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The incidence of virulence factors in mesophilic *Aeromonas* species isolated from farm animals and their environment

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SUMMARY

Sixty-one isolates of *Aeromonas* spp. from the faeces of pigs, cows and a variety of associated environmental sources were examined for the characteristics that are reputed to have roles in pathogenicity. Most isolates of *Aeromonas hydrophila* were cytotoxic (96·4 %) and were capable of producing cell elongation factor (75 %) and haemagglutinins (67·9 %). In contrast few of the *Aeromonas caviae* isolates produced these three markers (13·6 %, 27·3 % and 36·4 % respectively). In general, *Aeromonas sobria* occupied an intermediate position (36·4 %, 27·3 % and 54·5 %), but they did produce the highest mean invasion index for HEp-2 cells. Statistical analysis revealed significant associations between the carriage of these factors and it was clear that many isolates of aeromonads from water and animals possessed the full battery of putative virulence factors.

INTRODUCTION

The mesophilic *Aeromonas* spp. have been implicated as causative agents of human gastro-enteritis. Epidemiological studies have suggested their involvement in food and water-borne outbreaks producing conditions varying in severity from mild diarrhoea to life-threatening cholera-like illness [1]. Laboratory investigations have confirmed their ability to attach to intestinal epithelial cells [2–4]. Sanyla, Singh and Sen [5] reported that some strains of *Aeromonas hydrophila* were enterotoxigenic in ligated rabbit ileal loops. Current evidence suggests that there may be at least three mechanisms by which *Aeromonas* spp. could produce this enteropathogenic effect. Ljungh, Popoff and Wadström [6] reported a cytotonic enterotoxin which did not cross-react with cholera toxin: a heat-labile cytotonic enterotoxin immunologically similar to cholera toxin has been identified [7, 8] and a cytotoxic haemolysin with enterotoxic activity has been observed [9, 10]. *In vitro* experiments with HEp-2 cells have also indicated that certain strains of *A. hydrophila* and *A. sobria* are invasive [11, 12]. The demonstration of all these

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virulence factors thus supports the epidemiological evidence that these aeromonads may be human enteropathogens.

We have recently reported on the incidence of mesophilic aeromonads in the faeces of cows and pigs and in the agricultural environment [13]. As the reservoir of aeromonads in healthy livestock and their environment may represent a source of human infection it was decided to examine these isolates for the virulence factors thought to be involved in human enteropathogenesis. We also investigated the inter-relationship between these virulence markers and their association with other phenotypic characters.

MATERIAL AND METHODS

Bacterial isolates

Sixty-one isolates of *Aeromonas* spp. from pig and cow faeces and a variety of associated environmental sources were examined for virulence characters. The isolates comprised *A. sobria* [11], *A. hydrophila* [28], *A. caviae* [22] plus three control aeromonads, *A. hydrophila* AH2 (courtesy of Dr T. Chakraborty) used by Chakraborty and colleagues [14] to demonstrate cytotonic enterotoxin (elongation factor) in Chinese hamster ovary cells (CHO-KI), *A. hydrophila* V6053, cytotoxic against Vero cells and enterotoxic in animal models and *A. caviae* V6065, non-cytotoxic in Vero cells (both courtesy of Dr T. J. Donovan). Isolates were maintained at −70 °C in tryptone soya broth with 15% (w/v) glycerol or 'Protect Bacterial Preserver' system (Technical Service Consultants Limited) until required.

Virulence markers

Cytotoxin and cytotonic enterotoxin production by isolates was detected using CHO-KI monolayers in microtitre trays. The method employed to seed the trays was based on that of Giugliano, Mann and Draser [15]. Preformed monolayers were preferred to the freshly seeded 1200 cells/well method employed by Chakraborty and colleagues [14] as preliminary experimentation demonstrated that cells in scantily seeded cultures exhibited an innate tendency to elongation. A cell suspension containing 2×10^5 cells/ml was prepared in single strength Ham-F12 medium (Flow Laboratories Limited) supplemented with penicillin (200 units/ml), streptomycin (100 μg/ml), glutamine (0·29 mg/ml), 10% (w/v) foetal calf serum (Gibco) and $NaHCO_3$ (0·11% w/v) with phenol red (0·001%) as indicator. Sterile flat-bottomed lidded microtitre trays, 96 wells (NUNC), were seeded with 100 μl of the cell suspension (2×10^4 cells) per well. Trays were incubated at 37 °C in a humidified atmosphere of air and 5% CO_2 for 24 h. Trays were checked microscopically for monolayer formation and then inverted to remove growth medium which was replaced with 100 μl 199 maintenance medium (Wellcome) supplemented as for Ham-F12 medium but using 1% foetal calf serum.

Preparation of culture filtrates for toxin assays

Test organisms were grown as overnight shake cultures in tryptone soya broth supplemented with 0·6% (w/v) yeast extract and 3% (w/v) casamino acids.

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Culture supernatants were prepared and assayed for cytotoxic and cytotonic effects by the method of Chakraborty and colleagues [14].

Unheated and heated filtrates were assayed in parallel in the same microtitre tray. Samples (20 μl) of the neat filtrates and doubling dilutions prepared in supplemented 199 maintenance medium were added to the CHO-KI monolayers in microtitre trays. Trays were incubated overnight in a humid atmosphere of air and 5% CO_2 at 37 °C.

