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Adherence to HEp-2 cells and enteropathogenic potential of *Aeromonas* spp.

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SUMMARY

Aeromonas strains (total = 60) of clinical, water and food origin were tested for adherence to HEp-2 cells. Environmental strains were selected (except for *A. caviae*) to include primarily those expressing other virulence-associated properties. Adhesion was markedly species-dependent (*A. veronii* biotype sobria, 15 of 26 [58%], *A. caviae*, 4 of 12 [33%] and *A. hydrophila*, 2 of 8 [11%]). *A. veronii* biotype sobria were adhesive, irrespective of source (62 and 54% for clinical and environmental strains, respectively). Adherent strains of this species were enterotoxin-positive and most (13 of 15) grew at 43 °C. *A. caviae* isolated from clinical specimens contained a higher proportion (75%) of adherent strains than environmental strains (13%). Virulent subsets of *A. veronii* biotype sobria and *A. caviae* are adherent to HEp-2 cells. The HEp-2 assay is a useful model for investigating mechanisms of adherence and enteropathogenicity of virulent *Aeromonas* species.

INTRODUCTION

Aeromonas species are now recognized as important human enteropathogens. They are found in most aquatic environments and a wide range of foods [1, 2]. Only some strains from the great variety of environmental strains are able to infect the human gastrointestinal tract. They belong primarily to three main genetic hybridization groups corresponding to the phenotypic species *A. veronii* biotype sobria (formerly *A. sobria*), *A. hydrophila* and *A. caviae* [3]. Gastroenteritis-associated strains usually possess virulence-associated properties, such as the ability to produce enterotoxin(s), cytotoxin(s), haemolysins and an array of proteases, and/or invasive ability [1, 4, 5]. However, not all strains with these properties may be virulent. For the effective delivery of toxins or invasion, they must also be able to adhere to the intestinal mucosa. The different clinical manifestations of aeromonas-associated gastroenteritis probably result from various combinations of virulence factors. Aeromonas-associated secretory diarrhoea is likely to follow colonization of the small intestine by enterotoxin-producing strains [4, 6].

Little is known about aeromonas colonization factors, the receptors they recognize or the relative contributions of filamentous and outer membrane adhesins [7–9]. Adhesive ability to the HEp-2 cell line (human, laryngeal epithelial

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cell carcinoma) has proved a useful *in vitro* model of adhesion to intestinal cells for a number of bacterial enteropathogens, such as *Escherichia coli* and *Yersinia*, *Edwardsiella*, *Campylobacter*, *Salmonella* and *Shigella* species. Although it cannot be assumed that tissue culture cells possess the same surface receptors for bacterial adherence as those found on human intestinal cells *in vivo*, strong positive correlations have been found between adhesion *in vitro* and virulence for these bacterial enteropathogens [10–16].

There are few studies of aeromonas adhesion to HEp-2 cells. Carrello and others showed that a greater proportion of faecal strains (12 of 34, 34%) adhered to the cell line compared to randomly selected water strains (0 of 29, 0%) [17]. A preliminary report suggests that the pattern of species adhering to HEp-2 cells parallels adhesion to formalized human ileal tissue [18]. Moreover, Nishikawa and colleagues showed that strains which were highly adherent to a human embryonic intestinal cell line (INT407) were also adherent to HEp-2 cells [19]. Namdari and Bottone reported that *A. caviae* strains, isolated as the sole potential enteric pathogen from the faeces of children with watery diarrhoea, were adhesive to HEp-2 cells (8 of 14, 57% > 10 bacteria per cell) [20]. Overall, these studies suggest that the HEp-2 cell adhesion assay may be useful for the detection and investigation of at least some enteropathogenic aeromonas. To confirm this, *Aeromonas* strains from clinical specimens, predominantly diarrhoeal faeces, and selected water and food strains, which in the main expressed other putative virulence factors, were examined for their ability to adhere to HEp-2 cells.

MATERIALS AND METHODS

Bacterial strains

Aeromonas strains were obtained from Tasmania ($n = 35$) and mainland Australia ($n = 24$) [21, 22]. One clinical strain (AH6) was from the USA [23]. Strains were of clinical (80% diarrhoeal faeces), water and food origin. They were maintained in minimal maintenance media (MMM) at room temperature. All strains were speciated using the API 20E system (Analytab Products, La Balme–Les Grottes, Montalieu-Vercieu, France) and additional biochemical tests, as previously described [22]. Classification of strains into the species, *A. veronii* biotype sobria (*A. sobria*) (S), *A. hydrophila* (H), and *A. caviae* (C) was based on the criteria of Popoff [3, 24]. Strains differing in more than two tests from the typing scheme were not classified (NC).

