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Mannose-resistant adhesion of motile *Aeromonas* to INT407 cells and the differences among isolates from humans, food and water

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SUMMARY

Aeromonas has been recognized as an important enteropathogen, but factors related to its virulence have not been clarified. For most enteric pathogens, attachment is a prerequisite for infection and for the effective delivery of toxins to the intestinal epithelial cells. We examined a total of 273 strains isolated from stool, food and environmental specimens by an assay for mannose-resistant adhesion to INT407 cells *in vitro*. Seventeen of the 102 faecal isolates were adhesive strains with more than 10 bacteria adhering per cell, while only 2 of the 118 isolates from foods and river water adhered to the cells ($P < 0{\cdot}001$). It is possible that the adhesion might serve as a marker for discrimination between the pathogenic and nonpathogenic isolates. The 8 highly adhesive strains with more than 20 adhering organisms per cell were scrutinized for the mechanism of adhesion. No correlation was apparent between the adhesion to INT407 cells and hydrophobicity. It was noted that fucose inhibited the adhesion of four strains as well as haemagglutination by them. Electronmicroscopic studies showed the presence of flexible and curvilinear fimbriae in only 2 of the 8 highly adhesive strains.

INTRODUCTION

Aeromonas organisms are considered to be autochthonous inhabitants of aquatic environments. Their roles in enteric infection in immunologically normal adults [1] and children [2] have been the subject of a number of studies. The illnesses reported range from mild to dysentery-like diarrhoea [3] and include travellers' diarrhoea [1, 4, 5]. However the evidence associating *Aeromonas* with diarrhoea is largely circumstantial and epidemiological. In some well-controlled studies, *Aeromonas* spp. were isolated at significantly higher frequency from diarrhoeal stools than from normal ones [2, 6–8]; in other studies, the rates of isolation of *Aeromonas* spp. from the two groups were similar to one another [9–11].

The organisms are commonly isolated from various foods and environmental specimens but it is not known whether all such strains are pathogenic [8]. Indeed, it seems apparent that the asymptomatic carriage of *Aeromonas* in humans in developing countries reflects the non-pathogenic nature of some of these environmental strains [11]. Methods for the discrimination between isolates that are pathogenic in man and those from the environment is clearly needed. Although β-haemolysin might seem to be a likely pathogenic factor [12, 13], failure of

haemolytic strains to cause diarrhoea in human volunteers suggests that haemolysin *per se* is not the sole determinant of virulence [14].

It has been well recognized that the adhesion of bacteria to mucosal surfaces is an important step in the pathogenesis of most infections in humans and animals. *Escherichia coli* and *Vibrio cholerae* induce diarrhoea by elaboration of enterotoxins after adhesion to the intestinal mucosa [15]. Both adhesins and enterotoxins were necessary for production of diarrhoea in volunteers fed orally with *E. coli* [16]. Although most strains of *A. hydrophila* and *A. sobria* produce β-haemolysin as a possible enterotoxin [8], few strains may possess adhesin.

In order to test this hypothesis and to advance our understanding of the pathogenicity of *Aeromonas*, we have examined the ability of the organisms to adhere to cells in tissue culture cells in an attempt to correlate such an ability with the species, pathogenicity and origin.

MATERIALS AND METHODS

Strains of Aeromonas

The strains used are shown in Table 1. These organisms, all from Osaka City, were obtained during a previously described epidemiological study of *Aeromonas* [8]. They were isolated from stool specimens submitted for shigella examination from food handlers etc., from river water, and from various foods. Stock cultures of the organisms were stored at room temperature in half-strength nutrient agar (Nissui, Tokyo, Japan). To prepare inocula, all strains were seeded into nutrient broth (Eiken, Tokyo) and incubated statically for 18 h at 30 °C. A 1000-fold dilution of each culture was made in Eagle's minimal essential medium (EMEM, Nissui) supplemented with 1% D-mannose, 2 mM L-glutamine and 2% fetal calf serum, and used as an inoculum. This suspension gave a concentration of about 10^6 c.f.u./ml.

Epithelial cells

Human embryonic intestinal cells (INT407) and HEp-2 cells were purchased from Dai-Nippon Pharmaceutical Co., Osaka, Japan. The cell lines were cultured in flat-bottom 25 cm^2 tissue culture flasks in EMEM supplemented with 10% (v/v) fetal calf serum (Flow Laboratories, North Ryde, Australia), but without antibiotics, at 37 °C in a humidified atmosphere of 5% CO_2. Cell cultures were split at a ratio of 1:4 at weekly intervals after detachment of the cells with trypsin (0·5 g of Difco 1:250 trypsin per litre) in a buffered solution of EDTA (0·2 g of disodium EDTA per litre in PBS).