Included in each batch of assays were filtrates of the three control strains AH2, V6053 and V6065. Other batch controls used were (a) uninoculated CHO-KI cells (b) tryptone soya broth at pH 6·7, and tryptone soya broth with pH adjusted to 5·4 (c) and 8·5 (d). The latter two controls were included as it was found that the original pH 6·7 was altered on growth of the organism to points within this range.

Assays were stained with Giemsa prior to reading by inverted microscopy. The cytotoxic and cytotonic titres of each preparation were calculated by determining the reciprocal of the highest dilution of the unheated or heated filtrates which produced a cytopathic effect. The end points for the titration of cytotoxic effect were unequivocal. The elongation effect however, was not so clear and was only positively scored if two independent observers agreed on the interpretation. For the Chi squared analysis of the incidence of cytotoxic and elongation factor titres were scored as + or −.

Invasion of human epithelial (HEp-2) cells

The assay method used was that of Watson and colleagues [11], but with the following modifications. Three days prior to assay isolates were cultured from storage at −70 °C on to Brain Heart Infusion Agar (Oxoid) and sub-cultured twice on the same medium at 30 °C to encourage fimbrial production. The inoculum for the assay was prepared from bacterial suspensions made to a 0·5 McFarland standard diluted 1 in 200 in Eagle MEM containing 5% foetal calf serum. Bacteria recovered from the HEp-2 cell lysate were checked for sensitivity to gentamicin by placing one drop of Eagles MEM containing gentamicin (10 μg/ml) on to a lysed horse blood agar seeded with the respective isolate.

Viable bacterial counts from the lysates were performed by the method of Miles and Misra [16] on Columbia Agar incubated at 37 °C overnight. Assays were performed on two separate occasions for each isolate tested and the mean counts/ml of lysate used as the invasion index. In each batch of assay a control invasive strain, *Escherichia coli* E12632/0 (invasive in the sereny test) and a non-invasive strain, *E. coli* 2744/4 (both courtesy of Dr B. Rowe), were included together with control monolayer lysates to check for bacterial contamination.

Haemagglutination tests

The method used was based on that described by Burke and colleagues [17]. Isolates to be tested were sub-cultured for 3 consecutive days on Brain Heart Infusion Agar incubated at 30 °C to encourage production of fimbriae. Human group O and fresh rabbit blood cells were obtained on the day of the test. 1% (w/v) D(+) galactose (G), L(−) fucose (F) and D(+) mannose (M) (Sigma and

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BDH-purest forms available) were used to examine inhibition of haemagglutination. Reactions were scored (+) haemagglutination or (−) no haemagglutination. Controls employed were mixtures of the respective blood type and phosphate buffered saline (PBS, OXOID), and samples of the respective blood cell type containing the individual sugar and PBS.

Congo red binding

The method used was based on a method devised by Ishigura and colleagues (18) to investigate congo red binding in *A. salmonicida*. Aeromonads were grown at 25 °C for 5 days on Congo Red Agar (Tryptone Soya Agar with 30 μg/ml congo red). Binding of congo red to individual cells produced colonies coloured either pale orange, a deeper salmon pink or red which were scored as +, ++, and +++ respectively.

Phenotypic characters

The data used in this study were obtained from 58 biochemical, enzymatic and antibiotic susceptibility reactions performed as follows: API 20E (API-bioMérieux (UK) Ltd, Basingstoke, Hants). Each strip was set up according to the manufacturer's instructions with the exception that (a) incubation was at 30 °C for 5 days with daily reading; (b) Voges–Proskauer (V–P), tryptophane deaminase and indole production were tested after incubation for 48 h; (c) acid production from amygdalin was not used in computer analysis of phenotypic properties.

Additional biochemical tests

Glucose (1% w/v) in peptone water containing Andrade's indicator was inoculated and examined for gas production in inverted durham tubes after incubation at 30 °C for 48 h. Acid production from arbutin, salicin and cellobiose was examined in peptone water containing the appropriate sugar (1% w/v). Incubation was in air at 30 °C for 5 days and tests were read daily. Acid production from lactose was tested on MacConkey agar incubated at 30 °C overnight.

As lysine decarboxylase production in API 20E may be unreliable [19], all lysine reactions were checked by heavy inoculation of Möllers lysine broth (Taylor modification, Oxoid), overlayered with sterile liquid paraffin (> 1 cm depth). Tests were incubated for 5 days at 30 °C and read daily. Gluconate broth was used to detect gluconate oxidase as described by Gray [19]. Aesculin hydrolysis was detected by the agar plate method of Lee and Donovan [20]. A maximum of eight strains were spot inoculated and incubated at 30 °C for 48 h.

Elastin breakdown was determined using the method of Scharmann [21] and fibrinolysin production by the method of Marandon and Oeding [22]. Strains were spot inoculated, eight per plate, and incubated for 5 days or 48 h respectively at 30 °C. A positive reaction was visible as a clearing of the opaque agar around the area of growth.

β-haemolysis of rabbit red blood cells (RBC): columbia agar plates containing thrice washed fresh rabbit blood (6% v/v) were used to spot inoculate up to eight strains per plate, and incubated for 24 h at 30 °C. β-haemolysis was indicated by total lysis of the red cells, usually extending beyond the area of growth. Partial

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haemolysis, often confined beneath the area of growth, and no haemolysis were scored as negative.

Enzymatic analysis

The enzymatic profile of each strain was examined using the API ZYM kit (API-bioMérieux (UK) Ltd), as described by Gray [23].