Exotoxin assays

The ability of strains to produce enterotoxin (Ent), haemolysin (Hly) and cytotoxin (Cyt) was determined by assaying overnight cell-free tryptone soya broth plus yeast extract (Oxoid, Basingstoke, England) (TSB+YE) culture supernatants in the suckling mouse assay, in a titration *v.* rabbit erythrocytes, and for their effects on Vero cell cultures, respectively, as described elsewhere [22, 25].

Growth at 43 °C

Cultures were streaked from turbid TSB+YE broth onto blood ampicillin agar (30 mg l^{-1}) (BAA) (Becton Dickinson, Brisbane, Australia) and incubated at

43 °C. Growth was examined after 36 h and was scored from 0 (no growth) to 4+ (heavy growth).

Adhesion assay

Strains for the adhesion assay were sub-cultured onto solid media, BAA, brain heart infusion agar (BHIA) (Oxoid), or tryptone soya agar (TSA) (Oxoid) and incubated at 37 °C for 20–24 h. Colonies were diluted to a final suspension of $\sim 5 \times 10^5$ c.f.u./ml in Eagle Minimal Essential Medium containing 1% fetal bovine (calf) serum (MEM+FCS) (Cytosystems, Castle Hill, Australia). Semi-confluent monolayers of HEp-2 cells were grown for 20 h on 12 mm glass cover-slips (Vitromed, Basel, Switzerland) in 24-well plates (Corning Glass Works, Corning, New York, USA). These were washed twice with ~ 1 ml Hanks balanced salt solution (HBSS), before the addition of bacterial cells. Strains under test were inoculated (1 ml) into triplicate wells, containing HEp-2 cells on cover-slips (approximately 5–10 bacteria per HEp-2 cell). A positive control strain was added, in duplicate only, and the one remaining well received MEM+1% FCS (1 ml) containing no bacteria, as a negative control. After incubation (90 min, 37 °C, 5% CO_2), non-adherent bacteria were removed by washing four times with HBSS (1–2 ml). The monolayers were fixed with 3:1 methanol:acetic acid (~ 1 ml, 5 min) and stained in the wells with May–Grunwald and Giemsa stains (Gibco, Grand Island, New York, USA). The cover-slips were mounted on glass slides, using DePex mounting medium (BDH Gurr, Poole, England).

Adhesion was assessed by bright-field microscopy. For each monolayer, 25 infected cells were chosen at random and the number of cell-associated bacteria counted. The proportion of a further 100 cells per monolayer which were infected was determined. Each strain thus had a total of 75 counts of infected cells, from which a mean and standard error of the mean (SEM) were calculated. The resultant mean number of bacteria per infected cell was multiplied by the average of the three proportions, yielding the average number of bacteria per HEp-2 cell overall. A strain with a score of zero to one bacteria per cell was considered 'non-adhesive'. Strains with scores of one to 10 bacteria per cell were 'low adherers': > 10 to 20 bacteria per cell the strains were 'adherent'; > 20 bacteria per cell were defined as 'highly adherent'.

The assay could be modified to reduce incubation time with no significant loss of adhesion, as follows. After addition of the bacterial suspension, the plate was centrifuged (150 g, 10 min) (Sorvall RT6000, Newtown, Connecticut, USA) and the assay incubation time reduced to 50 min. The monolayers were then washed, fixed, stained and mounted as usual.

Statistical analysis

The significance of variation in adhesive ability between groups of strains, was determined using χ^2-test.

RESULTS

Source, speciation and virulence properties of strains tested

Details of the source and speciation of the strains tested for adhesion are shown in Table 1. The *A. veronii* biotype sobria and *A. hydrophila* strains were selected particularly for their expression of virulence factors, such as the ability to produce

Table 1. *Source and species of* Aeromonas *strains tested for adhesion*

Source (No. of strains)	Species*			
	veronii biotype sobria	*hydrophila*	*caviae*	NC†
Clinical (30)	12	11	4	3
Water (17)	7	4	5	1
Food (11)	6	2	3	—
Unknown (2)	1	1	—	—
Total 60	26	18	12	4

* Includes atypical strains (differing by one taxonomic test).
† NC, not classifiable strains (differing by two or more tests).

Table 2. *Virulence properties of strains tested for adhesion*

Species	Exotoxin ⩾ 2*		Growth 43 °C†	
	No.	(%)	No.	(%)
veronii biotype sobria	23	(88·5)	23	(88·5)
hydrophila	13	(72·2)	11	(61·1)
caviae	0	(0)	4	(25)
NC	3	(75)	4	(100)
Total (60)	49	(81·7)	42	(70)

* Enterotoxin, positive suckling mouse assay; Haemolysin, titre ⩾ 16; Cytotoxin, ⩾ 50% of Vero cells affected.
† Moderate (2+) growth at 36 h.

two or more exotoxins and grow at high temperature (43 °C), as shown in Table 2.