Test for adhesion

For the INT407 adhesion assay, the method of Scaletsky and co-workers [17] was used with some modifications. An aliquot of 0·5 ml of a suspension of approximately 2×10^5 cells per ml of EMEM with 10% fetal calf serum was placed in each chamber of a four-chambered, tissue culture slide (Nunc, Naperville, IL, USA). After overnight incubation, semiconfluent monolayers of INT407 cells grown on the slide were washed with Hanks' balanced salt solution (Hanks', Nissui). An aliquot of 0·5 ml of each inoculum was introduced into each chamber well and incubated for 30 min at 37 °C (infection period). Unattached bacterial

Table 1. *Numbers of strains of aeromonas used in this study*

Source	*A. hydrophila*	*A. sobria*	*A. caviae*
Stool	38 (19)*	40 (2)	45 (0)
Foods	39 (18)	25 (6)	26 (0)
River	20 (8)	20 (0)	20 (0)
Total	97 (45)	85 (8)	91 (0)

* Numbers in parentheses indicate the numbers of strains for which adhesive ability could not be examined because of the extremely cytotoxic nature of the strains.

cells were then eliminated from the monolayers by four washings *in situ* with 0·5 ml of Hanks', and then 0·5 ml of EMEM supplemented with 1% D-mannose and 2% fetal calf serum was added to each chamber. After incubation for 3 h at 37 °C (multiplication period), the slides were washed four times with Hanks'. The cells were then fixed in methyl alcohol for 5 min and stained for 20 min with 10% Giemsa diluted with Sorensen buffer M/15 (pH 6·8). The stained slides were washed with water, dried, and the number of aeromonads adhering to each of 50 epithelial cells was then counted under a light microscope. Numbers of bacteria exceeding 50/cell were recorded as 50.

Inhibition by carbohydrates

Fucose, galactose, and mannose were dissolved individually at 1% (w/v) in EMEM, and these solutions were used instead of EMEM in the test for adhesion.

Fluorescence staining of accumulated actin

The accumulation of actin was examined by the method of Knutton and colleagues [18]. The HEp-2 cells were fixed for 20 min in phosphate-buffered 3% formalin after the test for adhesion. Fixed and washed cells were permeabilized by treating the chamber slides with 0·1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. After three washes in PBS, the slides were treated with a solution of 5 μg/ml of fluorescein isothiocyanate-phalloidin (Sigma, St Louis, MO, USA) in PBS for 20 min to stain filamentous actin specifically. The slides were washed three times in PBS and covered with glycerol-PBS. Specimens were examined by incident-light fluorescence under a Nikon microscope. A strain of *E. coli* positive for the actin reaction was kindly provided by Dr S. Knutton of University of Birmingham and was used as a positive control.

Haemagglutination test

Human group O blood was collected, placed immediately in Alserver's solution, and stored at 4 °C for no longer than 7 days before use. Blood cells were washed three times and resuspended in PBS at 3% (v/v). *Aeromonas* strains were grown for 18 h on trypticase soy-agar slants and suspended in PBS.

Haemagglutination on slides was assessed at room temperature after mixing 20 μl of the suspension of blood cells with the same volume of a suspension of bacteria (10^{11} c.f.u./ml). Haemagglutination was monitored after 3 min. L-fucose, D-galactose, and D-mannose (Nakarai, Kyoto, Japan) were included individually at 1% (w/v) in the suspension of blood cells. Inhibition was scored as positive when haemagglutination was abolished.

Table 2. *Numbers of strains of aeromonas in relation to the ability to adhere to INT407 cells, by species and by source of isolates*

Adhesion (bacteria/cell)	Stool			Foods			River		
	H*	S	C	H	S	C	H	S	C
0–10	17	26	42	21	17	26	12	20	20
10–20	1	5	3	0	2	0	0	0	0
20–	2	6	0	0	0	0	0	0	0

* H, *A. hydrophila*; S, *A. sobria*; C, *A. caviae*.

Bacterial hydrophobicity

The salt aggregation method of Lindahl and colleagues [19] was used for quantification of hydrophobic surface properties. In brief, aliquots of 25 μl of a bacterial suspension were mixed with different concentrations of ammonium sulphate in 0·002 M sodium phosphate (pH 6·8). The test was performed on glass slides and read after 2 min. Presence of bacterial clumps in the mixture was read as a positive result at the given concentration of ammonium sulphate and scored as the relative cell-surface hydrophobicity. A lower salt aggregation value corresponded to greater hydrophobicity of the cell surface.