Antibiotic susceptibility tests

Sensitivity testing was performed on DST agar (Oxoid) containing 6·5% (v/v) lysed horse blood using a standard disk diffusion technique, results being read after overnight incubation at 30 °C. Disks containing the following concentration (μg) of antibiotic were employed; amoxycillin (10), tetracycline (10), sulphamethoxazole (25), trimethoprim (1·25), cephradine (30), gentamicin (10), cefuroxime (30), mezlocillin (75), neomycin (10), chloramphenicol (25). *E. coli* NCTC 10418 was used to control each batch of sensitivity tests. All intermediate results were regarded as resistant for the purpose of subsequent analyses.

Statistical analyses were carried out using SAS version 5·16 (SAS Institute Inc., SAS Circle; P.O. Box 8000, Cary, NC 27511, USA).

RESULTS

The incidence of the virulence factors

The incidence of cytotoxin, cytotonic enterotoxin (elongation factor), congo red binding, haemagglutinins and ability to invade human epithelial cells in 61 isolates and three control strains are reported in Tables 1–3. In the case of cytotoxin the differences between species, *A. hydrophila* (96·4%), *A. sobria* (36·4%) and *A. caviae* (13·6%) was highly significant ($x^2 = 36{\cdot}272$, D.F. 2, $P < 0{\cdot}0001$). Varying degrees of elongation in CHO-KI cells was noted in the dilution immediately after the cytotoxic end point with 1 of 4 *A. sobria*, 16 of 27 *A. hydrophila* and all 3 *A. caviae*. There was also noticeable elongation of the few cells remaining intact at the cytotoxic end point, although this did not always occur. Reference strain V6053 showed elongation of remaining cells at the cytotoxic end point but not thereafter, whilst AH2 showed elongation in the dilution after the cytotoxic end point.

Presence of an elongation factor

There were significant differences between species in the expression of an elongation factor ($x^2 = 13{\cdot}805$, D.F. 2, $P = 0{\cdot}001$). Three of 11 (27·3%) *A. sobria* produced a factor resistant to heating at 56 °C for 20 min which caused elongation of the normally paddle-shaped cells of the CHO-KI monolayer. This effect was only demonstrable in neat culture filtrates. Twenty-one of 28 (75%) isolates of *A. hydrophila* possessed an elongation factor with a range of 1–4 units. Six of 22 (27·3%) *A. caviae* caused elongation of CHO-KI cells at a titre of one unit. The elongation factor was not demonstrable in *A. caviae* V6065, *A. hydrophila* V6053 nor in *A. hydrophila* AH2. Strain AH2 produced cytotoxic effect at a titre of four units in the heated filtrate.

Twenty-eight of the 30 (93·3%) isolates with demonstrable elongation factor

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Table 1. *The prevalence of virulence markers in 11 isolates identified as* Aeromonas sobria

Isolate number	Source	Virulence properties: Reciprocal titre cytotoxin	Reciprocal titre elongation factor	pH after OSC*	Degree of congo red binding†	Agglutination inhibition human 'O' RBC‡	Agglutination inhibition rabbit RBC‡	Invasive index CFU/ml
130	Reen water§	0	0	8·40	++	$F^+G^-M^+$	$F^+G^-M^+$	$1{\cdot}0\times10^6$
132	Cow faeces	512	0	8·65	+	$F^-G^-M^+$	$F^-G^-M^+$	$2{\cdot}0\times10^6$
133	Cow faeces	512	0	8·50	+	$F^-G^-M^+$	$F^-G^-M^+$	$7{\cdot}6\times10^5$
152	Reen water	0	1	5·40	+++	—	—	$7{\cdot}5\times10^3$
170	Cow slurry	128	1	5·30	++	—	—	$1{\cdot}3\times10^4$
175	Reen water	0	0	7·68	++	$F^+G^-M^+$	$F^+G^+M^+$	$6{\cdot}6\times10^7$
182	Reen water	0	1	5·81	++	—	—	$2{\cdot}9\times10^3$
190	Reen water	> 2048	0	8·92	++	—	—	$3{\cdot}4\times10^6$
191	Reen water	0	0	8·57	++	$F^-G^-M^+$	$F^-G^-M^+$	$4{\cdot}5\times10^5$
203	Cow faeces	0	0	7·45	+++	—	—	$6{\cdot}8\times10^2$
211	Cow faeces	0	0	8·67	++	—	$F^-G^-M^+$	$4{\cdot}6\times10^5$

* Overnight shake culture.
† +, pale orange colony coloration; ++, salmon pink coloration; +++, red coloration.
‡ Fucose galactose mannose; + or − superscripts indicate inhibition or no inhibition of agglutination by the sugar; −, no haemagglutination.
§ Reen water, natural drainage water.

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Table 2 *The prevalence of virulence markers in 28 isolates identified as* Aeromonas hydrophila