HEp-2 cell adhesion assay

Initially strains to be tested for adhesion were cultured on BAA (37 °C, 20 h). However, the number of adherent bacteria per cell significantly increased when strains were cultured on TSA or BHIA. Seventeen of 22 strains tested in a comparative experiment showed higher adhesion following growth on BHIA. Several strains were assayed repeatedly on BAA and BHIA and the increase in adhesive ability was consistent. In all the experiments reported below, strains were assayed following growth on BHIA. All strains were tested on at least two occasions. Variation in adhesive ability occurred between experiments, but it was rare for a strain to vary beyond the limits of its adhesion category (i.e. non-adherent, poorly adherent, adherent or highly adherent). Adherent strains showed clear binding to the HEp-2 cell cytoplasm above any background binding to the glass cover-slips (Fig. 1).

Some strains produced cytotoxic effects on the cell line. However, the bacterial inoculum and time of incubation meant these were minimal and did not interfere with the assay for the vast majority of strains. Only 3 of the 60 strains produced cytotoxic effects severe enough to warrant modification of the assay to shorten the length of time the cells were exposed to bacteria, as outlined in the Materials and

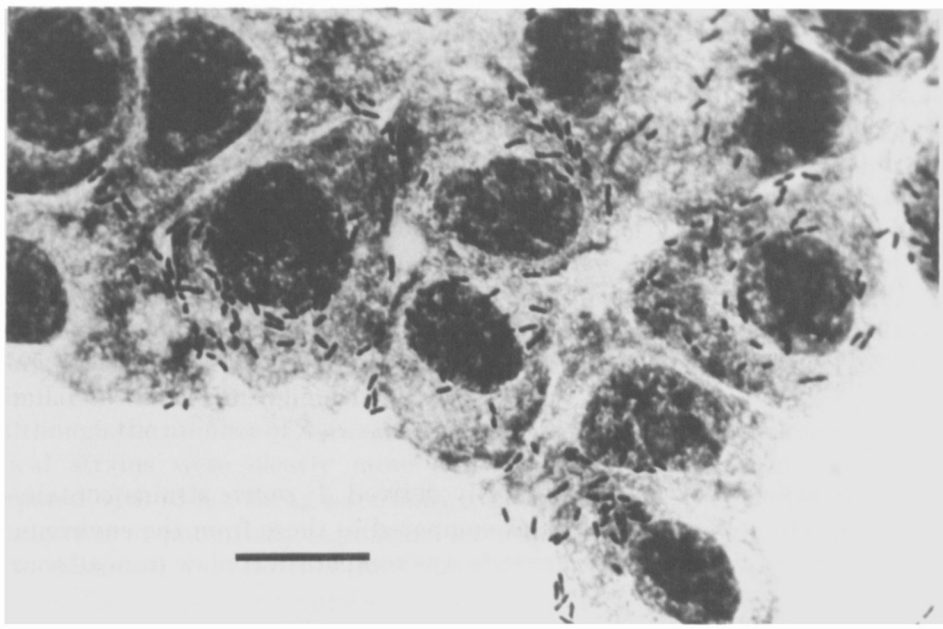

Fig. 1. Adhesion of *Aeromonas veronii* biotype sobria (faecal strain) to HEp-2 cells. Bar = 10 μm.

Table 3. *Adhesion in relation to species and source*

Species	Source*	No. tested	Adherent strains† No.	Adherent strains† %
veronii	C	13	8	61·5
biotype sobria	F+W	13	7	53·8
hydrophila	C	12	1	8·3
	F+W	6	1	16·6
caviae	C	4	3	75
	F+W	8	1	12·5
Total		60	21	35

* C, clinical strains; F+W, food and water strains.
† $\geqslant$ 10 bacteria per HEp-2 cell.

Methods section. Thus, all strains, despite selection in favour of cytotoxin production, were able to be evaluated for adhesive ability to the cell line.

Adhesion in relation to source and species

A similar proportion (40 and 41%, respectively) of all the clinical and water strains were adherent. Those from food included fewer adhesive strains (18%). *A. veronii* biotype sobria contained the highest proportion (15 of 26, 58%) of adhesive strains, including all the highly adhesive strains (> 20 bacteria per cell). Four (33%) of the 12 *A. caviae*, only 2 (11%) of the 18 *A. hydrophila* and none of the non-classified strains were adherent. The proportion of adherent *A. veronii* biotype sobria was significantly different to the proportion of adherent *A. hydrophila* ($P < 0{\cdot}005$). Table 3 summarizes the results of adhesion in relation to species and source of strains. A majority of *A. veronii* biotype sobria strains were

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Table 4. *Adhesion in relation to virulence properties*

Species* (No. of adherent strains)		Exotoxins†			Growth‡ 43 °C
		Ent+	Cyt+	Hly+	
A. veronii biotype sobria	(15)	15	10	11	13
A. hydrophila	(2)	0	2	0	1
A. caviae	(4)	0	1	0	2
Total	21	15	12	11	16

* ⩾ 10 bacteria per HEp-2 cell.