Electronmicroscopy

A 10 μl amount of a washed bacterial suspension was applied to carbon-coated grids. The grids were blotted dry with filter paper and stained with potassium phosphotungstate (2%, pH 7·0) for 1 min. They were again blotted and examined in a transmission electronmicroscope (JEM-100B).

RESULTS

Forty-five strains (46·4%) of *A. hydrophila* and eight strains (9·4%) of *A. sobria* could not be assessed for adhesion since they caused detachment of the monolayers of INT407 from the slides (Table 1). Consequently, a total of 220 strains was available for the adhesion assay *in vitro*. The distribution of adhesive capacity in relation to the species and source of the various isolates is shown in Table 2. Seventeen of the 102 faecal isolates were adhesive strains with more than 10 bacteria adhering per cell. By contrast, only 2 of the 118 food and river isolates adhered to cells (Fisher's exact test, $P < 0{\cdot}001$). The proportion of adherers with more than 10 bacteria adhering per cell was significantly greater for strains of *A. sobria* (17·1%) than for strains of *A. hydrophila* (5·7%) and *A. caviae* (3·3%). *A. sobria* accounted for 34·5% of isolates examined, but it accounted for 57·9% of adhesive strains.

Eight highly adhesive strains with more than 20 bacteria adhering per cell were scrutinized in detail as representatives of adhesive strains (Fig. 1, Table 3). The cell surface hydrophobicity varied from strain to strain. Seven strains caused haemagglutination, but one non-haemagglutinative isolate (no. 79) was also highly adhesive when incubated with INT407 cells. In the case of four strains (nos. 62, 106, 107, 189), fucose inhibited both the adhesion to INT407 cells and haemagglutination. By contrast, two strains (nos. 17, 21) showed adhesion that was immune to inhibition by the tested sugars, even though their haemag-

Table 3. *Haemagglutinative ability and cell-surface hydrophobicity of various highly adhesive strains and the effects of carbohydrates on the adhesion to INT407 cells*

Strain	Species*	Hydro-phobicity†	Haemagg-lutination‡	Adhesion (bacteria/cell) F	G	M
17	S	0·02	F+G+M+	31·1	49·2	33·0
21	S	0·4	F−G+M+	22·0	30·8	28·8
22	S	0·4	F−G−M−	22·9	38·2	29·2
62	S	2·8	F+G−M−	1·6	30·6	24·8
79	S	3·8	NA	26·0	23·4	20·9
106	H	3·0	F+G−M−	3·6	26·2	32·2
107	S	1·2	F+G−M−	7·0	41·8	32·0
189	H	0·4	F+G−M−	8·0	49·2	47·1

* S, *A. sobria*; H, *A. hydrophila*.

† Hydrophobicity is indicated by results of the aggregation test, in terms of the molar concentration of ammonium sulphate (see text).

‡ F, fucose; G, galactose; M, mannose; NA, non-hemagglutinative. +indicates inhibition of hemagglutination; −indicates no inhibition.

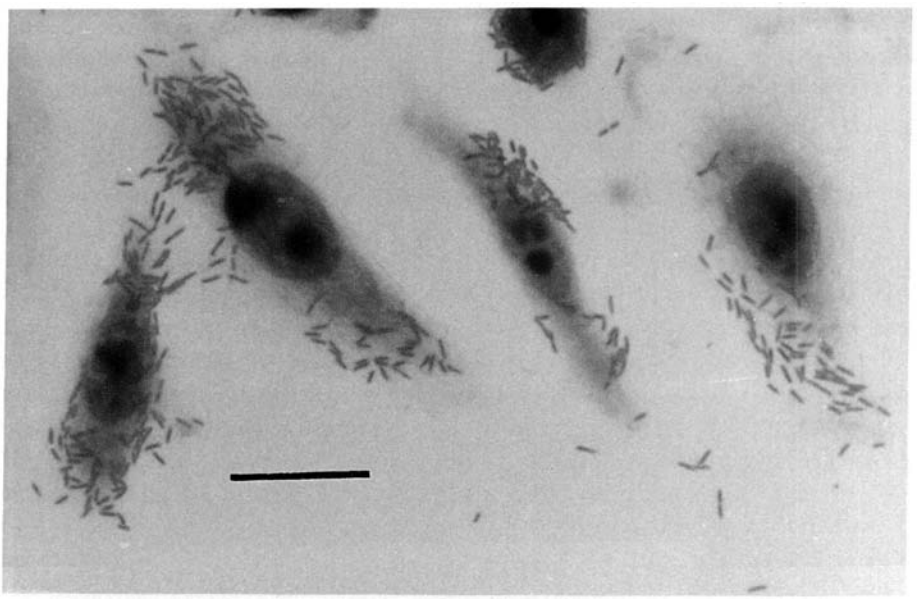

Fig. 1. Light micrograph of INT407 cells after incubation with *A. hydrophila* 106, demonstrating adherent bacteria. Bar, 10 μm.

glutination was blocked by these carbohydrates. Electronmicroscopic examinations revealed the presence of supple and curvilinear fimbriae on only two strains (nos. 106, 189, Fig. 2). These eight highly adhesive strains adhered well to HEp-2 cells as well as INT407 cells; however, no actin accumulation was observed at the sites of adhesion (data not shown).

