BACTERIAL PATHOGENICITY

Identification of a 43-kDa outer-membrane protein as an adhesin in *Aeromonas caviae*

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***Aeromonas* spp. are associated with intestinal and extra-intestinal infections. However, the virulence factors of *A. caviae* remain, for the most part, poorly known. This study examined the interactions involved in the adherence of *A. caviae* isolates Ae56, Ae391 and Ae398 to HEp-2 cells. All strains expressed high levels of aggregative adherence. Maximum adhesion occurred with bacteria grown at 22°C, but transmission electron microscopy did not reveal the presence of fimbrial structures on the bacterial cell surface. Outer-membrane proteins (OMPs) extracted from isolate Ae398, grown at 22°C and 37°C, showed similar SDS-PAGE protein profiles. Most proteins were < 60 kDa. A major 43-kDa protein was seen only in the boiled OMP extract. The biotinylated 43-kDa protein bound specifically to HEp-2 cells. Microbeads coated with the 43-kDa protein were also adherent to HEp-2 cells, and anti-43-kDa protein antibody blocked adherence of 43-kDa protein-coated latex beads. These data suggest that the 43-kDa OMP functions as an adhesin in *A. caviae*.**

Introduction

Aeromonas spp. comprise a widespread group of gram-negative bacteria that can be isolated from many sources such as food, drinking water, sewage, environmental water and human clinical specimens [1–3]. Virulence mechanisms are not well understood and confusion over the taxonomy of the genus has compounded difficulties in the identification of potential disease-causing strains. However, substantial evidence points to *Aeromonas* spp. as the causative agents of sporadic diarrhoea, dysentery and extra-intestinal infections that may be life-threatening [4, 5]. Two hybridisation groups (HGs), *A. hydrophila* (HG1) and *A. veronii* biovar *sobria* (HG8) were thought to be the main causes of aeromonas-associated illness. However, some investigations also implicate *A. caviae* (HG4) as a significant enteropathogen [6, 7]. It has been recognised that adherence to host cells is an important step in the pathogenesis of enteropathogenic bacteria, including *Aeromonas* spp. [8–11]. Although recent studies indicate that some filamentous structures, outer-

membrane proteins (OMPs) and lipopolysaccharide (LPS) could be intestinal adhesins, the colonisation mechanisms of enteropathogenic *Aeromonas* strains are poorly characterised, especially in *A. caviae* [12–15]. The pili of aeromonads have been described by morphological criteria as short, rigid (S/R) and long, wavy (L/W) structures. S/R pili are common and numerous among environmental isolates. In contrast, L/W pili are few in number, but are the dominant type observed on clinical strains [8]. L/W pili have been identified as aeromonas colonisation factors and also as haemagglutinins [12, 13]. Furthermore, an A6-type haemagglutinin (Ae-HAG) from *A. hydrophila* has been reported as a possible colonisation factor. A6-HAG is a 43-kDa OMP that binds to H antigen expressed on the surface of most human erythrocytes [16, 17]. In this study OMPs were investigated as possible colonisation factors in *A. caviae* adherence to HEp-2 cells.

Materials and methods

Bacterial strains and culture conditions

Three clinical isolates of *A. caviae* (Ae56, Ae391 and Ae398) from children with gastro-enteritis were studied. They were isolated in the Bacteriology Department

at the Pedro Ernesto Hospital of Rio de Janeiro University [18]. *A. hydrophila* strain Ae7 was chosen to serve as a fimbriate control. *Escherichia coli* DH5 α (K12) was included in this study as a non-adherent avirulent negative control. Long-term storage of strains was in skim milk (Difco, Detroit, MI, USA) at -70°C and short-term storage was in minimal maintenance medium at 15°C . The *A. hydrophila* and *E. coli* strains were cultured in Tryptic Soy Broth (TSB, Difco) at 22°C for 48 h and at 37°C for 24 h, respectively. The *A. caviae* strains were subcultured from -70°C storage on to blood agar and incubated at 37°C overnight. A sweep of colonies was inoculated into TSB without glucose and incubated at 37°C for 24 h or at 22°C for 48 h [19]. For OMP purification, a broth culture was used to inoculate 6 L of TSB which was incubated statically at 22°C or 37°C . For adherence assays, the culture density was estimated both by serial dilution and plating on to Mueller-Hinton Agar (Difco) for viable counting, and by the measurement of OD at 640 nm.

