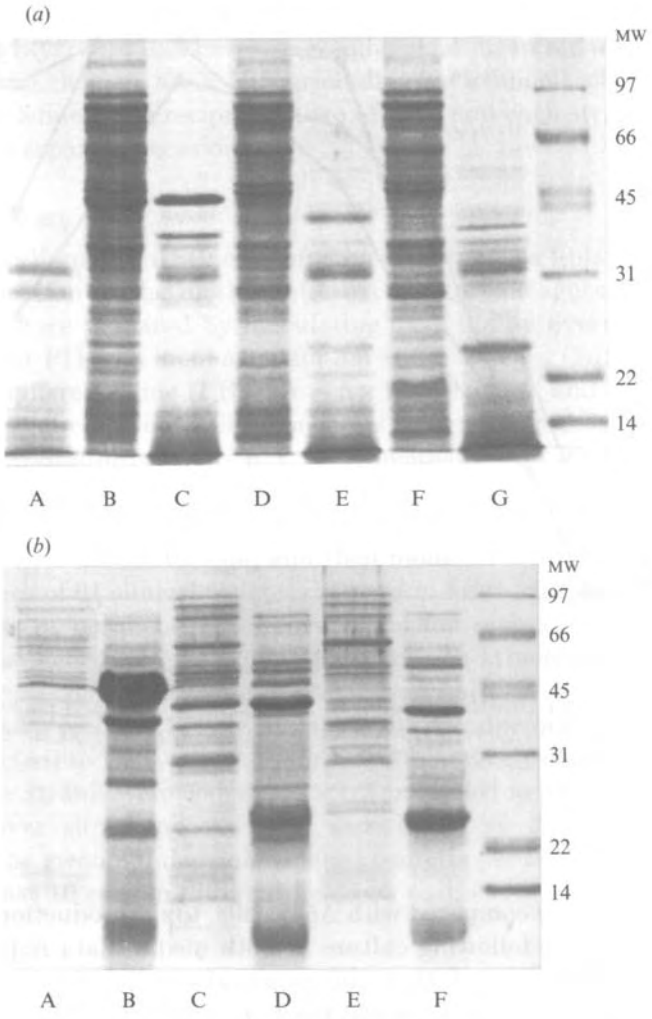

Fig. 2. Whole cell (*a*) and outer membrane (*b*) protein profiles of *A. sobria*, *A. hydrophila* and *A. caviae*, respectively. In (*a*) lane A contains proteins precipitated from ileostomy fluid, lanes B, D and F contain proteins prepared following growth in broth, and lanes C, E and G contain proteins expressed in pooled ileostomy fluid. The far right lane contains molecular weight markers. The order of lanes is the same in (*b*), except that the proteins precipitated from ileostomy fluid are omitted.

reference strains, 2 (HG 2 and HG3) produced significantly increased amounts of cytotoxin in broth compared with PIF (Fig. 3). The remaining HG reference strains did not produce cytotoxin in either broth of PIF, with the following exceptions: HG 1, HG 9 and HG 10 produced 1:64, 1:64 and 1:16 cytotoxin in broth, respectively; the corresponding figures in PIF were 1:32, 1:64 and 1:8.

Bacterial adherence

Twenty-three out of 61 (38%) *Aeromonas* spp. isolates were adherence-positive following culture in broth. Twenty-one of these adhered to A-549 cells, 16 to Vero cells, and 14 adhered to both cell types. Significantly fewer *A. caviae* (4 strains,

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Table 1. *Results of cytotoxin assay, using Vero cells, according to species type following culture in broth and ileostomy fluid. Results are shown for the 55 clinical isolates for which a definite phenospecies was known. Titres are expressed as reciprocal values. Toxin positive is defined as titre* $\geqslant 4$. *Titres in ileostomy fluid are corrected values (two doubling dilutions) to allow for cytotoxicity of sterile ileostomy fluid*

Species (*n*)	Broth			Ileostomy fluid			% toxin titre $\geqslant$ 4-fold higher in broth
	Median titre	Range of titres	% toxin positive	Median titre	Range of titres	% toxin positive	
A. hydrophila (15)	4	0–256	60	2	0–32	47	40
A. sobria (14)	16	2–64	79	8	0–32	71	21
A. caviae (26)	2	0–2	0	0	0–2	0	0