DISCUSSION

Some methods for discrimination between isolates of *Aeromonas* that are pathogenic for man and those from the environment is clearly necessary. Since

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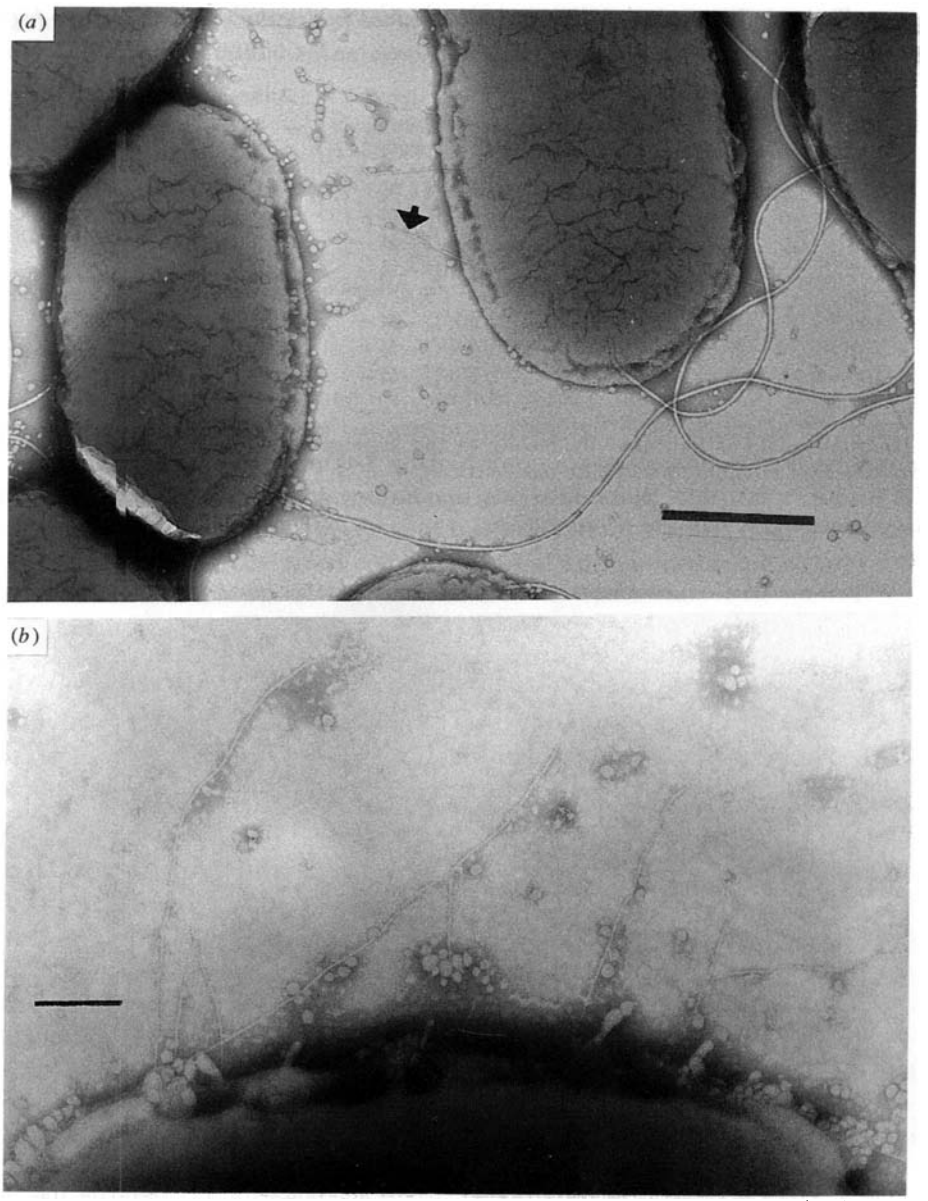

Fig. 2. Electron photomicrograph of *A. hydrophila* 189. (*a*) Flexible fimbria is indicated by an arrow. Bar, 0·5 μm. (*b*) Flexible fimbriae are shown. Bar, 0·2 μm.