Isolate number	Source	Virulence properties: Reciprocal titre cytotoxin	Reciprocal titre elongation factor	pH after OSC*	Degree of binding on congo red agar†	Agglutination inhibition human 'O' RBC‡	Agglutination inhibition rabbit RBC‡	Invasive index CFU/ml
4	Pig faeces	32	4	6·73	++	$F^+G^-M^+$	—	$6{\cdot}0 \times 10^{6}$
12	Cow faeces	16	2	5·68	+	$F^-G^-M^+$	$F^-G^-M^+$	$1{\cdot}2 \times 10^{4}$
25	Pig faeces	1024	1	8·46	+	$F^+G^-M^+$	—	$1{\cdot}7 \times 10^{5}$
29	Pig faeces	8	2	6·44	++	$F^+G^-M^+$	$F^+G^-M^+$	$2{\cdot}3 \times 10^{5}$
33	Pig faeces	128	2	5·65	+	$F^+G^-M^+$	—	$1{\cdot}1 \times 10^{5}$
39	Pig faeces	1024	0	8·34	+	$F^+G^-M^+$	—	$3{\cdot}9 \times 10^{5}$
58	Pig faeces	128	4	5·70	+	$F^+G^-M^+$	—	$7{\cdot}5 \times 10^{4}$
63	Pig faeces	8	2	5·59	+	$F^+G^-M^+$	$F^-G^-M^+$	$3{\cdot}1 \times 10^{4}$
66	Pig faeces	1	1	5·73	+	$F^+G^-M^+$	—	$8{\cdot}7 \times 10^{4}$
75	Cow faeces	32	2	5·90	+	$F^+G^-M^+$	–	$9{\cdot}9 \times 10^{4}$
79	Pig faeces	4	1	6·35	+	$F^+G^-M^+$	$F^-G^-M^+$	$1{\cdot}5 \times 10^{8}$
84	Chlorinated water	16	1	5·84	++	—	—	$3{\cdot}1 \times 10^{2}$
104	Chlorinated water	4	1	5·79	++	—	—	$4{\cdot}0 \times 10^{1}$
112	Reen water§	128	2	5·64	+	$F^+G^-M^+$	$F^-G^-M^+$	$9{\cdot}8 \times 10^{5}$
118	Pasture	8	1	5·99	+	—	—	$9{\cdot}0 \times 10^{1}$
134	Soil	16	1	5·45	+	—	—	$1{\cdot}6 \times 10^{3}$
137	Chlorinated water	32	2	5·21	+	—	—	$3{\cdot}7 \times 10^{2}$
138	Fly	16	2	5·77	++	$F^+G^-M^+$	$F^+G^-M^+$	$1{\cdot}3 \times 10^{6}$
139	Fly	16	2	6·01	+	—	—	$3{\cdot}0 \times 10^{9}$
146	Chlorinated water	256	4	5·89	++	$F^+G^-M^+$	$F^-G^-M^+$	$4{\cdot}2 \times 10^{3}$
150	Reen water	32	1	5·64	+	—	—	$1{\cdot}1 \times 10^{3}$
168	Cow faeces	128	0	8·50	++	$F^-G^-M^+$	$F^-G^-M^+$	$1{\cdot}1 \times 10^{4}$
173	Reen water	0	0	7·48	+++	—	—	$4{\cdot}8 \times 10^{2}$
178	Reen water	1024	0	8·00	++	$F^+G^-M^+$	$F^-G^-M^+$	$2{\cdot}1 \times 10^{6}$
204	Cow faeces	256	0	8·80	+	$F^-G^-M^+$	$F^-G^-M^+$	$1{\cdot}5 \times 10^{6}$
206	Cow faeces	> 2048	0	8·38	++	$F^-G^-M^+$	$F^-G^-M^+$	$6{\cdot}1 \times 10^{3}$
212	Cow faeces	32	2	5·90	++	—	—	$4{\cdot}2 \times 10^{2}$
214	Cow faeces	512	0	8·70	+	$F^-G^-M^-$	$F^-G^-M^+$	$1{\cdot}1 \times 10^{5}$
AH2	Control	512	0	8·71	++	$F^+G^-M^+$	$F^-G^-M^+$	$3{\cdot}4 \times 10^{8}$
V6053	Control	512	0	8·61	++	$F^+G^-M^+$	$F^+G^-M^+$	$1{\cdot}7 \times 10^{4}$

* Overnight shake culture.

† +, pale orange colony coloration; ++, salmon pink coloration; +++, red coloration.

‡ Fucose galactose mannose; + or – superscripts indicate inhibition or no inhibition of agglutination by the sugar; –, no haemagglutination.

§ Reen water, natural drainage water.

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Table 3. *The prevalence of virulence markers in 22 isolates identified as* Aeromonas caviae

Isolate number	Source	Virulence properties: Reciprocal titre cytotoxin	Reciprocal titre elongation factor	pH after OSC*	Degree of binding on congo red agar†	Agglutination inhibition human 'O' RBC‡	Agglutination inhibition rabbit RBC‡	Invasive index CFU/ml
2	Pig faeces	0	0	8·54	++	—	—	$1·7 \times 10^3$
5	Pig faeces	0	0	8·39	++	—	—	$1·0 \times 10^5$
6	Pig faeces	0	0	9·56	++	—	—	$1·0 \times 10^1$
11	Pig faeces	0	0	8·33	++	$F^+G^-M^+$	—	$6·3 \times 10^2$
14	Pig faeces	0	0	8·46	++	—	—	$8·1 \times 10^3$
16	Pig faeces	0	0	8·58	++	—	$F^-G^-M^-$	$1·0 \times 10^3$
36	Pig faeces	1024	1	8·40	++	—	—	$2·0 \times 10^4$
52	Pig faeces	0	1	5·27	+	$F^+G^-M^+$	$F^+G^-M^+$	$2·2 \times 10^6$
56	Pig straw	32	1	4·78	+	$F^+G^-M^+$	$F^+G^-M^+$	$1·7 \times 10^3$
59	Pig faeces	4	1	4·79	+	$F^+G^-M^+$	$F^+G^-M^+$	$2·9 \times 10^5$
98	Pig faeces	0	1	5·21	+	$F^+G^-M^+$	$F^+G^-M^+$	$2·3 \times 10^6$
99	Cow faeces	0	0	8·72	++	—	—	$5·9 \times 10^4$
111	Reen water§	0	0	8·70	++	—	—	$4·7 \times 10^3$
114	Reen water	0	0	8·63	++	—	—	$2·1 \times 10^2$
120	Chlorinated water	0	0	8·72	++	—	—	$6·8 \times 10^3$
126	Pig faeces	0	0	8·63	++	$F^+G^-M^+$	—	$3·4 \times 10^3$
128	Reen water	0	0	8·71	++	—	—	$1·2 \times 10^3$
164	Chlorinated water	0	0	8·58	++	—	—	$8·5 \times 10^2$
185	Chlorinated water	0	0	8·76	++	—	—	$5·6 \times 10^3$
202	Cow faeces	0	1	5·85	+++	—	—	$1·2 \times 10^2$
207	Cow faeces	0	0	8·83	++	—	—	$1·2 \times 10^3$
215	Cow faeces	0	0	8·67	++	$F^+G^-M^+$	—	$1·8 \times 10^7$
V6065	Control	0	0	8·92	++	—	—	$1·3 \times 10^5$