Adhesion to HEp-2 cells

HEp-2 cells (ATCC CCL23), derived from human epidermoid larynx carcinoma, were grown under standard conditions in air with CO_2 5% at 37°C in Eagle's Minimal Essential Medium (MEM, Sigma) supplemented with fetal calf serum (FCS; Gibco BRL, NY, USA) 5%. For the qualitative adhesion assay, HEp-2 cells were grown to semi-confluency for 24 h on circular glass coverslips (diameter 12 mm) in 24-well tissue-culture plates (Nalge Nunc International, Denmark). Bacteria grown in TSB at 22°C or 37°C were harvested by low speed centrifugation, washed twice in 10 mM Na-phosphate-buffered saline, pH 7.4 (PBS) and resuspended in MEM containing FCS 5%. One ml of bacterial suspension, containing 10^7 cfu (OD_{640} 0.2), was added to duplicate coverslip cultures of HEp-2 cells. The infected monolayers were incubated at 37°C for 90 min in air with CO_2 5%. Non-adherent bacteria were removed from monolayers by washing three times with PBS. The cells were fixed with methanol:acetic acid (3:1) for 15 min and stained with Giemsa 10% for 1 h. The coverslips were mounted on glass slides and viewed by light microscopy [8]. For the quantitative adhesion assay, HEp-2 cells were grown to confluency for 48 h in 24-well tissue-culture plates. Bacterial suspensions, prepared as described above, were inoculated on to the HEp-2 monolayers. After incubation at 37°C for 90 min in air with CO_2 5%, the monolayers were washed three times with PBS. Then 200 μl of Triton X-100 1% in PBS were added to each well and the plates were rocked at 37°C for 30 min. MEM (800 μl) was added and pipetted up and down vigorously to thoroughly mix the bacteria and lyse the HEp-2 cells. The suspensions were diluted serially in PBS and plated on to Mueller-Hinton Agar to determine the number of cell-associated bacteria. Adherence was expressed as the percentage of the

inoculum recovered from the HEp-2 cells after incubation for 90 min.

OMP extraction

For OMP extraction, *A. caviae* Ae398 was grown at both 22°C for 48 h and 37°C for 24 h. The cultures were centrifuged at low speed for 15 min at 4°C . The bacterial pellet was resuspended in 70 ml of a solution containing 10 mM Tris-HCl, pH 7.8, 5 mM EDTA, pH 7.8, and 1 mM β -mercaptoethanol (Sigma). The cells were disrupted by four 15-s bursts at the full power of an ultrasonic disintegrator. Cell debris was removed by low-speed centrifugation (2000 g , 20 min) and membranes were pelleted from the supernatant fraction by centrifugation at 100 000 g for 1 h at 4°C . The membrane pellet was resuspended in 20 ml of Sarkosyl (Sigma) 0.5% and 1 mM β -mercaptoethanol in distilled water and incubated at 4°C overnight. The suspension was then centrifuged at 100 000 g for 1 h at 4°C and the final outer-membrane pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.8 [19]. The protein content of the OMP suspension was determined by the modified method of Lowry [20] with bovine serum albumin (BSA, Sigma) as standard.

Biotinylation of OMPs

The OMPs were dialysed against 100 mM sodium bicarbonate solution (pH 9.0) at 4°C overnight, then 10 μl of 100 mM biotin (Sigma) were added to 1 ml of OMP solution (1 mg/ml) at 4°C for 1 h. The OMP solution was then dialysed against PBS at 4°C overnight [21].

HEp-2 cell-associated biotinylated OMP assay

Biotinylated OMP solution prepared as described above was added to confluent HEp-2 monolayers in a 24-well tissue-culture plate. After incubation for 90 min at 37°C in air with CO_2 5%, the monolayers were washed three times in PBS. Lysis buffer (200 μl) pH 7.5 – 25 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, 1 mM MgCl_2 , phenylmethylsulphonylfluoride (PMSF) 100 $\mu\text{g}/\text{ml}$ and Igepal (Sigma) 0.1% – were added to each well and rocked at 4°C for 30 min. The lysed cells were removed by centrifugation at 5000 g at 4°C for 15 min. The supernate was submitted to SDS-PAGE and analysed by the Western blot technique.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed as described by Laemmli [22] with a discontinuous acrylamide 10% gel (Mini-protein II, BioRad). Five μl of OMP extract (1 mg/ml) or 5 μl of HEp-2 cell-associated biotinylated OMPs (1 mg/ml) were mixed with an equal volume of $2 \times$ SDS-PAGE sample buffer (125 mM Tris-HCl, SDS 4%, glycerol 20%, β -mercaptoethanol 2%, bromophenol blue 0.002%) and boiled for 5 min before