Aeromonas strains produce a variety of biologically active extracellular substances, including haemolysins [12, 13] and enterotoxins [20–22], a number of studies have concentrated on the roles of exotoxins in enteropathogenicity, and β-haemolysin is considered to be a likely pathogenic factor [13]. However, epidemiological investigations suggest that production of haemolysin can serve as a marker for discrimination between species but does not distinguish between pathogenic and nonpathogenic strains, because most strains of *A. hydrophila* and *A. sobria* are haemolytic regardless of their source [8]. Failure of haemolytic strains to cause diarrhoea in human volunteers suggests that haemolysin *per se* is not the sole

determinant of virulence [14]. Current experiments revealed that mannose-resistant adhesive strains were found at higher levels among strains from faecal specimens than among strains from samples of food and river water. It is possible that adhesion may be a marker for stool isolates.

The physical condition of the people from whom the adhesive strains were isolated was not available to us since the stools were brought into our laboratory for the routine check of shigella, and the etiological significance of the aeromonads was also obscure. However, mannose-resistant adhesion to cells in tissue culture is recognized as a characteristic of virulence of diarrhoeagenic pathogens [23]. It seems to be possible that the adhesive strains of *Aeromonas* are enteropathogenic for man. Aeromonads can commonly be recovered from various kinds of food and water in considerably high numbers; they seem to form part of the natural flora [24, 25]. In spite of the pervasiveness of the organisms, outbreaks of *Aeromonas* infection hardly ever occur. If the capacity for adhesion is vital for the bacteria, the rarity of highly adherent strains in foods and water may account for a part of this phenomenon. The significance of the adhesion in enteritis should be studied further in aeromonads.

It was reported that accumulation of actin occurred at the sites of adhesion of shigella to cells in tissue culture [26], and of enteropathogenic or enterohaemorrhagic *E. coli* to such cells [18]. No actin reaction in the presence of adhering aeromonads was observed in this study. These strains attach to the cells but may not cause the attaching and effacing lesions in the membrane to the same extent as enteropathogenic *E. coli* [27].

Expression of cell surface hydrophobicity and negative charge seem to enhance the interaction of *Salmonella typhimurium* with epithelial cells [28]. It has also been shown that strains of *E. coli* with colonization factor antigens have high surface hydrophobicity [29]. We previously examined 626 strains of *Aeromonas* isolated from various sources for hydrophobicity. However, no relationship between hydrophobicity, species and the source of the organism could be demonstrated [30]. Moreover, no tendency for adhesive strains to have comparatively high surface hydrophobicity was recognized in this study.

The ability of an enteric bacterium to adhere to human intestinal epithelium is often ascribed to haemagglutinin [31]. Fucose inhibited both the adhesion to INT407 cells and haemagglutination of four strains. However, many haemagglutinative strains did not adhere to epithelial cells. By contrast, one non-haemagglutinative strain adhered to the cells. In addition, the adhesion of two strains was not inhibited by the carbohydrates that block haemagglutination by these strains (Table 3). Hence, purification and characterization of the adhesin are required if we are to develop diagnostic practical methods for the identification of adhesive strains in the clinical laboratory. So-called L-type fimbrial structures [32] could be found in only two strains. It has been reported that only a small percentage of clinical non-O1 *V. cholerae* isolates, another member of the Vibrionaceae, are fimbriated [33]. Although it is possible that our failure to see fimbriae is technical, non-fimbrial adhesive factors such as outer membrane proteins might be involved in the adhesion [34].

Adhesion of about half of the strains of *A. hydrophila* could not be evaluated because of their marked cytopathic effect. In a preliminary experiment, antisera specific for β-haemolysin [35], which were kindly donated by Dr S. Kozaki of

Osaka Prefecture University, did not neutralize this effect. Furthermore, the organisms exhibited this effect irrespective of their titer of haemolysin (data not shown). Although the factors contributing to this phenomenon could not be clarified, they do not seem to include the haemolysin.

There are a few reports about adhesion of aeromonads [32, 36]. However, mannose-sensitive adhesions might be included in their experiments since the adhesion assays were performed in media without mannose. On *E. coli*, it is recognized that mannose-sensitive adhesins are probably of minor importance in intestinal colonization [37]. To our knowledge, this is the first report of mannose-resistant adhesion of motile strains of *Aeromonas*. It is indicated that the adhesion to INT407 cells lead to a method for discriminating between faecal and environmental aeromonads. Further investigations are in progress to confirm the significance of adhesion in the enteropathogenicity and clarify the adhesin.

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