* Overnight shake culture.
† +, pale orange colony coloration; + +, salmon pink coloration; + + +, red coloration.
‡ Fucose galactose mannose; + or − superscripts indicate inhibition or no inhibition of agglutination by the sugar; —, no haemagglutination.
§ Reen water, natural drainage water.

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elicited this response only when the final pH of the overnight shake culture was acid. Conversely only 2 of 31 (6·5%) showed this response at an alkaline pH. The three control organisms all produced an alkaline pH after overnight shake culture.

Binding of congo red

The binding of congo red to cells was used as an indication of their ability to produce the surface S-layer. The degree of binding to the intact organism from agar containing congo red was estimated. Using this method 9 of 11 (81·8%) *A. sobria* bound congo red to an extent of + + or greater, as did 18 of 22 (81·8%) *A. caviae*, but only 11 of 28 (39·2%) *A. hydrophila* showed this level of binding. All organisms tested took up the dye to some extent, yet there was a significant difference between species in the degree of binding ($x^2 = 11{\cdot}607$, D.F. 2, $P < 0{\cdot}01$).

Haemagglutinins

More *A. hydrophila* (67·9%) than *A. sobria* (54·5%) or *A. caviae* (36·4%) showed haemagglutination in one or both of the red cell indicator systems. Inhibition of haemagglutination by the fucose, galactose and mannose demonstrated eight different inhibition patterns. There was no predominant pattern of inhibition which could be related to individual species (human cells $x^2 = 12{\cdot}057$, D.F. 6, $P = 0{\cdot}061$: rabbit cells, $x^2 = 17{\cdot}004$, D.F. 8, $P = 0{\cdot}03$). Although non-haemagglutination of both cell types was more strongly associated with *A. caviae*, differences in haemagglutination inhibition patterns between the three species using the two cell types were slight. Only one strain showed a haemagglutinin which was inhibited by galactose.

Invasion of HEp-2 cells

The control organism *E. coli* E12632/0 gave an invasive index of more than 5×10^{11} organisms/ml of lysate and the non-invasive control organism *E. coli* 2744/4 a mean count of $1{\cdot}3 \times 10^4$/ml of lysate. It can be seen from Tables 1–3 that 6 of 28 (21·4%) *A. hydrophila*, 4 of 11 (36·4%) *A. sobria* and 3 of 22 (13·6%) *A. caviae* gave an invasion index of 1×10^6 organisms/ml or greater. However, analysis of differences between the three species using a one-way analysis of variance showed that while means of the $\log_{10}$ of the invasive index was greater in *A. sobria* than *A. hydrophila* or *A. caviae* and was greater in *A. hydrophila* than *A. caviae* these differences were not significant ($F = 2{\cdot}89$, $P > 0{\cdot}05$).

Associations between virulence markers and their relationships to other phenotypic characters

Analyses of the inter-relationship between markers of virulence and between markers of virulence and other phenotypic characters were examined using Spearman correlation coefficients. Possession of a cytotoxin showed highly significant positive relationships with elongation factor ($P < 0{\cdot}0001$), lysine decarboxylase ($P < 0{\cdot}0002$), Voges Proskauer ($P < 0{\cdot}0001$), chymotrypsin ($P < 0{\cdot}0001$), β-haemolysis ($P < 0{\cdot}0001$), gas from glucose ($P < 0{\cdot}0004$), elastase ($P < 0{\cdot}0001$), gluconate oxidase ($P < 0{\cdot}0001$) and acid from sorbitol ($P < 0{\cdot}0002$). Cytotoxin production was also less significantly associated with haemagglutination of human group O blood ($P < 0{\cdot}002$) and the acidity of overnight cultures

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Table 4. *Analysis of relationship between log invasion index and agglutination of human O blood, and rabbit blood, using a least significance difference test*