electrophoresis. Separated proteins in the gel were stained with Coomassie Brilliant Blue in methanol 40%, acetic acid 10% in distilled water. In some analyses, the OMPs were mixed within $2 \times$ SDS-PAGE sample buffer without β -mercaptoethanol and were not boiled. After SDS-PAGE, HEp-2 cell-associated biotinylated OMPs were transferred to a nitrocellulose membrane in a Mini-Transblot Cell (BioRad) by electrophoresis in running buffer, pH 8.3 (25 mM Tris, 192 mM glycine, SDS 1%) for 90 min at 100 mV/400 mA [23]. Membranes were blocked with skim milk 5% in Tris-buffered saline, pH 7.5 (TTBS; 20 mM Tris, 500 mM NaCl, Tween-20 0.05%) for 2 h, washed three times in TTBS and incubated with 1 in 5000 streptavidin-peroxidase (Sigma) for 30 min. After washing, they were developed in a solution of H_2O_2 0.3%, 3-3' diaminobenzidine 1 mg/ml, imidazole (Sigma) 1 mg/ml.

43-kDa OMP isolation and antibody production

SDS-PAGE of OMP extract (500 μ g) was performed as described above. The 43-kDa protein was cut out and eluted from gel slices with a Electro-Eluter (Model 422, BioRad) and the running buffer, pH 8.3, at 20 mA for 6 h. The 43-kDa protein concentration was assayed as described previously [20]. A New Zealand White rabbit was immunised by multiple subcutaneous injections with 150 μ g of purified 43-kDa OMP emulsified with complete Freund's adjuvant (Sigma). Subsequently, three intramuscular injections were given weekly in incomplete Freund's adjuvant (Sigma). The rabbit was bled directly *via* the heart at day 10 after the last injection. The antiserum was pooled and stored at -20°C until used [24]. The IgG fraction of the antiserum was obtained by multiple salting out with 33% saturated ammonium sulphate and purified on a column of Superose (10/30) by high-performance liquid chromatography (HPLC; Pharmacia LKB Biotechnology, Uppsala, Sweden) [25]. The purification procedure was monitored by SDS-PAGE analysis and the concentration of IgG antibody was determined by absorbance at 280 nm. The specificity of the IgG for the 43-kDa OMP was checked by the Ouchterlony immunodiffusion method [25].

HEp-2 cell-associated 43-kDa OMP assay

Carboxylated microspheres (1 μm^3 , Sigma) were coated with the 43-kDa OMP or BSA. The concentration of coated beads was *c.* 1.8×10^{10} beads/ml, and the amounts of 43-kDa OMP and BSA bound to each microsphere were estimated as 20 fg [26]. Then, 50 μ l of a suspension of protein-coated beads were added to HEp-2 semi-confluent monolayers in 24-well tissue culture plates. After incubation at 37°C for 90 min in air with CO_2 5%, non-adherent beads were removed from monolayers by washing three times in PBS. The cells were fixed with glutaraldehyde (Merck) 0.2% and viewed by light microscopy. For adherence inhibition

assays, 43-kDa OMP-coated beads (1.8×10^{10} beads/ml) were pretreated with anti-43-kDa OMP antibody (200 μ g/ml) at 37°C for 1 h and added to the HEp-2 cell monolayers. In the other assay, HEp-2 cells were pretreated with OMP solution (1 mg/ml) and exposed to the 43-kDa OMP-coated beads. The adhesion assays were performed as described above.

Electron microscopy

All three *A. caviae* strains were examined for the presence of fimbriae by transmission electron microscopy (TEM). Cell suspensions were fixed in phosphate-buffered glutaraldehyde 0.2% (pH 7.2) at 4°C for 15 min and a drop was placed on formvar-coated copper grids (150 mesh, Sigma) for 5 min. The grids were negatively stained with potassium phosphotungstate 1%, washed with water for 1 min, dried and then viewed in a Zeiss 906 transmission electron microscope. For immunolabelling, the grids were inoculated in the presence of BSA 5% w/v in Tris buffer, pH 8.2 (20 mM Tris, Tween 20 0.05%) for 30 min to prevent non-specific labelling, and then treated with anti-43-kDa OMP antibody (1 in 40 dilution) for 1 h at room temperature, washed three times in PBS and then incubated in the presence of 5 nm gold-labelled goat anti-rabbit IgG (1 in 20 dilution) for 30 min at room temperature. After incubation, the grids were washed in the same buffer, negatively stained as before and viewed as above. Grids treated with pre-immune serum (1 in 40 dilution) were used as a control.