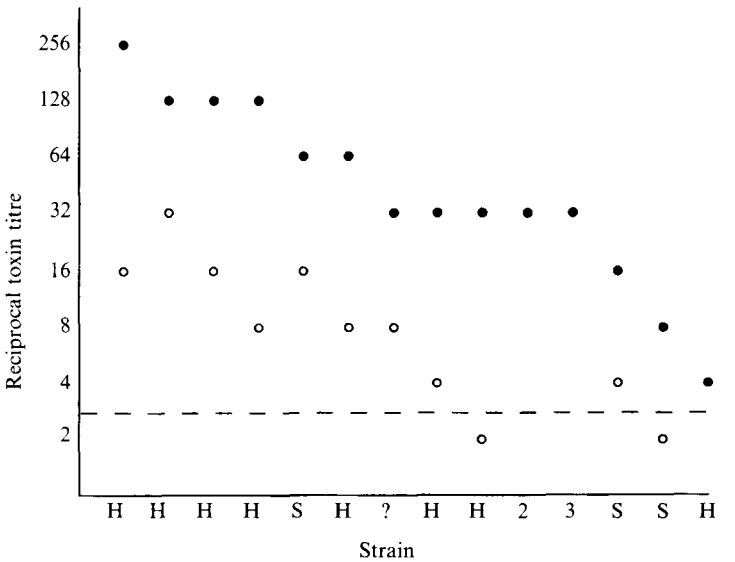

Fig. 3. Strains producing at least fourfold more cytotoxin following culture in broth compared with ileostomy fluid. Letters on the x-axis indicate the *Aeromonas* spp. identity: *A. hydrophila* (H); *A. sobria* (S); DNA hybridization group reference strains (2, 3); and one unidentifiable strain (?). One *A. hydrophila* (far right) and reference strains 2 and 3 produced no cytopathic effect after culture in ileostomy fluid. The dashed line represents the cut-off point for strains to be considered as toxin positive (titre $\geqslant 4$). ●, Broth; ○, ileostomy fluid.

15%) were adherent compared with *A. hydrophila* (8 strains, 53%) and *A. sobria* (9 strains, 64%) (χ^2 9·6, $P < 0{\cdot}01$). There was no significant association between cytotoxin production and adherence.

Each of the 6 adherence-positive strains examined bound in higher numbers to Vero cells following culture in PIF compared with broth (Table 2). For the 6 non-adherent strains, the average number of bacteria observed bound to Vero cells was approximately 2 or less, and no differences were detectable following culture in the 2 media. The differences in adherence between broth and ileostomy-grown

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Table 2. *Comparison of numbers of bacteria adhering to Vero cells following culture in broth and ileostomy fluid. Results are expressed as means (standard errors of means) of values derived from three separate experiments*

Strain	Mean no. of adherent bacteria/cell (SEM)		% increase in adherent cells in fluid
	Broth	Ileostomy fluid	
A. caviae S18	7·8 (1·3)	8·2 (0·9)	5
A. hydrophila S43	6·5 (1·5)	8·3 (0·8)	28
A. sobria S22	7·2 (1·4)	10·1 (1·6)	40
A. hydrophila S32	7·5 (1·1)	13·5 (2·8)	80
A. hydrophila M15	2·0 (0·3)	5·8 (0·7)	190
A. hydrophila F14	3·7 (0·3)	13·2 (1·8)	258

bacteria were consistently recorded by the 2 observers, each of whom was blinded to the identity of the strains.

DISCUSSION

There is now good evidence that the *in vitro* use of biological fluids as culture media provides valuable models of *in vivo* conditions. The expression of iron-repressible OMPs by coagulase-negative staphylococci grown *in vitro* in pooled human peritoneal dialysate closely resembled that observed in bacteria recovered from peritoneal chamber implants in an animal model [20–22]. Also, parent and mutant staphylococcal strains, which were found to differ markedly in their abilities to accumulate on surfaces when examined *in vitro* in conventional complex growth media, were indistinguishable in repeat experiments using pooled human serum. The virulence of these 2 strains was identical in an animal endocarditis model [23]. It is apparent, therefore, that the behaviour of bacteria grown in human body fluids is significantly altered when compared with that observed in conventional complex media. In this study we have demonstrated marked differences in the physiology of *Aeromonas* spp. cultured in broth compared with PIF.

In order to overcome the problem of marked variations in bacterial behaviour between different ileostomy fluid specimens noted elsewhere, we used a pooled fluid [16]. It was not possible to filter the ileostomy fluid, as a first step; therefore an equal volume of normal saline was added to emulsify particulate matter. The pH of collected ileostomy fluid specimens was approximately 5·0–5·5, and such acidic conditions were found to be bactericidal in preliminary experiments (data not shown). It has been shown previously that *A. caviae* in particular are killed when media become acidic during incubation, due to bacterial fermentation of sugars [17]. Hence, the pH of the fluid was adjusted to 7·0 at which point all tested strains were able to grow. pH adjustment by addition of sodium bicarbonate, as opposed to sodium hydroxide, caused marked precipitation, presumably of insoluble metallic bicarbonates. All examined *Aeromonas* spp. achieved similar growth yields in PIF compared with brain heart infusion broth, although reduced cell viability was observed in the former medium after extended incubation periods. The whole cell protein profiles of PIF grown bacteria indicated grossly reduced expression of proteins compared with broth cultured cells. Such protein

repression has been noted after bacterial culture in other human body fluids and is believed to reflect nutrient limitation [20, 21, 24]. Several novel proteins were expressed in bacteria cultured in PIF; for example, protein bands of 24 kDa and 36 kDa were seen in the OMP profiles of *A. caviae* cultured in PIF but not in broth (Fig. 2*a*, lanes F and G). OMPs have been described in both Gram-positive and Gram-negative bacteria which are produced in response to specific nutrient limitations, such as iron for example [20, 21, 24]. Also, it was apparent that ileostomy fluid-derived proteins, of approximate molecular weights 12 kDa and 33 kDa, bound to the bacteria during incubation.