Comparison of agglutination patterns		Human O blood			Rabbit blood		
		Difference between means of log invasion index	Lower 95% confidence limit	Upper 95% confidence limit	Difference between means of log invasion index	Lower 95% confidence limit	Upper 95% confidence limit
$F^+G^+M^+$	$F^+G^-M^+$	—	—	—	2·266	−1·033	5·566
$F^+G^+M^+$	$F^-G^-M^+$	—	—	—	2·446	−0·749	5·640
$F^+G^+M^+$	No agglutination	—	—	—	3·905*	0·778	7·032
$F^+G^+M^+$	$F^-G^-M^-$	—	—	—	4·820*	0·455	9·185
$F^+G^-M^+$	$F^-G^-M^+$	0·361	−0·917	1·639	0·180	−1·249	1·608
$F^+G^-M^+$	No agglutination	1·948*	1·127	2·768	1·639*	0·369	2·908
$F^+G^-M^+$	$F^-G^-M^-$	0·452	−2·572	3·476	2·553	−0·746	5·853
$F^-G^-M^+$	No agglutination	1·587*	0·344	2·829	1·459*	0·494	2·424
$F^-G^-M^+$	$F^-G^-M^-$	0·091	−3·074	3·255	2·374	−0·821	5·569
$F^-G^-M^-$	No agglutination	1·496	−1·513	4·505	−0·914	−4·041	2·213

* Indicates comparisons significant at the 0·05 level.

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($P < 0{\cdot}001$). Highly significant inverse relationships were found between cytotoxin and fermentation of cellobiose ($P < 0{\cdot}0002$) and β-glucuronidase ($P < 0{\cdot}0001$).

The presence of an elongation factor showed highly significant positive correlation with production of cytotoxin ($P < 0{\cdot}0001$), β-haemolysin ($P < 0{\cdot}0001$), elastase ($P < 0{\cdot}0001$), chymotrypsin ($P < 0{\cdot}0001$) and acid from sorbitol ($P < 0{\cdot}0007$). Production of an acid pH during overnight shake culture was highly significantly related to elongation of CHO-KI cells ($P < 0{\cdot}0001$).

Haemagglutination of human group O blood showed a highly significant positive correlation with fermentation of sorbitol ($P < 0{\cdot}0001$), chymotrypsin ($P < 0{\cdot}0006$) and β-haemolysin production ($P < 0{\cdot}0008$). An inverse correlation was recorded between binding of congo red and agglutination of human O blood ($P < 0{\cdot}0002$). Haemagglutination of human O and rabbit blood showed a highly significant relationship ($P < 0{\cdot}0001$). The log invasive index was significantly correlated with the agglutination of human ($P < 0{\cdot}0001$) and rabbit erythrocytes ($P < 0{\cdot}0002$).

The relationship between haemagglutination, non-haemagglutination and the log invasion index was also analysed using analysis of variance. This showed that the log invasion index was significantly different between agglutinating and non-agglutinating isolates in human blood ($F = 8{\cdot}10$, D.F. 3, $P < 0{\cdot}0001$) and rabbit blood ($F = 4{\cdot}71$, D.F. 4, $P < 0{\cdot}0024$). Comparisons of difference in the $\log_{10}$ invasion index between different patterns of inhibition in the two cell types were tested by the Least Significance Difference (LSD) test, the results of which are shown in Table 4. These analyses showed that, with the exception of the $F^-G^-M^-$ pattern of the single isolate with rabbit blood, isolates which caused haemagglutination of either of the cell types exhibited significantly higher invasion indices than non-agglutinating isolates. Further, with the exception of the single isolate of inhibition type $F^-G^-M^-$ and the $F^+G^+M^+$ pattern in rabbit blood, there are no significant differences in the log invasion index between different haemagglutination inhibition patterns in either of the cell types. Thus the suggestion that haemagglutinating isolates had a higher invasion index and were therefore more likely to be invasive than non-agglutinating isolates was confirmed. Invasiveness was not related to any particular sugar inhibition pattern however.

The relationship between the log invasion index, and the ability of strains to ferment sorbitol and rhamnose and produce chymotrypsin were also examined using Student's T-test where variances were assumed to be unequal and calculations were performed to give approximate degrees of freedom. These results demonstrate that isolates fermenting sorbitol ($t = 3{\cdot}7299$, D.F. 43·9, $P < 0{\cdot}0005$) or producing chymotrypsin ($t = 4{\cdot}4372$, D.F. 42·4, $P < 0{\cdot}0001$) have significantly higher log invasion indices than isolates that do not show these characters. In contrast, strains which ferment rhamnose have significantly lower log invasion indices than those which fail to ferment this sugar ($t = 5{\cdot}6932$, D.F. 25·3, $P < 0{\cdot}0001$).

DISCUSSION

It has been suggested that environmental [24] and animal isolates [19] may be important reservoirs of aeromonads that are capable of causing human infection. Aeromonads have been isolated from a variety of food products [25–27], and as

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aeromonads multiply during chill-storage of foods [26, 27], this has generated speculation that there may be an increasing hazard of human food poisoning from this source. Indeed there have been several recent reports implicating food in aeromonad-associated diarrhoeas [1, 28, 29].

Analysis of the incidence of virulence markers in the aeromonad isolates revealed a highly significant association ($P < 0{\cdot}0001$) between cytotoxin production and elongation effect in CHO-KI cells. While some isolates produced cytotoxin independently of elongation factor and vice versa, most produced both or neither of these markers. Although possession of cytotoxin has been correlated to production of enterotoxic effect in animal models [30, 31]; strains which produce cytotoxin but are enterotoxin negative, have been noted [32, 33]. However, Charkraborty and colleagues [14] reported that the elongation effect observed in CHO cells was due to a cytotonic enterotoxin and not a cytotoxin. Further, as had been previously shown [34, 35], the production of these toxins was significantly related to species, with *A. hydrophila* and *A. sobria* being more likely to produce them than *A. caviae*.