Results

Non-fimbrial adherence to HEp-2 cells

A. caviae isolates (Ae56, Ae391, Ae398) showed a higher percentage of adherence than *E. coli* DH5a to HEp-2 cells (Table 1). All isolates formed small clusters of aggregated bacteria (≥ 10 bacteria/cell) on the cell surface. Bacterial aggregates adhered to cell monolayers with a typical 'stacked-brick' appearance (Fig. 1). All the *A. caviae* isolates were more adherent to HEp-2 cells when the bacteria were grown at 22°C than when they were grown at 37°C . In quantitative assays, a mean of 32.5% and 3.4% of the initial inoculum of strains grown at 22°C and 37°C , respectively, adhered to HEp-2 cells after incubation for 30 min (Table 1). The results of electron microscopy showed that all the *A. caviae* isolates, whether grown at 37°C or 22°C , did not form fimbrial structures on the bacterial cell surface when compared with the fimbriate control *A. hydrophila* Ae7. Fig. 2 shows an electron micrograph of *A. caviae* isolate Ae398 grown at 22°C for 48 h.

Purification of OMPs and binding to HEp-2 cells

Preparation of OMPs by the Sarkosyl method was found to be effective for the *A. caviae* isolates; *c.* 10–

Table 1. Comparison of adherence of *A. caviae* strains grown at 22°C and 37°C to HEp-2 cells

Strain* no.	Mean bacterial count (cfu/cell monolayer) at	
	22°C	37°C
Ae56	2.7 SD 0.1 × 10 ⁶ (27%) [†]	2.95 SD 0.05 × 10 ⁵ (2.95%)
Ae391	3.2 SD 0.2 × 10 ⁶ (32%)	3.25 SD 0.01 × 10 ⁵ (3.25%)
A398	3.85 SD 0.05 × 10 ⁶ (38.5%)	4.05 SD 0.05 × 10 ⁵ (4.05%)
<i>E. coli</i> DH5a [‡]	NT	4.7 SD 0.1 × 10 ⁴ (0.47%)

NT, not tested.

*Inoculum 10⁷ cfu/ml.[†]Data are means and SD for three assays done in triplicate. Adherence data are expressed in percentages of inoculum.[‡]Non-adherent *E. coli* control. **Fig. 1.** Aggregative adherence of *A. caviae* isolate Ae398 to HEp-2 cells showing a stacked-brick appearance on the cell surface. Light microscopy × 550. **Fig. 2.** TEM of negatively stained *A. caviae* isolate Ae398 grown under fimbrial expression conditions (22°C for 48 h). No fimbrial structures are seen on the bacterial cell surface.

20 mg and 2–4 mg of OMPs were obtained from 6 L of bacterial culture incubated at 22°C for 48 h and at 37°C for 24 h, respectively. No qualitative differences were observed in the OMP profiles by SDS-PAGE of *A. caviae* Ae398 grown at 22°C or 37°C (Fig. 3a).

Fig. 3. SDS-PAGE of OMPs from *A. caviae* isolate Ae398. Mol. wt standards are indicated in kDa (lane 1). (a) Similar protein profiles were observed when bacteria were grown at 22°C for 48 h (2) and 37°C for 24 h (3). (b) Protein profiles after different treatments: OMP in sample buffer with β -mercaptoethanol (lane 4) and without β -mercaptoethanol (5); OMPs boiled in sample buffer with β -mercaptoethanol (2) and without β -mercaptoethanol (3); purified 43-kDa OMP (6).

Most proteins were <60 kDa, the predominant band being a 43-kDa protein. This 43-kDa protein was observed only in the boiled OMPs and was not influenced by β -mercaptoethanol treatment (Fig. 3b). Western blotting analysis revealed specific binding of the biotinylated OMP extract to HEp-2 cells. Only the 43-kDa OMP of *A. caviae* bound selectively to HEp-2 cell surface molecules (Fig. 4).