We found that cytotoxin production was common in *A. hydrophila* (60%) and *A. sobria* (79%), but absent in *A. caviae*, as noted elsewhere [6, 7, 15]. Toxin production was reduced in 40% and 21% of *A. hydrophila* and *A. sobria*, respectively, in PIF compared with broth, and some strains were toxin-negative in PIF but toxin-positive in broth. It would be interesting to examine whether such strains are more prevalent in environmental *Aeromonas* spp. and in strains recovered from asymptomatic patients, than in those recovered from patients with gastroenteritis. It is puzzling why cytotoxin production is frequently found in *A. hydrophila* and *A. sobria*, but not in *A. caviae*, despite good evidence that the latter are enteropathogenic [4, 10–12]. Namdari and Bottone observed that cytotoxin production by *Aeromonas* spp. was both time and culture medium dependent [12]. In particular, *A. caviae* produced toxins in glucose-free, double strength tryptone soya broth (TSB), but not in single strength TSB nor in double strength TSB with glucose; toxin was produced only after incubation for at least 16 h. Enterotoxin activity by *A. caviae* was also demonstrated by these authors [12]. However, the inability of *A. caviae* to produce cytotoxin in PIF, particularly considering that we have examined clinically significant isolates, raises doubts as to the role of such toxins as virulence markers.

Grey and Kirov found that although the numbers of adherent aeromonas bacterial cells were medium dependent (only 2 conventional media were examined), the proportion of adhesive strains within a collection was minimally affected by the choice of medium [15]. Similarly, we observed increased adherence by 6 aeromonas strains previously classified as adherence-positive, but not by 6 non-adherers, in PIF compared with brain heart infusion broth. We were careful to exclude observer bias by the use of coded multiple repeats. Our inability to demonstrate medium dependence for the adherence of the latter group may reflect a lack of sensitivity of the assay, although we believe we would have detected increases of the magnitude seen in the adherence-positive strains (up to 2–2·5 fold). Nevertheless, the number of strains examined in broth and PIF in parallel in the present study remains small, and further studies are underway to confirm our findings. Given the marked changes in envelope proteins noted in this study in bacteria cultured in PIF compared with broth, it is perhaps not surprising to observe differences in the adherence of *Aeromonas* spp. grown in these two emdia. The large range of increases in adherence by PIF grown *Aeromonas* spp. that we recorded may reflect the differential expression of one or more protein adhesins. Interestingly, Ho and colleagues reported that of 2 pili in *A. hydrophila* one was constitutively expressed, but the other was environmentally regulated; pilus expression was particularly observed in bacteria grown at 22 °C, in liquid media

and under iron-regulated conditions [25]. It is of general concern that studies of virulence determinants may be hampered by changes in bacterial expression during repeated sub-culture, and it is seldom practical to exclude this possibility.

We compared Vero and A-549 cells, which are derived from monkey kidney and human epithelium respectively, in the present study [26]. We have experience of the successful use of both of these cell lines in the routine bacteriology laboratory to assay for cytotoxin production by *Clostridium difficile* in faeces. Vero cells have been used elsewhere to assay for cytotoxin production by *Aeromonas* spp., but we are unaware of reports of A-549 cells in this context [7, 14]. For our studies of the applicability of PIF as a culture medium to demonstrate expression of virulence determinants, we wanted to examine the relationship between adherence and toxin production using a single cell line, in order to exclude an extra variable. Although we observed some increased adherence to A-549 cells compared with Vero cells in early experiments, we opted to use the latter in adherence studies with PIF grown bacteria. This was because we encountered repeated problems in obtaining semi-confluent cell layers with A-549 cells after a uniform period of incubation, leading to the postponement of several experiments. In addition, Vero cells were uniformly more susceptible to aeromonas toxins than A-549 cells. Vero cells are not the most appropriate choice of cell line for *in vitro* experiments which seek to simulate conditions found *in vivo*. However, previous studies of virulence determinants in *Aeromonas* spp. have used a variety of cell lines, and to date there is no consensus on the most appropriate choice [7, 14, 15]. Human-derived epithelial cell lines such as A-549, HEp-2 or INT 407 cells would complement the use of human gut fluids as culture media, and we advocate that they are employed in future studies. We appreciate that the modifications which were made to the collected ileostomy fluid, and the fact that intestinal pathogens exist primarily in the environment of the brush border where they interact with other bacteria, means that batch culture in PIF is a less than ideal substitute for the conditions found *in vivo*. We were unable to demonstrate differences in the expression of presently recognized virulence determinants by *A. caviae* in PIF compared with conventional broth media. However, the alterations in bacterial physiology which were observed in PIF compared with broth support a place for this kind of experimental approach in the future study of virulence determinants in infection.

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