An association between cytotoxin and haemagglutination in human diarrhoeal isolates has been noted by other workers [2]. Analysis of the association between these two markers in the isolates examined in the present study gave Spearman correlation coefficients of 0·206 for rabbit erythrocytes and was not significant ($P = 0{\cdot}11$), in the case of human red blood cells however, the value was 0·395 ($P < 0{\cdot}0016$). If one were to use a very strict criterion ($P < 0{\cdot}0001$) to accept significance because of the large number of calculations carried out then this association would be rejected, but only just, nevertheless it can be calculated from Tables 1–3 that 67·6% of the cytotoxin producers were also capable of causing haemagglutination. Although haemagglutination of both cell types was significantly related ($P < 0{\cdot}0001$) this study confirmed previous reports concerning differences in the ability to haemagglutinate different cell types [4, 36, 37].

The degree of uptake of the dye congo red by the 61 isolates showed inverse relationships to the other virulence markers examined. Significant inverse relationships were seen with haemagglutination of human O cells ($P < 0{\cdot}0002$) and cytotoxin production ($P < 0{\cdot}0001$). The association found between greater degrees of congo red binding and negative reactions for elongation factor, invasive potential and haemagglutination of rabbit blood, reflects the lower binding capacity of the *A. hydrophila* isolates tested in this study. These observations support the findings of Statner and George [38], that congo red uptake was not a useful marker of virulence in the motile aeromonads, and all isolates bound congo red to some extent.

Analysis of the relationship between virulence markers and other phenotypic characters showed that production of cytotoxin and elongation factor were significantly correlated with positive tests for chymotrypsin, β-haemolysis, elastase and acid from sorbitol, and cytotoxin further significantly correlated to positive tests for lysine decarboxylase, V–P, gas from glucose and gluconate oxidase. These were all characters strongly associated with *A. hydrophila* and *A. sobria*. Furthermore, the strong relationship observed between cytotoxin production and failure to ferment cellobiose and lack of β-glucuronidase again reflected the phenotypic make up of *A. hydrophila*. The significant correlation found between high log invasion indices and production of acid from sorbitol and

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chymotrypsin illustrates their association with *A. hydrophila* and *A. hydrophila* and *A. sobria* respectively. Although the number of isolates fermenting rhamnose were small, those fermenting this sugar were significantly less invasive than other strains.

A. sobria and *A. hydrophila* were more likely to show haemagglutination than *A. caviae* with the two blood cell types used. In particular, isolates agglutinating human O cells showed a significant positive relationship to fermentation of sorbitol, chymotrypsin and β-haemolysin production which further emphasizes the relationship to species.

The degree of congo red binding showed inverse relationships to phenotypic characters. Thus isolates that bound congo red poorly were likely to be V–P positive ($P < 0{\cdot}0005$) and to produce acid from sorbitol, β-haemolysin, elastase and gluconate oxidase ($P < 0{\cdot}0001$), all properties strongly associated with *A. hydrophila*. Statner and George (38) have also reported that the ability to bind congo red was not related to positive reactions for V–P or lysine decarboxylase.

It can be seen from Tables 2 and 3 that the control strains V6053 (cytotoxin positive) and V6065 (cytotoxin negative) gave titres of 512 and 0 respectively in the cytotoxin assay. The results from the cell-elongation assay were inconclusive, however, the heated filtrates from control positive strains AH2 and V6053 both failing to give any measurable titres. Indeed the heated filtrate of strain AH2 still exhibited low titre cytotoxic activity, as had been previously demonstrated by Bunning and colleagues [39]. The interpretation of the end point for the cell-elongation was also difficult. The results achieved from this assay thus have to be treated with caution.

Chakraborty and colleagues [14] consider that the cytotoxic, cytotonic enterotoxic and haemolytic activities of *A. hydrophila* are due to three different proteins resulting from the expression of three distinct genes. Other workers have come to different conclusions however. Bunning and colleagues [39] reported that cell-elongation could be observed in assays of unheated culture filtrates at dilutions immediately after the cytotoxic end-point. They also failed to detect any ADP-ribosyl transferase activity in the assays and concluded that cytotonic enterotoxin-like elongation of CHO cells was either due to sub-lethal doses of cytotoxin, which might survive heat-inactivation, or perhaps due to the effects of endogenous growth factors released from lysed cells. In the present study elongation of CHO-KI cells was noted in the dilution immediately after the cytotoxic end-point in 1 out of 4 cytotoxic *A. sobria*, 16 out of 27 cytotoxic *A. hydrophila* and all 3 cytotoxic *A. caviae*. There was also noticeable elongation in the few cells remaining at the cytotoxic end-point in some, but not all cytotoxic strains. Jiwa [4] notes that elongation in CHO-KI cells could be observed in the assays prior to the cytopathic effect and considered that it represented a distinct cytotonic enterotoxic activity. While Bunning and colleagues [39] reported that cell-elongation activity could be neutralized by anti-serum to haemolysin, Chopra and colleagues [8] found non-haemolytic isolates which produced elongation of CHO-KI cells, and gave positive reactions for enterotoxin in the suckling mouse test. In the present study elongation factor was detected in strains which produced the full range of cytotoxin titres from 0 to 2028 units, and 4 out of 26 non-β-haemolytic isolates produced cell-elongation. It is possible, of course, that differences in phenotypic expression occurring under the different growth

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conditions used in these tests could account for these discrepancies. Overall there was a highly significant correlation ($P < 0{\cdot}0001$) between the production of a heat labile cytotoxin and β-haemolysin. This confirms the observations of Cumberbatch and colleagues [30] who postulated that these activities were either the expression of the same protein or subject to the same genetic control. Asao and colleagues [9] were able to purify a haemolysin which was cytotoxic, enterotoxic and heat-labile and Chakraborty and colleagues [40] have demonstrated that deletion of the aerolysin (β-haemolysin) gene removed haemolytic and cytotoxic activity. Reintegration of the gene restored both functions. Poor correlation between haemolysis of rabbit erythrocytes and enterotoxin production as measured by the suckling mouse test, has been reported by Okitsu and colleagues [41]. The situation is further complicated by reports that aeromonads possess at least two different haemolysins with cytotoxic activity [42] and that *A. hydrophila* may also produce another cytotoxin which is devoid of haemolytic activity [10].