Adhesion of 43-kDa OMP-coated beads to HEp-2 cells

Each 1 mg of OMP extract yielded *c.* 150 μ g of purified 43-kDa protein. The 43-kDa OMP-coated beads adhered to HEp-2 cells by interacting with the cell surface. The attachment was confirmed by light microscopy (Fig. 5a). Control BSA-coated beads did not interact with HEp-2 cells, even when high concentrations of beads (10^{10} beads/ml) were used (data not shown). The 43-kDa OMP-coated beads did not interact with the cell surface when they were pretreated with anti-43-kDa OMP antibodies (Fig. 5b). Similar results were obtained with HEp-2 cells pretreated with OMP extract solution, confirming the specificity of 43-kDa OMP binding to cell surface (data not shown).

Distribution and immunolocalisation of 43-kDa OMP on bacteria

A uniform distribution of the 43-kDa OMP over the *A. caviae* cell surface was demonstrated by immunogold staining (Fig. 6). The 43-kDa OMP was observed on the cell surface of all the *A. caviae* isolates tested. In the absence of anti-43-kDa OMP antibodies, gold-labelled goat anti-rabbit IgG did not interact with the bacterial cell surface.

Fig. 4. Western blotting analysis of lysates of HEp-2 cells after interaction with biotinylated OMPs following lysis buffer solubilisation (see *Materials and methods*) and then stained by the biotin-avidin-peroxidase technique. Specific binding of biotinylated 43-kDa protein to cell surface receptors is shown.

Fig. 5. Light microscopy ($\times 520$) showing (a) the interaction of 43-kDa OMP-coated beads with HEp-2 cells (inset $\times 1030$); (b) inhibition of 43-kDa OMP-coated bead adherence by anti-43-kDa OMP antibody.

Fig. 6. Immunogold electron microscopy (IEM) of *A. caviae* isolate Ae398, stained with anti-43-kDa OMP antibody. IEM demonstrated a uniform distribution of the 43-kDa protein over the cell surface.

Discussion

Although attachment to the intestinal mucosa is likely to be an important pathogenic stage for diarrhoea-causing bacteria, little is known about the adhesive mechanisms of aeromonads. Studies have shown a correlation between bacterial adherence *in vitro* and infectivity *in vivo* [10]. Some studies have described adherence of *Aeromonas* strains to cultured cells such as HEp-2, Caco-2 and INT407 [9, 10, 27]. Although it cannot be assumed that HEp-2 cells, derived from human laryngeal epithelial carcinoma, possess surface receptors identical to these of human enterocytes, a correlation between the high-level adherence to HEp-2 cells and aeromonas enteropathogenicity has been observed [9, 28]. On the other hand, *A. caviae* strains were also reported to express weak adhesive properties to HEp-2 cells [8, 28]. The present study demonstrated that all the *A. caviae* isolates tested showed a high percentage of adherence to HEp-2 cells. The high percentage of isolation of *A. caviae* from children with gastro-enteritis may reflect the enteropathogenic potential of this species. Bacterial growth temperature influenced *A. caviae* adherence to HEp-2 cells and quantitative expression of the 43-kDa OMP on the cell surface. All the *A. caviae* isolates showed greater adherence when grown at 22°C than at 37°C, as observed similarly with *A. veronii* biovar *sobria* strains [9]. This difference correlated with higher expression of the 43-kDa OMP at 22°C than at 37°C.

The aggregative adherence pattern was first observed in *E. coli* strains. Enteroadherent-aggregative (EA-Agg) *E. coli* have been incriminated as important agents of diarrhoea, inducing damage to the intestinal epithelium, and characterised by a shortening of the villi, haemorrhagic necrosis of the villus tips and a mild inflammatory submucosa response [29]. Previous investigations have also described the aggregative adherence pattern to HEp-2 and Caco-2 cells for *Aeromonas* spp. [11, 30]. All the *A. caviae* strains investigated in this study exhibited the aggregative adherence pattern and readily formed aggregates able to adhere to HEp-2 cells with a stacked-brick appearance.