† Enterotoxin, positive suckling mouse assay; Haemolysin, titre ⩾ 16; Cytotoxin, ⩾ 50% of Vero cells affected.

‡ Moderate (2+) growth at 36 h.

adhesive irrespective of source. Clinically derived *A. caviae* strains contained a greater proportion of adhesive strains compared to those from the environment. The proportion of adherent *A. hydrophila* was comparatively low from all sources.

Adhesion in relation to virulence factors

Table 4 shows the virulence properties of the 21 adhesive strains. The relatively few adherent *A. hydrophila* and *A. caviae* were essentially negative for virulence-associated factors under the conditions of assay, compared with the high proportion of *A. veronii* biotype sobria which were able to produce two or more exotoxins and grow at 43 °C.

DISCUSSION

This study has demonstrated that, as for other enteropathogenic bacteria, adhesion of *Aeromonas* strains to HEp-2 cells is probably also strongly correlated with ability to cause diarrhoea.

A. veronii biotype sobria and faecal *A. caviae* comprised the majority (90%) of adherent strains. The association of high-level adhesion with these species confirms the findings of Carrello and colleagues with faecal *Aeromonas* strains [17], and parallels the few studies of aeromonas binding to an intestinal cell line and human ileal tissue [18, 19]. The overall proportions of adhesive strains in each species found in our study are remarkably similar to those of Carrello and colleagues, despite varying conditions of assay. They tested young (3·5 h, 37 °C) tryptone soya broth cultures. This similarity, our observations with the different solid media (BAA and BHIA) and the low levels of background adhesion, suggest that although the efficiency of adhesion may be increased by varying culture conditions, there would be little change in the overall proportion of adhesive strains.

Enterotoxigenic *A. veronii* biotype sobria is the most commonly implicated *Aeromonas* species in diarrhoeal stools in Tasmania and elsewhere [22, 26, 27]. Evidence suggests that it may be the most virulent. It is has been found most lethal in mouse pathogenicity studies [28, 29], and is more often invasive in tissue

culture assays than the other species [30–32]. Our finding that > 60% of strains of this species from symptomatic patients were adherent, or highly adherent, to HEp-2 cells and that virtually all these adherent strains expressed two or more other virulence-associated factors, further identifies this species as an important enteropathogenic subset. Similar strains are present in local waters and foods, though in much lower proportions overall than among the clinical strains. We found *A. veronii* biotype sobria comprised < 15% of Tasmanian water isolates [22]. It is rare, or usually found as the minority *Aeromonas* species, in foods [2, 33, 34]. Adherent strains of this species are present in food and water. This study has shown that when environmental strains are selected by known virulence criteria, rather than being chosen at random, the proportion of adherent strains is similar to that of the clinical strains.

Although the number of *A. caviae* strains tested in this study was small ($n = 12$), clinical strains were clearly more adhesive than environmental ones (75% compared with 13%). Being essentially exotoxin-negative in our standard assays, no selection of potentially significant environmental strains of this species was made. Namdari and Bottone have presented evidence supporting the role of *A. caviae* as a paediatric enteric pathogen [20, 35]. Our finding confirms their observation that strains from diarrhoeal faeces are adhesive to HEp-2 cells. These strains may produce cytotoxins under certain conditions, but even if most do not elaborate toxins, there remains the possibility of a mode of pathogenesis similar to those of *E. coli* enteropathogenic and enteroadherent strains [36].

Enterotoxigenic *A. hydrophila* did not adhere well to HEp-2 cells. They may also be less adhesive *in vivo*, as the other studies have suggested [18, 19]. This may account for the apparent lower virulence of this species. However, in all studies some adhesive *A. hydrophila* strains were found.

Carrello and co-workers have suggested that adherence to HEp-2 cells is pilus-mediated. Removal of pili of long, flexible morphology, by shearing or trypsinization, resulted in a marked decrease (> 70%) in adhesive ability (three strains) [17]. Hokama and Iwanaga have recently purified and characterized pili of similar morphology isolated from an '*A. sobria*' (Ae1) strain and showed them to be a colonization factor of Ae1. Antibody against this pilus reacted with 49% of '*A. sobria*' strains (clinical and environmental combined), or 69% of strains of clinical origin, but only 13% of *A. hydrophila* (all sources), 16% of clinical. The distribution of this adhesive pilus is similar to the proportions of HEp-2 adhesive strains found in this study (58 and 11% respectively, for the selected strains of these species) [8].

We conclude that the HEp-2 assay is a useful model for the investigation of aeromonas enteropathogenicity. The assay is relatively easy to perform and could aid further investigations into the adhesive mechanisms of *Aeromonas* sp., particularly the role of pilus-adhesins.

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