The significant association between elongation effect (and by implication cytotonic enterotoxin) and the production of an acid pH in overnight shake cultures appears to contrast with earlier reports on the pH dependance of aeromonad toxin detection [43–45]. Fehlhabner and Scheiber [45] for example found that enterotoxic activity was minimal at acid pH's. Only 18·9% of strains gave a positive response at pH 6·0 in the suckling mouse test, compared with 100% positive at pH 7·4 and 76·9% positive at pH 10·0. Other workers [30, 34] have regarded the cytotonic-like effects observed in various cell lines to be due to protease(s). It has been demonstrated that although protease production by *A. hydrophila* was accompanied by a fall in pH values [46], protease activity was greater at neutral to alkaline than at very acid pH's [46, 47]. Casein digests, such as casamino acids used in the present study, have been found to be potent inducers of proteases (48). It is possible therefore, that the elongation effect seen in CHO-KI cells may be due to sub-optimal activity of a proteolytic enzyme(s). Whether this also has haemolytic effect has to be established.

Haemagglutination of different animal erythrocyte systems and particularly sugar inhibition of haemagglutination, have been used to type strains of mesophilic aeromonads [2]. There is evidence that haemagglutination patterns vary under different test conditions [2, 4], so that putatively different haemagglutination types may merely reflect different amounts of the same haemagglutinin produced under different *in vitro* conditions. Geographic variation in haemagglutination inhibition patterns has also been noted (17). In the present study non-agglutinating isolates were more likely to be *A. caviae* than *A. hydrophila* or *A. sobria* and this confirms previous findings [17, 37]. Significant differences in the distribution of haemagglutinin types between the three species of aeromonads have been shown by some workers, the $F^-G^-M^+$ pattern for example being typical of *A. sobria* [49].

Burke and colleagues [24] have used haemagglutination inhibition patterns as epidemiological markers to implicate water as an important source of human enteric aeromonads. Sanyal, Agarwal and Annapurna [50] however, found no association of haemagglutination inhibition patterns with individual species among 40 enterotoxigenic isolates from diverse sources. In the present study the pattern $F^-G^-M^+$ however, was restricted to *A. sobria* and *A. hydrophila* and to

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isolates from cattle and reen water. Most of the haemagglutinating isolates proved to be sensitive to fucose inhibition, particularly when human erythrocytes were used in the test. The variety of haemagglutination patterns recorded in this study suggests that as with other species the various aeromonad haemagglutinins have a number of different receptor sites on the red cell and presumably intestinal cell membranes.

In vitro studies in HEp-2 cells have indicated that certain strains of aeromonads may have invasive potential [11, 12], earlier histological observations had also suggested that they possessed this property [51]. Watson and colleagues [11] set an arbitrary criterion in this test, considering that organisms which produced cell lysates with 5×10^6 CFU/ml were invasive. Applying this criterion they found that of the 18 invasive strains (26·1 % of total isolates tested) 16 were *A. sobria* and none of the *A. caviae* examined was invasive. When this criterion is applied to the 61 isolates in the present study only 1 of 11 *A. sobria*, 3 of 28 *A. hydrophila* and 1 of 22 *A. caviae* are classified as invasive. However, 36·4 % of *A. sobria*, 21·4 % of *A. hydrophila* and 13·6 % of *A. caviae* gave lysates with $> 1 \times 10^6$ CFU/ml, and the means of the log invasion indices for the three species were 5·28, 4·66 and 3·87 respectively. In general therefore, the results of this study support the contention that *A. sobria* is the most invasive and *A. caviae* is the least able to penetrate and grow in human cells in tissue culture. These findings are consistent with the observation that *A. sobria* is the most frequent aeromonad isolated from cases of bacteraemia [52].

Lawson, Burke and Chang [12] had noted that the efficiency of HEp-2 cell invasion by aeromonads was somewhat lower (minimum of 3·5 fold) than that observed for a control invasive *E. coli* strain. This was confirmed in the present study, the control invasive *E. coli* having an invasive index at least 200 times greater than the most invasive aeromonad.

In conclusion, this study has shown differences in the incidence of virulence characteristics in agricultural isolates of the three species of mesophilic aeromonads. Most isolates of *A. caviae* were non-cytotoxic and failed to produce cell-elongation effects or haemagglutination. They were also less cell-invasive than the other two species. In contrast most isolates of *A. hydrophila* were cytotoxic and produced cell-elongation and haemagglutination. *A. sobria* produced the highest mean log invasion index in HEp-2 cells, but had a lower incidence of the other three virulence factors than *A. hydrophila*. Statistical analysis revealed significant associations between the carriage of these factors and it was clear that many isolates of aeromonads from water and animals possessed the full battery of putative virulence factors.

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