Filamentous structures were described as colonisation factors in *A. hydrophila* and *A. veronii* biovar *sobria* strains [13]. L/W pili, bundle-forming pili and filamentous networks showed maximal expression under the same conditions (22°C for 48 h) as those that induce high adherence to cultured cells [9, 31]. However, TEM did not reveal the presence of fimbrial structures on the cell surface of highly adherent *A. caviae* strains grown under these conditions. The data suggested that instead of fimbriae, OMPs might be involved in the adherence process. Merino *et al.* suggested that LPS and OMP might be other important adhesins for binding of *Aeromonas* strains to HEp-2 cells [15]. The findings of the present study are at

variance with that described recently of type IV pili in *A. caviae* strains isolated from clinical sources in Australia [32], possibly because they are from a different geographic location [6]. Furthermore, it is possible that, if filamentous structures are expressed on the cell surface of *A. caviae*, they exist only in very small numbers under the conditions tested here. On the other hand, adhesion of the Australian *A. caviae* strains to HEp-2 cells was only in part mediated by filamentous structures [32].

OMPs from *A. caviae* Ae398, grown at 22°C and 37°C, showed similar protein profiles in SDS-PAGE. The 43-kDa OMP was observed only in boiled extract of OMPs, independently of the presence of β -mercaptoethanol, indicating either a link of this 43-kDa protein to a heavier molecular structure or its aggregation to other proteins.

A. hydrophila strain A6 was found to produce a haemagglutinin that was inhibited by L-fucose [16]. The haemagglutinin was also described as a pore-forming 43-kDa OMP in artificial planar bilayer membranes [17, 33]. The 43-kDa OMP from *A. caviae* isolate Ae398 may also be a pore-forming OMP. However, none of the *A. caviae* isolates showed either haemagglutinating activity to human erythrocytes or L-fucose-sensitive adherence to HEp-2 cells (data not shown). Interaction of OMP extract with HEp-2 cells revealed a specific binding of the 43-kDa protein to the HEp-2 cell surface, suggesting that this protein could be an adhesin of *A. caviae*. Furthermore, the 43-kDa OMP-coated beads adhered to the HEp-2 cell surface. The 43-kDa OMP exhibited a uniform distribution over the cell surface of all *A. caviae* isolates tested. Both anti-43-kDa OMP antibody and OMP extract inhibited adherence of 43-kDa protein-coated latex beads. From these experiments, it was concluded that a 43-kDa OMP is involved in *A. caviae* adherence. Complementary studies are currently in progress to enhance understanding of the role of the 43-kDa protein and other structures in the adherence of *A. caviae* to host cells.

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References

1. Araujo RM, Arribas RM, Pares R. Distribution of *Aeromonas* species in waters with different levels of pollution. *J Appl Bacteriol* 1991; **71**: 182–186.
2. Freitas AC, Nunes MP, Milhomen AM, Ricciardi ID. Occurrence and characterization of *Aeromonas* species in pasteurized milk and white cheese in Rio de Janeiro, Brazil. *J Food Protect* 1993; **56**: 62–65.
3. Gluskin I, Batash D, Shoseyov D *et al.* A 15-year study of the role of *Aeromonas* spp. in gastroenteritis in hospitalised children. *J Med Microbiol* 1992; **37**: 315–318.
4. Janda JM. Recent advances in the study of the taxonomy,

- pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. *Clin Microbiol Rev* 1991; **4**: 397–410.
5. Pazzaglia G, Sack RB, Bougeois AL, Froehlich J, Eckstein J. Diarrhea and intestinal invasiveness of *Aeromonas* strains in the removable intestinal tie rabbit model. *Infect Immun* 1990; **58**: 1924–1931.
 6. Namdari H, Bottone EJ. Cytotoxin and enterotoxin production as factors delineating enteropathogenicity of *Aeromonas caviae*. *J Clin Microbiol* 1990; **28**: 1796–1798.
 7. Wilcox MH, Cook AM, Eley A, Spencer RC. *Aeromonas* spp as a potential cause of diarrhoea in children. *J Clin Pathol* 1992; **45**: 959–963.
 8. Carrello A, Silburn KA, Budden JR, Chang BJ. Adhesion of clinical and environmental *Aeromonas* isolates to HEp-2 cells. *J Med Microbiol* 1988; **26**: 19–27.
 9. Kirov SM, Jacobs I, Hayward LJ, Hapin RH. Electron microscopic examination of factors influencing the expression of filamentous surface structures on clinical and environmental isolates of *Aeromonas veronii* biotype *sobria*. *Microbiol Immunol* 1995; **39**: 329–338.
 10. Nishikawa Y, Hase A, Ogawassara J, Scotland SM, Smith HR, Kimura T. Adhesion to and invasion of human colon carcinoma Caco-2 cells by *Aeromonas* strains. *J Med Microbiol* 1994; **40**: 55–61.
 11. Thornley JP, Shaw JG, Gryllos IA, Eley A. Adherence of *Aeromonas caviae* to human cell lines HEp-2 and Caco-2. *J Med Microbiol* 1996; **45**: 445–451.
 12. Hokama A, Honma Y, Nakasone N. Pili of an *Aeromonas hydrophila* strain as a possible colonization factor. *Microbiol Immunol* 1990; **34**: 901–915.
 13. Hokama A, Iwanaga M. Purification and characterization of *Aeromonas sobria* pili, a possible colonization factor. *Infect Immun* 1991; **59**: 3478–3483.
 14. Kirov SM, Sanderson K. Characterization of a type IV bundle-forming pilus (SFP) from a gastroenteritis-associated strain of *Aeromonas veronii* biovar *sobria*. *Microb Pathog* 1996; **21**: 23–34.
 15. Merino S, Camrubi S, Tomás JM. Effect of growth temperature on outer membrane components and virulence of *Aeromonas hydrophila* strains of serotype O:34. *Infect Immun* 1992; **60**: 4343–4349.
 16. Atkinson HM, Adams D, Savvas RS, Trust TJ. *Aeromonas* adhesin antigens. *Experientia* 1987; **43**: 372–374.
 17. Quinn DM, Wong CYF, Atkinson HM, Flower RLP. Isolation of carbohydrate-reactive outer membrane proteins of *Aeromonas hydrophila*. *Infect Immun* 1993; **61**: 371–377.
 18. Freitas AC, Souza SMS, Macedo LC, Pinto EC, Pereira SS. *Aeromonas* species associated with gastroenteritis in children: prevalence, characteristics and virulence properties. *Rev Microbiol* 1998; **29**: 152–157.
 19. Bölin I, Norlander L, Wolf-Watz H. Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. *Infect Immun* 1982; **37**: 506–512.
 20. Markwell MAK, Haars SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978; **87**: 206–210.
 21. Ternynck T, Avrameas S. Techniques immuno-enzymatiques. Paris, Les Editions INSERM. 1991: 108.
 22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
 23. Towbin H, Gordon J. Immunoblotting and dot immunoblotting – current status and outlook. *J Immunol Methods* 1984; **72**: 313–340.
 24. Dias Filho BP, Benchimoli M, Andrade AFB, Angluster J, de Souza W. Purification and immunocytochemical localization of neuraminidase from *Trichomonas foetus*. *Parasitology* 1999; **118**: 17–25.
 25. Harlow E, Lane D. Storing and purifying antibodies. In: Antibodies: a laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory. 1998: 283–318.
 26. Jouve M, Garcia M-I, Courcoux P, Labigne A, Gounon P, Le Bouguénec C. Adhesion to and invasion of HeLa cells by pathogenic *Escherichia coli* carrying the *afa-3* gene cluster are mediated by the AfaE and AfaD proteins, respectively. *Infect Immun* 1997; **65**: 4082–4089.
 27. Bartková G, Čiznár I. Adherence pattern of non-piliated *Aeromonas hydrophila* strains to tissue cultures. *Micróbios* 1994; **77**: 47–55.
 28. Grey PA, Kirov SM. Adherence to HEp-2 cells and enteropathogenic potential of *Aeromonas* spp. *Epidemiol Infect* 1993; **110**: 279–287.
 29. Vial PA, Robins-Browne R, Lior H *et al*. Characterization of Enteroadherent-Aggregative *Escherichia coli*, a putative agent of diarrheal disease. *J Infect Dis* 1998; **158**: 70–79.
 30. Neves MS, Nunes MP, Milhomem AM. *Aeromonas* species exhibit aggregative adherence to HEp-2 cells. *J Clin Microbiol* 1994; **32**: 1130–1131.
 31. Kirov SM, Hayward LJ, Nerrie MA. Adhesion of *Aeromonas* sp. to cell lines used as models for intestinal adhesion. *Epidemiol Infect* 1995; **115**: 465–473.
 32. Kirov SM, Sanderson K, Dickson TC. Characterisation of a type IV pilus produced by *Aeromonas caviae*. *J Med Microbiol* 1998; **47**: 527–531.
 33. Quinn DM, Atkinson HM, Bretag AH *et al*. Carbohydrate-reactive, pore-forming outer membrane proteins of *Aeromonas hydrophila*. *Infect Immun* 1994; **62**: 4054–4058.